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Rapid Phenotypic Reversion of Zidovudine-Resistant Feline Immunodeficiency Virus without Loss of Drug-Resistant Reverse Transcriptase

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We have selected and plaque purified zidovudine (3′-azido-3′-deoxythymidine [AZT])-resistant mutants from an infectious molecular clone of feline immunodeficiency virus (FIV). The patterns of cross-resistance and drug susceptibilities of these mutants were similar to those of the AZT-resistant FIV that we previously selected in vitro from a wild-type FIV population and to those of the most common AZT-resistant clinical isolates of human immunodeficiency virus type 1. Two AZT-resistant mutants of FIV, one selected from a normal population and one selected from the molecular clone, each reverted rapidly to an AZT-sensitive phenotype when passaged in the absence of drug. Sequence analysis of the reverse transcriptase (RT)-encoding region from the plaque-purified AZT-resistant FIV revealed a single base change at position 2939, resulting in a Glu-to-Lys substitution at amino acid 202 of the RT. Similar analyses of plaque-purified revertants showed that the phenotypic reversion was not the result of a genotypic reversion at this position and that no additional mutations existed within the RT-encoding region of the revertants. Moreover, RTs purified from the mutant and revertant were both resistant to the 5′-triphosphate of AZT. These results indicate the complexity of AZT resistance and suggest the presence of additional factors, outside the RT-encoding region, which may contribute to AZT resistance.

Chemotherapy of AIDS has been complicated by the emergence of drug-resistant variants of human immunodeficiency virus type 1 (HIV-1). The most widely used therapeutic agents are the nucleoside analogs zidovudine (3′-azido-3′-deoxythymidine [AZT]), 2′,3′-dideoxyinosine (ddI), and 2′,3′-dideoxycytidine (ddC). The active forms of these nucleoside analogs are the corresponding 5′-triphosphates, which act as DNA chain terminators and inhibitors of the HIV-1 reverse transcriptase (RT) (3). AZT has been shown to improve the clinical status of patients with AIDS and AIDS-related complex (4). However, HIV-1 variants that are less sensitive to AZT have been isolated from patients following prolonged therapy with AZT (8, 11, 12, 24, 25, 29). Similarly, ddI-resistant variants have been isolated from patients following treatment with ddI (26). At least one of the AZT-resistant variants obtained from a patient on AZT therapy may display cross-resistance to ddI (8). HIV-1 mutants that are resistant to ddC (5) as well as to nonnucleoside inhibitors of the HIV-1 RT, namely, nevirapine (BIRG-587) and TIBO compounds (1, 18), have also been isolated. In contrast to the prolonged therapy necessary for selection of mutants resistant to nucleoside analogs, resistance to the nonnucleoside drugs occurs rapidly (23).

There are many questions about drug resistance that must be answered before strategies can be formed to improve AIDS therapy. Of central importance is whether the drug-resistant phenotype alters pathogenicity and/or infectivity of the virus. This will be difficult to determine in studies of HIV-1 but might be addressed experimentally with a suitable animal model. Other important concerns are whether the drug-resistant mutants are stable and whether their emergence can be diminished or eliminated with combination chemotherapy. Answers to these questions might be best addressed with in vitro systems that enable selection and manipulation of drug-resistant mutants.

We have used feline immunodeficiency virus (FIV) in model systems that provide an opportunity to characterize important features of drug resistance. FIV is a lentivirus that causes a natural AIDS-like disease in domestic cats (19, 20). FIV also causes an AIDS-like immune suppression following experimental infection of specific-pathogen-free cats (7, 28, 30). The RT encoded by FIV is similar to the HIV-1 RT in physical properties, catalytic activities, and sensitivity to the active forms of AZT, ddI, and ddC (2, 15–17). We have used FIV for in vitro studies of antiviral drugs (2, 17) and the emergence of drug-resistant mutants (22). With this FIV system, we reported the first in vitro selection of AZT-resistant mutants (22). These mutants are similar to the AZT-resistant clinical isolates of HIV-1 reported by Larder et al. (11, 12) in that they are resistant to AZT and other 3′-azidonucleosides, but they remain sensitive to ddI, ddC, and several other inhibitors (22). Like the RT of AZT-resistant HIV-1, purified RT from AZT-resistant FIV failed to show resistance to the 5′-triphosphate of AZT (11, 12, 22). Subsequently, in vitro selection of AZT-resistant variants of HIV-1 was reported (6, 10).

The availability of in vivo and in vitro systems for studies of FIV affords a unique opportunity to address the important issues pertaining to drug resistance. Studies are in progress to evaluate the pathogenicity and infectivity of AZT-resistant FIV in cats. In the work reported here, we used in vitro systems to determine the stability of AZT-resistant mutants. We observed rapid phenotypic reversion of AZT-resistant mutants that had been derived either from a normal population of FIV or from a molecular clone of FIV.

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MATERIALS AND METHODS

Chemicals. dTTP, aminoethylcarazole, phosphonoformate (PFA), and ddC were purchased from Sigma Chemical Co., St. Louis, Mo. AZT and the 5′-triphosphate of AZT (N_3dTTP) were provided by Phillip A. Furman and Wayne Miller, respectively, Burroughs Wellcome Co., Research Triangle Park, N.C. 2′,3′-Dideoxy-2′,3′-didehydrothymidine (D4T) and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) were provided by H.-T. Ho, Bristol Myers-Squibb Co., Wallingford, Conn. ddI was provided by the Developmental Therapeutics Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases. 3′-Azido-2′,3′-dideoxyuridine (AZdU) was provided by Gary Williams, Triton Biosciences, Inc., Alameda, Calif. Poly(rA)-oligo$(dT)_{10}$ was purchased from Pharmacia LKB, Piscataway, N.J. [*methyl*-^{3}H]dTTP was obtained from Dupont-New England Nuclear, Boston, Mass. International Bio Technologies phenol for DNA extractions was purchased from VWR Scientific. The *Taq* DyeDeoxy Terminator cycle sequencing kit was purchased from Applied Biosystems, Foster City, Calif. All other chemicals were reagent grade or better.

Cells and virus. The Petaluma strain of FIV (20, 30) and virus derived from a molecular clone of FIV, 34TF10 (27), were used in these studies. AZR-1 is the AZT-resistant mutant of FIV that was described previously (22). Virus was grown and maintained in Crandell feline kidney (CrFK) cells as previously described (17). AZT-resistant mutants of FIV were maintained in medium that contained 10 μM AZT, and the medium was replaced every 2 days. Cells were routinely tested with a Gen Probe *Mycoplasma* detection kit (Gen Probe Inc., San Diego, Calif.) and found to be free of *Mycoplasma* contamination.

Enzymes and enzyme assays. RT was purified from virions of wild-type, mutant, and revertant FIV by a method that had been developed in this laboratory (16, 22). Assays for RT were performed as reported previously (16, 17) with poly(rA)-oligo$(dT)_{10}$ as the template-primer. Double-reciprocal plots were used to determine kinetic constants (K_m and K_i) as reported previously (2, 15).

FIA. FIV infectivity in the presence or absence of various inhibitors was determined by a focal infectivity assay (FIA) as described previously (22). Briefly, uninfected CrFK cells were infected with 20 to 60 focus-forming units of wild-type or AZT-resistant FIV per well in the presence of various concentrations of inhibitor. After 4 days, media were removed and the cells were fixed with methanol. Infectious foci were detected by reacting the cells with a polyclonal antiserum that had been obtained from FIV-infected specific-pathogen-free cats (generously provided by N. C. Pedersen, School of Veterinary Medicine, University of California, Davis) and then peroxidase-conjugated anti-cat immunoglobulin (Organon Teknika). The peroxidase stain was developed with H_2O_2 and aminoethyl-carbazole, and infectious foci appeared as areas of red cells against a background of unstained cells. Data were plotted as percentage of control foci (no drug) versus inhibitor concentration. Concentrations required to inhibit focus formation by 50% (IC_{50} values) were obtained directly from the linear portion of these plots, using a computer-generated regression line. Within an experiment, each datum point represents the mean of four determinations. Results from two or more independent experiments were used to derive IC_{50} ± standard deviation.

Nucleic acid preparation. Total cellular DNA containing proviral DNA was extracted from CrFK cells that were infected with AZT-resistant or wild-type FIV. Infected cells were washed with TE (10 mM Tris-HCl [pH 7.6] containing 1 mM EDTA) and then treated with 0.1 mg of proteinase K per ml and 0.5% sodium dodecyl sulfate overnight (12 to 16 h) at 37°C. The cell lysates were phenol extracted and treated with RNase A (0.1 mg/ml) for 3 h at 37°C. The RNase A-treated aqueous phase was then subjected sequentially to phenol-chloroform-isoamyl alcohol (25:24:1) extraction, chloroform-isoamyl alcohol (24:1) extraction, and ethanol precipitation. The DNA from this procedure was dissolved in distilled water and used for amplification by the PCR.

PCR amplification and nucleotide sequence analysis. Amplification of the RT-encoding region of the *pol* gene was performed by the Perkin-Elmer/Cetus GeneAMP PCR protocol. Each 100-μl reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM each deoxyribonucleoside 5′-triphosphate, 4 mM $MgCl_2$, 0.2 μM each primer, 2.5 U of AmpliTaq DNA polymerase, and 10 to 20 μg of target DNA. The sense primer (5′-GGA AAT AGA AAG AAT TCG GGA AAC-3′) and the reverse complement primer (5′-GGC AAC ATT AGC TTT ACC CCT GTT GG-3′) were chosen to amplify a 2,271-bp fragment that contained nucleotides 1860 through 4131. This segment included the entire RT- and RNase H-encoding regions. The PCR was run for 30 cycles, each cycle consisting of 30 s of denaturation at 94°C, 30 s of annealing at 62°C, and 2 min of extension at 72°C. After PCR, the product was run on a 0.8% low-melting-point agarose electrophoresis gel and visualized with ethidium bromide. A 2,271-bp fragment was gel purified with Geneclean II (Bio 101, Inc., La Jolla, Calif.). This DNA was directly sequenced with a Taq DyeDeoxy Terminator sequencing kit and analyzed on a model 373A automated DNA sequencer (Applied Biosystems).

RESULTS

Reversion of AZR-1. The stability of the AZT-resistant phenotype was determined for AZR-1, the AZT-resistant FIV that we selected previously (22). CrFK cells were infected with AZR-1 and maintained without AZT. When virus production was apparent, an IC_{50} for AZT was determined, and this virus was used to initiate a second round of infection. This method was used to initiate three successive cycles of infection. The IC_{50} values rapidly dropped, and by three cycles of infection in the absence of drug, AZR-1 had reverted to an AZT-sensitive phenotype. Representative data are shown in Fig. 1A and discussed in more detail below.

Because AZR-1 was selected from a genetically heterogeneous population, it was possible that this phenotypic reversion was due to the presence of a small amount of wild-type FIV that remained within the mutant population and was able to outcompete the mutant virus for replication in the absence of drug. Likewise, we cannot be certain that the mutant that we previously selected, AZR-1, was not already present at low levels in the swarm population of FIV Petaluma. Therefore, we felt it necessary to attempt selection of a drug-resistant mutant from a genetically homogeneous parent and then plaque purify an isolate of this mutant for use in reversion studies. This approach would facilitate study of genetic lesions responsible for drug resistance. Moreover, the use of mutants and revertants derived from a molecular clone would simplify DNA sequence analysis by eliminating heterogeneity which is present in the swarm population.

Selection of AZT-resistant FIV from a molecular clone. A full-length molecular clone of FIV, 34TF10, was transfected into CrFK cells, and within 10 days, the infected cells produced fully infectious virus (27). This genetically homogeneous population was grown in the presence of inhibitory concentrations

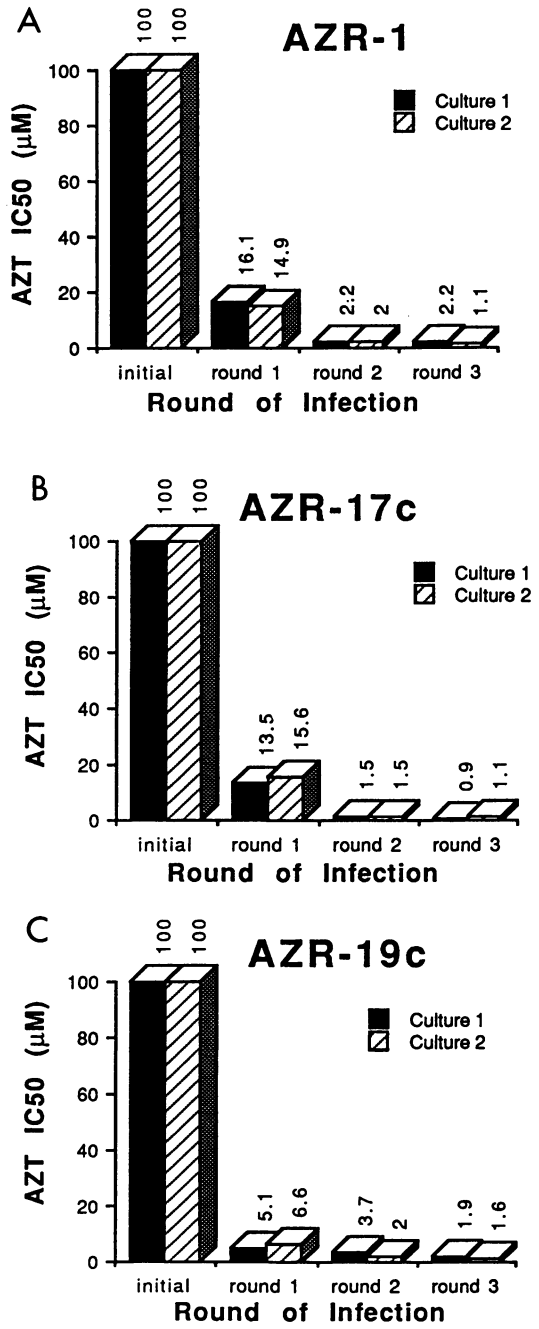

FIG. 1. Reversion of AZR-1, AZR-17c, and AZR-19c to AZT-sensitive phenotype. Groups of two 25-cm^2 flasks were infected with AZR-1, AZR-17c, or AZR-19c and maintained without AZT. When virus production was detected by FIA, an IC_{50} for AZT was determined, and this virus was used to begin a second round of infection. This procedure was used to initiate three successive rounds of infection.

of AZT. For the first round, culture supernatant that contained 148 focus-forming units was used to infect each of two 25-cm^2 flasks of CrFK cells. These infected cells were maintained in the presence of 10 μM AZT and were assayed weekly by FIA until virus production was detected. By 6 weeks, virus production was apparent, and a second round of infection was initiated. A lower-input multiplicity was used for this round of infection. Culture supernatants from each of the first-round cultures, each containing about 30 focus-forming units, were used to infect flasks of CrFK cells. As with the first round of infection, cultures were maintained in the presence of 10 μM AZT and were monitored for virus production by FIA. By 5 weeks after infection, virus production was apparent. At this point, two potentially unique AZT-resistant mutants, AZR-12c and AZR-13c, were obtained, one from each culture. The IC_{50} values for AZT were determined for each round of infection. For AZR-13c, after the first round of infection, the IC_{50} was approximately 55 μM. By the second round of infection, AZR-13c was highly resistant to AZT; the IC_{50} was greater than 100 μM. Similar results were obtained with AZR-12c.

TABLE 1. Sensitivity of AZT-resistant FIV to AZT

Mutant	IC_{50}[a] (μM)
AZT-resistant FIV derived from a molecular clone (FIV 34TF10)	
AZR-12c	>100
AZR-13c	>100
Plaque-purified AZT-resistant FIV derived from AZR-13c	
AZR-17c	>100
AZR-18c	>100
Single cell clones of AZR-13c	
AZR-19c	>100
AZR-20c	>100
AZR-21c	>100
AZR-22c	17.4
AZR-23c	3.1
AZR-24c	21.0
Plaque-purified AZT-resistant FIV derived from AZR-1 parent	
AZR-7	27
AZR-8	97
AZR-9	94
AZR-10	65
AZR-11	50

[a] As determined by FIA. Values are means of four or more determinations.

Plaque purification of AZT-resistant mutants. In an effort to minimize possible heterogeneity within the AZR-13c mutant population, a clone of virus that had descended from a single infectious particle was isolated. CrFK cells were seeded at a density of 1.5×10^4 cells per well in TC24 plates in medium containing 10 μM AZT and were infected with virus stocks that had been diluted to contain approximately one infectious particle per well. After 6 days, culture supernatants were transferred to uninfected cells in TC24 plates, and the original plates were immunostained to identify those wells that had contained a single focus of infection. Cells infected with progeny from a single infection event were transferred to a flask and maintained in medium containing 10 μM AZT. This method of plaque purification was used to generate five clones from AZR-1 (AZR-7, AZR-8, AZR-9, AZR-10, and AZR-11) and two clones from AZR-13c (AZR-17c and AZR-18c). The resistance to AZT of virus that was plaque purified from AZR-1 and AZR-13c was confirmed by FIA (Table 1). All of the plaque-purified clones demonstrated at least some degree of resistance to AZT. There is, however, variation in the IC_{50} values for AZT of the mutants that were plaque purified from AZR-1. The IC_{50} values for AZT ranged from 27 to 97 μM. This is evidence that the AZR-1 population was composed of individual viruses with various degrees of resistance to AZT. Both of the AZT-resistant mutants that were plaque purified from AZR-13c (AZR-17c and AZR-18c) had IC_{50} values for AZT that were greater than 100 μM.

TABLE 2. Sensitivities of FIV Petaluma and AZT-resistant mutants derived from it to antiviral compounds

Compound	Mean IC_{50} (μM) ± SD[a]		
	FIV Petaluma	AZR-1	AZR-7
AZT	1.4 ± 0.4	>100	32 ± 8
AZdU	72 ± 6	>200	>200
PMEA	0.5 ± 0.03	0.6 ± 0.2	0.3 ± 0.1
ddI	2.1 ± 1	2.1 ± 1	1.8 ± 1
ddC	6.0 ± 1	5.0 ± 2	3.7 ± 2
d4T	12 ± 4	16 ± 4	11 ± 1
PFA	60 ± 4	62 ± 14	98 ± 24

[a] As determined by FIA. Each datum point represents the mean of two or more experiments, with four determinations per experiment.

In another attempt to minimize virus heterogeneity, we generated clones of infected cells that had multiplied from a single infected cell. Six clones (AZR-19c, AZR-20c, AZR-21c, AZR-22c, AZR-23c, and AZR-24c) were generated from cells infected with AZR-13c; the susceptibility of these clones to AZT is shown in Table 1. Variation in the IC_{50} values for AZT was seen among these six clones, indicating that cells infected with AZR-13c produced virus with different degrees of resistance to AZT.

Drug susceptibilities of AZR-7, AZR-17c, and AZR-19c were determined to confirm that the phenotype of the mutant had not changed during plaque purification or clonal isolation of infected cells. The sensitivities to AZT, AZdU, ddI, ddC, D4T, PMEA, and PFA of these plaque-purified mutants were compared with those of AZR-1, AZR-13c, and the wild-type parents FIV (Petaluma) and FIV 34TF10 (Tables 2 and 3). AZR-1 and AZR-13c were mutant populations which may have contained individuals that differed in their drug susceptibilities. From Tables 2 and 3 it can be seen that the phenotype of the plaque-purified mutants remained similar to that of the mutant populations. In all cases, the mutants maintained wild-type sensitivities to all drugs except for the azido compounds AZT and AZdU.

Reversion of plaque-purified mutants. The phenotypic reversions of the plaque-purified mutant AZR-17c and the clonal isolate of infected cells, AZR-19c, were compared with that of AZR-1. CrFK cells were infected with AZR-1, AZR-17c, or AZR-19c and maintained without AZT. When virus production was apparent, an IC_{50} for AZT was determined, and this virus was used to begin a second round of infection. This method was used to initiate three successive cycles of infection. The IC_{50} values for virus isolates from each of these three cycles of infection are shown in Fig. 1; the data demonstrate that these mutants revert to an AZT-sensitive phenotype as rapidly as AZR-1 and that within three rounds of infection, the AZT sensitivity is equivalent to that of the wild-type virus. Revertants from AZR-17c were plaque purified by the standard method and used for sequence analysis. AZR-17c-R8 is one plaque-purified revertant that is described in more detail below. The IC_{50} for AZT for AZR-17c-R8 was 0.81 μM.

TABLE 3. Sensitivities of FIV 34TF10 and AZT-resistant mutants derived from it to antiviral compounds

Compound	Mean IC_{50} (μM) ± SD[a]		
	FIV 34TF10	AZR-17c	AZR-19c
AZT	2.0 ± 0.9	>100	>100
AZdU	66 ± 31	>200	>200
PMEA	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
ddI	1.8 ± 0.6	1.7 ± 1.6	1.4 ± 0.2
ddC	4.7 ± 2.3	6.5 ± 2.1	3.6 ± 0.2
d4T	21 ± 9	17 ± 10	11 ± 3
PFA	105 ± 9	95 ± 27	141 ± 12

[a] As determined by FIA. Each datum point presents the mean from two or more experiments, with four determinations per experiment.

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                        202
     Lys Glu Lys Val Glu Glu Leu Arg Lys
2927 AAA GAA AAG GTA GAA GAA TTA AGA AAA  FIV 34TF10
     --- --- --- --- A-- --- --- --- ---  AZR-17c
     --- --- --- --- A-- --- --- --- ---  AZR-17c R7
     --- --- --- --- A-- --- --- --- ---  AZR-17C R8
                     Lys
```

FIG. 2. Nucleotide and deduced amino acid sequences of the region of the *pol* gene surrounding position 2939. The entire RT-encoding region (nucleotides 2241 to 4000) from the 34TF10 clone of FIV (27), AZR-17c, and the two plaque-purified revertants from AZR-17c, R7 and R8, were sequenced, and the only change from the 34TF10 sequence was found at position 2939. The nucleotides immediately surrounding this region are shown.

Nucleotide sequence analysis. In our initial attempts to sequence AZT-resistant FIV, we PCR amplified the RT-encoding region of AZR-1 from infected CrFK cell DNA and subsequently cloned it into M13. Fifteen clones were selected for sequencing. Comparison of these data revealed too much heterogeneity within the mutant population to determine a consensus sequence. Moreover, considerable variation was also observed with clones derived from wild-type FIV Petaluma. To circumvent this problem, we used plaque-purified mutants selected from a molecular clone for nucleotide sequence analyses. For these analyses, the region of the *pol* gene that encodes the RT and RNase H domains was PCR amplified from the 34TF10 clone of FIV, from AZR-17c, and from plaque-purified revertants of AZR-17c. These products were sequenced by the dideoxy method. FIV 34TF10 DNA, which had been subcloned into pUC119, was used as a sequencing control. Sequence data obtained from plasmid 34TF10 were identical to sequence data from infectious 34TF10 that had been derived from plasmid. The data that we obtained were identical to the published sequence (27) except at nucleotide 3742, where we determined an A rather than a T in all of our clones. Our sequence analysis revealed an A in this position for all wild-type and mutant FIV isolates sequenced (data not shown).

Sequence analysis of the entire RT-encoding region of AZR-17c revealed only a single mutation at position 2939 compared with the wild-type FIV 34TF10. Figure 2 shows the region of the FIV *pol* gene where this lesion occurs. The substitution of an A for a G at this position resulted in the replacement of Glu-202 with Lys and produced a negative-to-positive amino acid charge alteration. The entire RT-encoding regions of the plaque-purified revertants of AZR-17c, R7 and R8, were also sequenced. The G-to-A mutation at position 2939 remained, and there were no other changes (relative to FIV 34TF10 or AZR-17c) detected within the RT-encoding region of these revertants.

RT. RTs were purified from wild-type (34TF10), a plaque-purified mutant (AZR-17c), and a plaque-purified revertant (AZR-17c-R8) of FIV. Kinetic parameters for inhibition by N_3dTTP were compared, and the results are shown in Table 4. The three enzymes were similar in K_m for dTTP, but the enzymes from AZR-17c and AZR-17c-R8 displayed considerable resistance to N_3dTTP. The inhibition constants for AZR-

TABLE 4. Kinetic constants for wild-type (FIV 34TF10), mutant (AZR-17c), and revertant (AZR-17c-R8) RTs[a]

RT	Mean ± SEM ($n \geq 3$)	
	K_m (μM) for dTTP	K_i (nM) for N_3dTTP[a]
FIV 34TF10	5.0 ± 0.1	6.1 ± 0.2
AZR-17c	6.9 ± 0.7	27 ± 2.1
AZR-17c-R8	5.1 ± 0.1	22 ± 0.8

[a] The mode of inhibition of each enzyme by N_3dTTP was competitive.

17c and AZR-17c-R8 were both about four- to fivefold higher than the inhibition constant for RT from the wild-type FIV 34TF10. The enzymes from the mutant and revertant also had four- to five-times higher K_i values for inhibition by N_3dTTP in reactions with primed M13 DNA (data not shown). These data are in agreement with the DNA sequence analyses and suggest that the reversion may be due to suppression of the AZT resistance mutation by a second mutation that occurs outside the RT-encoding region.

DISCUSSION

We have observed the rapid phenotypic reversion of AZT-resistant mutants of FIV that were derived from either a swarm population or a molecular clone. Rapid reversion was seen even with a plaque-purified mutant, AZR-17c, that had been derived from the molecular clone. It is apparent that revertants arise rapidly during replication of these mutants and that in the absence of AZT, the revertants have a strong selective advantage over the drug-resistant mutants.

We have passaged cell-free virus in these reversion studies to ensure that drug-resistant provirus is not involved. It is important to note that the in vivo stability of AZT-resistant mutants is expected to be different from this, because provirus derived from AZT-resistant variants should persist in cells even in the absence of AZT. Our data do not address the effect of AZT removal upon generation of drug-resistant virus from already established provirus.

In contrast to this rapid reversion of AZT-resistant FIV, Gao et al. have reported the selection of AZT-resistant mutants of HIV-1 that do not rapidly revert if they are grown in the absence of drug (6). We are not sure of the reason for this apparent difference, although Gao et. al used a different protocol for selection of mutants. Their protocol involves sequential rounds of selection in the presence of increasing concentrations of AZT and results in mutants that have three to four lesions in the RT-encoding region. The mutant that we have sequenced, AZR-17c, has a single base change in the RT-encoding region, and this may be why it is more readily reverted.

This is the first report of AZT-resistant virus that was derived from a molecular clone, and it establishes that these mutants can arise and be selected in cell culture. In our previous selection of AZR-1 from FIV Petaluma, we could not preclude the possibility that we were merely selecting for mutants that already existed in the swarm population of FIV Petaluma. The ability to select mutants from a molecular clone, and plaque purify them, also enables more detailed sequence analysis and mapping of mutants. As noted above, we have seen considerable heterogeneity in attempts to sequence proviral DNA from AZR-1 or FIV Petaluma.

Sequence analysis revealed a single base change, G to A, at position 2939, within the RT-encoding region of AZR-17c relative to the wild-type molecular clone from which it was derived. This resulted in a Glu-202-to-Lys change in the protein. The negative-to-positive charge alteration at this position is expected to cause substantial change in protein conformation. Analysis of purified RT reveals that this change results in an RT that is resistant to N_3dTTP. The mutation is in a region of high (>70%) homology between FIV and HIV-1, and the site is comparable to Glu-203 in HIV-1 RT. It does not correspond to any of the lesions correlated with AZT resistance in HIV-1 (6, 8, 12, 14).

Recombinant HIV-1 RTs that contain the four mutations that are commonly seen in AZT-resistant clinical isolates of HIV-1 (D67N, K70R, T215Y, and K219Q) had K_i values for inhibition by N_3dTTP that were similar to or only slightly higher than the value for wild-type RT (9, 13). The levels of resistance varied, depending on whether the p66 homodimeric or p66/p51 heterodimeric form of the enzyme was examined. However, when recombinant HIV-1 RT that contained only the T215Y mutation was analyzed, its K_i for inhibition by N_3dTTP was significantly higher than that of wild-type RT. This is surprising since HIV-1 viral mutants that contain only one or two RT substitutions express lower levels of AZT resistance. These HIV-1 data suggest the cooperation of a viral component with the RT in AZT resistance.

Two revertants that were derived from AZR-17c maintained the change at 2939 which is present in the mutant, and we did not detect any other base change within the RT-encoding region of the revertants. Moreover, when RT was purified from mutant and revertant, both enzymes were resistant to the active form of AZT compared with wild-type RT. There are several mechanisms that could explain the phenomenon of AZT resistance and reversion that we have observed. Resistance in AZR-17c may be due to two mutations, one of which lies outside the RT. Phenotypic reversion is realized when the mutation outside the RT is lost. Alternatively, the 2939 mutation is directly involved in AZT resistance, and a suppressor mutation that maps outside the RT produces the AZT-sensitive pseudo-revertants. The enzyme data, however, strongly support the second hypothesis, that suppressor mutations in the revertants map outside the RT-encoding region. Consequently, all of our data point to the involvement of another region of the FIV genome in AZT resistance. Further studies of these mutants might provide information to help explain the enzymatic basis for AZT resistance. Experiments are under way to further evaluate the role of the change at position 2939 in AZT resistance as well as to map areas outside the RT that may be involved in suppression of AZT resistance.

The tools that we have developed for plaque purification and selection of mutants from clones will enable detailed analysis of a set of independent AZT-resistant mutants of FIV. We are also applying these approaches to analysis of FIV mutants resistant to other antiviral compounds.

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