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Multiple-Drug-Resistant Mutants of Feline Immunodeficiency Virus Selected with 2′,3′-Dideoxyinosine Alone and in Combination with 3′-Azido-3′-Deoxythymidine

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Mutants of feline immunodeficiency virus (FIV) were selected in cell culture in the continuous presence of 10 μM (each) 3′-azido-3′-deoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI). These mutants (AIR-1 and AIR-3) displayed a 13-fold resistance to AZT but had less than a 2-fold decrease in susceptibility to ddI. Interestingly, the AIR mutants were cross-resistant to phosphonoformate (PFA) and were hypersensitive to 2′,3′-dideoxycytidine (ddC). Mutants of FIV were also selected in the presence of 10 μM ddI alone (DIS-1, DIS-2c), and these displayed a two- to fourfold decrease in susceptibility to ddI. Like the mutants selected with the combination of AZT plus ddI, DIS-1 and DIS-2c were cross-resistant to PFA and were hypersensitive to ddC. However, they remained as susceptible as wild-type FIV to AZT. Thus, the mutants selected with the combination of AZT plus ddI have phenotypes which reflect those obtained by selection with these drugs individually.

In order to study viral resistance to drugs used for therapy of AIDS, we have developed model systems using feline immunodeficiency virus (FIV). FIV is a lentivirus that causes a natural AIDS-like disease in domestic cats (8, 12–14) that can also be induced experimentally in specific-pathogen-free cats (1, 2, 19, 20). FIV and human immunodeficiency virus type 1 (HIV-1) are similar in morphology, protein composition, and genome arrangement. Of particular importance to chemotherapeutic studies is the fact that the reverse transcriptase (RT) of FIV is similar to that of HIV-1 in its physical properties, catalytic activities, and susceptibilities to several RT inhibitors (4, 9–11). Moreover, FIV is susceptible to all of the RT-targeted antiviral agents approved for use in the treatment of AIDS (15). FIV is also susceptible to phosphonoformate (PFA) but is not susceptible to other nonnucleoside inhibitors of HIV-1 such as the tetrahydroimidazo[4,5-1-j,k][1,4-benzodiazepin-2(1H)-one and -thione (TIBO) compounds or nevaripine (21).

We previously reported the selection of 3′-azido-3′-deoxythymidine (AZT)-resistant mutants of FIV in a cell culture system (15). One of the mutants has been characterized, and the similarities to AZT-resistant clinical isolates of HIV-1 are striking. Like most AZT-resistant HIV-1 clinical isolates, our FIV mutant was resistant to AZT, 3′-azido-2′,3′-dideoxyuridine, and 3′-azido-2′,3′-dideoxyguanosine, but remained susceptible to 2′,3′-dideoxyinosine (ddI), 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T), PFA (15), and 2′,3′-dideoxycytidine (ddC) (16). Here we report the use of the FIV system to select and characterize mutants that arise in cell culture in response to ddI alone or to the combination of AZT and ddI.

The following antiviral compounds were used in the studies. PFA and ddC were purchased from Sigma Chemical Co., St. Louis, Mo. AZT was provided by Phillip A. Furman of Burroughs Wellcome Co., Research Triangle Park, N.C. D4T and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) were provided by H.-T. Ho of Bristol Myers-Squibb Co., Wallingford, Conn. ddI was provided by the Developmental Therapeutics Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases.

The Petaluma strain of FIV (14, 20) and virus derived from a molecular clone of FIV, 34TF10 (18), were used for the selection of mutants. Virus was grown and maintained in Crandell feline kidney (CrFK) cells as described previously (11). Mutants of FIV were selected by infecting approximately 40,000 to 60,000 CrFK cells in 25-cm^2 tissue culture flasks with a cell-free culture supernatant containing 2,000 to 4,000 focus-forming units (FFU) of FIV. Before infection, the cells were incubated with either 10 μM ddI or the combination of 10 μM ddI plus 10 μM AZT for 1 h to allow the cells to convert the drugs to their active forms. All selections of virus mutants were performed in the continuous presence of drug(s). The culture medium was replaced with fresh medium and appropriate concentrations of drug(s) every 48 h. After the initial infection, cultures were monitored weekly for the presence of cell-free virus. After virus was detected, cell-free supernatants from the initial cultures were used to initiate a second round of selection by infection of uninfected CrFK cells, again in the continuous presence of the appropriate concentrations of drug(s). This second passage was done at a lower input multiplicity (500 FFU) to try to eliminate mixed populations of virus. The ddI-selected mutants underwent a third passage of selection by using cell-free culture supernatants from the second passage to infect at an even lower input multiplicity (100 FFU) uninfected CrFK cells. Virus was detected and assayed for infectivity in the presence or absence of various inhibitors by using a focal infectivity assay described previously (15). The concentrations required to inhibit focus formation by 50% (IC_{50}s) were determined.

Mutants of FIV selected with ddI. Several isolates were selected from wild-type FIV Petaluma by passage in the presence of 10 μM ddI. Two isolates were also selected in the presence of 50 μM ddI (data not shown). None of these variants displayed more than a two- to fivefold decrease in susceptibility to ddI relative to that of the wild-type FIV Petaluma strain (for example, see DIS-1 in Table 1). Upon

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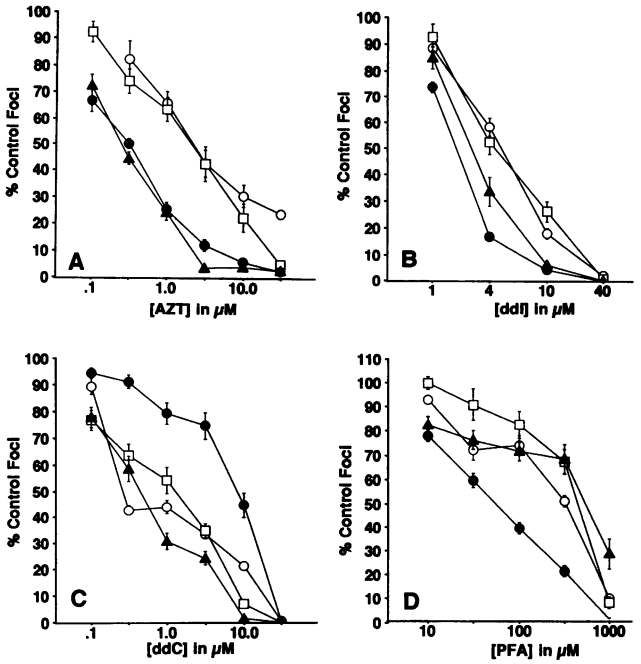

FIG. 1. Inhibition of wild-type FIV Petaluma and mutants derived from FIV Petaluma by AZT (A), ddI (B), ddC (C), or PFA (D). Mutants were selected in the presence of the combination of 10 μM AZT with 10 μM ddI, designated AIR-1 (○), or 10 μM ddI alone, designated DIS-1 (▲). Also shown are a plaque-purified clone obtained from AIR-1 in the presence of both 10 μM AZT plus 10 μM ddI, designated AIR-3 (□), and wild-type FIV Petaluma (●). Each datum point represents the results of two or more experiments with four determinations per experiment. Error bars represent the standard error of the mean and are omitted where the error was too small to be shown accurately.

subsequent passage of these mutants in the continuous presence of ddI, the IC_{50} of ddI often decreased, sometimes nearly to the wild-type level (data not shown). In order to minimize the inherent heterogeneity in the mutant populations selected from the wild-type Petaluma strain, we attempted to select ddI-resistant mutants from a more homogeneous parent stock. We infected CrFK cells with virus produced from infection of CrFK cells with a molecular clone of FIV Petaluma, FIV 34TF10 (18). For the initial round of selection, a cell-free culture supernatant that contained approximately 500 FFU of FIV 34TF10 was used to infect each of two 25-cm^2 flasks of CrFK cells that had been preincubated with 10 μM ddI. These infected cells were maintained in the presence of 10 μM ddI and were assayed weekly by a focal infectivity assay until virus production was detected. Virus was detectable by 3 weeks, and a second round of infection in the presence of 10 μM ddI was initiated with cell-free supernatant containing 50 FFU of virus from the initial infection. Virus from both rounds of selection were analyzed for their resistance to ddI. Again, we found a two- to fivefold increase in IC_{50} compared with that for the parent, FIV 34TF10.

Because of the apparent instability of the ddI-selected variants and the relatively low level of resistance found even when the virus was passaged in 50 μM ddI, we chose to investigate the combination of ddI and AZT.

Mutants of FIV selected with the combination of AZT and ddI. A mutant of FIV was selected with a combination of 10 μM ddI and 10 μM AZT by passage of wild-type FIV Petaluma in the continuous presence of the drug combination. This mutant, designated AIR-1, was resistant to AZT (Fig. 1A) and had a slight decrease in susceptibility to ddI (Fig. 1B). AIR-1 was cross-resistant to PFA (Fig. 1D) and was hypersensitive to ddC (Fig. 1C) but retained wild-type susceptibilities to both D4T and PMEA (Table 1). We passaged the cell-free AIR-1 virus population through another round of selection in the presence of ddI and AZT in order to determine whether these phenotypes would persist. The mutant obtained from this second round of selection, designated AIR-2, was phenotypically identical to AIR-1 (data not shown).

In order to determine whether the multiple phenotypes exist in individual virus particles or are due to a mixed population, we plaque purified an isolate from the AIR-1 population. Plaque purification was done as reported previously (16) in the continuous presence of 10 μM ddI and 10 μM AZT. AIR-3 is a single plaque isolate obtained from the AIR-1 population by this method. As shown in Fig. 1 and Table 1, AIR-3 retained the multiple phenotypes of the parental mutant AIR-1.

In the first round of selection with ddI and AZT, mutants emerged relatively slowly; virus was not detectable until 6 weeks after the initial infection. This is similar to the length of time required for the emergence of mutants when selection is done with AZT alone. In the second round of selection, mutants were detected within 3 weeks. In contrast, the ddI-selected virus emerged rapidly during each round of selection with ddI. Within 2 weeks of the initial infection of the first round, cell-free virus was detectable.

TABLE 1. Susceptibilities of FIV strains and drug-resistant variants to antiviral compounds as determined by FIA

Virus	Parent	IC_{50} (μM)[a]					
		AZT[b]	ddI	ddC	PFA	PMEA	D4T
FIV Petaluma		0.32 ± 0.01	1.9 ± 0.04	5.7 ± 0.4	64.7 ± 7.1	0.2 ± 0.02	5.7 ± 0.6
AIR-1	FIV Petaluma	4.2 ± 0.3	4.2 ± 0.3	0.8 ± 0.01	>400	0.2 ± 0.03	6.8 ± 0.7
AIR-3	AIR-1	2.1 ± 0.1	4.7 ± 0.3	0.9 ± 0.01	>400	0.12 ± 0.02	5.3 ± 1.0
DIS-1	FIV Petaluma	0.33 ± 0.01	2.9 ± 0.2	0.5 ± 0.01	>400	0.14 ± 0.02	5.5 ± 0.8
FIV 34TF10		0.13 ± 0.01	1.1 ± 0.01	2.3 ± 0.05	67.3 ± 11.2	0.16 ± 0.01	5.9 ± 0.9
DIS-2c	FIV 34TF10	0.11 ± 0.01	2.7 ± 0.1	0.4 ± 0.02	>400	0.2 ± 0.02	5.7 ± 0.8

[a] The values presented are from two or more experiments with four determinations per experiment and are reported as IC_{50}s ± the standard error of the mean. These values were determined by regression analysis of the linear part of the dose-response curves by the StatView data analysis program of Abacus Concepts.

[b] The IC_{50}s of AZT for FIV Petaluma and FIV 34TF10 reported here are lower than the values previously reported from our laboratory (15). We believe that this is due to variations in fetal bovine serum, because the absolute IC_{50}s vary among different lots of serum, but the relative IC_{50}s for mutants to those for the wild type do not change.

Multiple phenotypes associate with ddI selection. The AZT-resistant mutants that we characterized previously were not cross-resistant to PFA (15) or hypersensitive to ddC (16). In order to determine whether these phenotypes arose from ddI selection or were unique to the combination chemotherapy, we characterized the initial ddI-selected variants. DIS-1 and DIS-2c were both cross-resistant to PFA and were hypersensitive to ddC. As expected, they were indistinguishable from wild-type FIV in their susceptibilities to AZT, D4T, and PMEA. Results of the phenotypic analyses are summarized in Table 1.

The emergence of multiple-drug-resistant mutants of FIV reported here may have important implications for attempts to combat the emergence of drug resistance with combination chemotherapy. The combination of AZT with ddI is in clinical trials in human AIDS patients. Our data demonstrate that FIV mutants resistant to this combination can be readily selected in vitro. Given the high mutation rates of FIV and HIV-1, it is likely that mutants resistant to this combination will also arise in vivo, although there are other factors that may affect the types of mutants that do arise. For example, we cannot predict whether the mutations responsible for multiple drug resistance will alter either infectivity or pathogenicity when introduced into otherwise pathogenic strains of HIV-1 or FIV. The ability to follow the in vitro studies with in vivo pathogenesis studies in specific-pathogen-free cats (3) makes the FIV system particularly attractive for further studies of drug-resistant mutants that arise in response to the combination of AZT with ddI.

The mutants of FIV that were selected with the combination of AZT and ddI are phenotypically equivalent to that predicted from the sum of the phenotypes of mutants selected individually with each drug. This is not expected to be the case with all combinations of mutations, because some pairs of mutations may render the virus nonviable. This does not mean that mutants resistant to such combinations will not arise. It only ensures that any mutants that arise will contain mutations different from those selected with the single drug.

The mutants that we selected with ddI alone were different from the ddI-resistant mutants of HIV-1 that have been reported. They were also selected differently than those reported previously. For example, the ddI-resistant mutants of HIV-1 that were selected in vitro by Gao et al. (6, 7) were selected by a stepwise process. The drug concentration was initially below the minimum effective dose and was increased stepwise upon subsequent passage of the virus (7). Similarly, the ddC-resistant mutants that displayed cross-resistant to ddI were selected in this stepwise manner (6). In contrast, our mutants were selected in the continuous presence of high concentrations of drugs. The ddI-resistant variants of HIV-1 reported by St. Clair et al. (17) were obtained from AIDS patients who were initially receiving AZT therapy. The patients began to decline clinically, were removed from AZT therapy, and were placed on ddI therapy. It was subsequently found that AZT-resistant HIV-1 had emerged during AZT therapy. After the switch to ddI therapy, the level of AZT resistance diminished and ddI-resistant HIV-1 emerged. The ddI-resistant HIV-1 isolates obtained from these patients were cross-resistant to ddC (17). We are not sure whether the phenotypic differences between the ddI-resistant FIV and HIV-1 isolates are due to different properties of these two viruses or to the differences in selection procedures. We are using the FIV model systems to isolate and evaluate mutants arising from these other selection protocols.

The FIV mutants that we selected with AZT and ddI (AIR-1 and AIR-3) represent the first viral mutants selected in cell culture with this combination. Eron et al. (5) recently reported HIV-1 mutants that are resistant to both AZT and ddI. However, these mutants were constructed by site-directed mutagenesis with combinations of specific mutations known to individually confer AZT and ddI resistance.

We have previously reported the in vitro selection of AZT-resistant mutants of FIV that phenotypically mimic the clinical AZT-resistant variants of HIV-1 (15). Although the ddI-selected mutants that we reported here are somewhat different phenotypically from the ddI-resistant mutants of HIV-1 that have been reported, they are similar in their low-level resistance to ddI. We believe that the in vitro systems that we developed for FIV will continue to provide important information about resistance to AIDS chemotherapy. FIV has the further advantage of having an in vivo component in which drug-resistant mutants of FIV can be used for important studies of pathogenesis (3) that are not possible with HIV-1.

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