Mutants of Feline Immunodeficiency Virus Resistant to 3'-Azido-3'-Deoxythymidine

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We selected 3'-azido-3'-deoxythymidine (AZT)-resistant mutants of feline immunodeficiency virus (FIV) in a cat cell culture system. The characterization of one of these mutants was facilitated by the development of a focal immunoassay which could accurately measure FIV infectivity. This assay was used to quantify the susceptibility of FIV to various inhibitors. The AZT-resistant mutant was found to be cross-resistant to 3'-azido-2',3'-dideoxyuridine and 3'-azido-2',3'-dideoxyguanosine but remained sensitive to several other inhibitors (2',3'-dideoxyinosine, 2',3'-dideoxy-2',3'-didehydrothymidine, and phosphonoformate). These patterns of cross-resistance and sensitivity were similar to those of the AZT-resistant human immunodeficiency virus (HIV) that has recently been isolated from patients with AIDS (B. A. Larder and S. D. Kemp, Science 246:1155-1158, 1989). Like the AZT-resistant HIV, purified reverse transcriptase from mutant FIV failed to show resistance to the 5'-triphosphate of AZT. This mutant can be used in the FIV model system to study the mechanisms of drug resistance and to determine the pathogenicity of AZT-resistant mutants.

3'-Azido-3'-deoxythymidine (AZT) has demonstrated antiviral activity against human immunodeficiency virus type 1 (HIV-1) in vitro (9) and has been shown to improve the clinical status of patients with AIDS and AIDS-related complex (2). The use of AZT for the treatment of these patients is prevalent, but it has recently been shown that some isolates of HIV from patients who had been receiving AZT for 6 months or longer were less sensitive to the drug (5, 6). The emergence of these AZT-resistant strains of HIV has complicated considerations for use of antiviral agents in the treatment of AIDS.

In order to understand the mechanisms of AZT resistance, it would be useful to be able to generate drug-resistant mutants in the laboratory. Larder and his colleagues have constructed reverse transcriptase (RT) mutants of HIV by site-directed mutagenesis, and some of these are resistant to AZT (7, 8). However, these mutations do not necessarily mimic the ones that occur naturally. Attempts to select an AZT-resistant strain of HIV in cell culture have been unsuccessful (15).

We have used feline immunodeficiency virus (FIV) in attempts to study AZT resistance in a cell culture system. FIV is a lentivirus that causes an immune deficiency in cats that is very similar to AIDS in humans (13, 16). In addition, the RT of FIV is very similar to that of HIV in its physical and catalytic properties (11), as well as in its sensitivities to various inhibitors (10, 12). This virus is a potential model for both in vitro and in vivo studies of AIDS chemotherapy.

Using a naturally occurring FIV isolated from a cat never exposed to AZT, we have been able to isolate FIV mutants, in a tissue culture system, that are able to grow in the presence of high concentrations (10 to 50 μM) of AZT. In this article we describe the isolation and characterization of one of these AZT-resistant FIV mutants. We have also developed a focal immunoassay (FIA) to facilitate determination of the drug susceptibilities of these FIV isolates. The AZT-resistant mutant of FIV is very similar to the AZT-resistant HIV from clinical isolates in its sensitivities to several antiviral compounds.

MATERIALS AND METHODS

Chemicals. Phosphonoformate (PFA) was purchased from Sigma Chemical Co., St. Louis, Mo. AZT, the 5'-triphosphate of AZT (N3dTPP), and 3'-azido-2',3'-dideoxyguanosine (AZG) were provided by Wayne Miller, Philip A. Furman, and M. Nixon Ellis, respectively, all of Burroughs Wellcome Co., Research Triangle Park, N.C. 2',3'-Dideoxy-2',3'-didehydrothymidine (D4T) was provided by H.-T. Ho of Bristol Myers-Squibb Co., Wallingford, Conn. 2',3'-Dideoxyinosine (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 of Triton Biosciences, Inc., Alameda, Calif. Poly(rA)-oligo(dT)₅₀ was purchased from Pharmacia LKB, Piscataway, N.J.

Isotopes. [methyl-3H]dTTP was obtained from DuPont-New England Nuclear, Boston, Mass.

Cells and virus. The Petaluma strain of FIV (14, 16) was used for these studies. Wild-type and mutant strains of FIV were grown and maintained in Crandell feline kidney (CrFK) cells, and stocks of FIV were prepared as described previously (12, 16). Briefly, virus stocks were prepared from the culture medium of CrFK cells that had been infected with FIV and contained extracellular virus. Cells were removed from the medium by centrifugation, and the supernatant was stored frozen at -80°C, with dimethyl sulfoxide added to a concentration of 10%. These stocks were thawed and used to infect CrFK cells for subsequent studies. AZT-resistant FIV was maintained in medium that contained AZT, and the medium was replaced with fresh medium containing the appropriate concentration of AZT every 2 days.

Enzymes. RT was purified from virions of wild-type and
mutant FIV by the method that was reported recently (11). Briefly, the enzyme was purified from virions that had been disrupted with Triton X-100 by chromatography on DEAE-cellulose and phosphocellulose.

**Enzyme assays.** RT was assayed as reported previously (11, 12). One unit of RT is defined as the amount of enzyme required to catalyze the incorporation of 1 nmol of dTMP per h into poly(rA)-oligo(dT)12 at 37°C. Double-reciprocal plots were used to determine kinetic constants (K_m and V_max) by the method described previously (10).

**FIA.** FIV infectivity in the presence or absence of various inhibitors was determined by an FIA that was based on the FIA for HIV developed by Chesebro and Wehrly (1). CrFK cells were seeded into 24-well microtiter plates at a density of 1.5 × 10^4 cells per well and incubated for 1 h at 37°C in growth medium or growth medium containing a drug. This incubation was to allow the cells to convert drugs to their active forms. These cells were then infected with 20 to 40 focus-forming units of wild-type or mutant FIV per well and incubated at 37°C. After 4 days, the cells were fixed for 5 min with methanol and washed twice with TNE (0.01 M Tris hydrochloride [pH 7.5] containing 0.15 M NaCl and 0.002 M EDTA). Cells were then washed once with TNE that contained 10% fetal bovine serum. Immunostaining was performed by incubating the cells for 30 min with 0.15 ml of a 1/300 dilution of polyclonal antiserum that had been obtained from specific-pathogen-free cats that had been infected with FIV. Cells were washed twice with TNE that contained 10% fetal bovine serum to remove excess antiserum and were then incubated for 40 min with 0.15 ml of a 1/600 dilution of horseradish peroxidase-conjugated anti-cat immunoglobulin (Organon-Teknika). Cells were washed again with TNE, and foci of infected cells were visualized by reacting the antibody-bound monolayers for 20 min in the dark with a solution of aminoethyl carbazole (1 part per 19 parts buffer) and H_2O_2 (1 μl per 2 ml of buffer) in 0.05 M sodium acetate buffer, pH 5. Foci appeared as groups of red cells against an unstained background and were counted under a dissecting microscope at 10 to 40 × magnification. For the purpose of these drug susceptibility experiments, a group of four or more infected cells was defined as a focus of infection. The number of infected cells per focus did not vary as a function of increasing drug concentration. Secondary foci were not observed with increased incubation, for times up to 6 days. Light was reflected off a piece of ground glass to reduce the refractility of the uninfected cells. Data were plotted as percentage of control plaques (no drug) versus inhibitor concentration. Concentrations required to inhibit focus formation by 50% (IC_{50} values) were obtained directly from these plots.

**RESULTS**

**Selection of AZT-resistant mutants.** AZT-resistant mutants of FIV were selected by infection of CrFK cells with FIV in the presence of inhibitory concentrations of AZT. For the initial round of selection, cells were infected at a relatively high input multiplicity, although we had no quantitative assay to determine the exact multiplicity of infection. Undiluted virus stock (2 ml) that had been obtained from culture supernatant fluid was used to infect a 25-cm^2 flask of CrFK cells. From the onset of infection, cultures were maintained in 10 or 50 μM AZT. We had previously shown that at concentrations of 10 μM or greater, FIV replication is completely suppressed, as determined by measurement of virion-associated RT. These cultures were monitored weekly for RT activity. After 5 weeks in culture, RT activity was detectable but still significantly lower than in cultures that had been grown in the absence of AZT.

In the second round of infection, a lower input multiplicity was used in an attempt to eliminate mixed populations of virus that might have been present. Undiluted culture supernatant (1 ml) from CrFK cells infected with FIV in the first round in the presence of AZT was used to infect a new 25-cm^2 flask of CrFK cells. The amount of culture supernatant that was used for infection in the second round of selection was half that used in the initial selection, but from RT assays, we estimated the multiplicity to be about 20-fold lower than that of the first inoculum. These cultures were maintained in 10 or 50 μM AZT and were monitored weekly for RT activity. Ten weeks of culture were required before RT activity was detectable; at this time, the FIV was used to initiate a third round of infection. An even lower input multiplicity was used in an effort to select a single population of virus. For this round of infection, 0.2 ml of undiluted culture supernatant from the CrFK cells that had been infected with FIV in the presence of AZT during the second round of selection was used to infect a 25-cm^2 flask. From RT assays, the multiplicity was estimated to be about 250-fold lower than that used in the initial selection. Cultures were maintained in 10 or 50 μM AZT and were assayed weekly for RT activity. After 15 weeks, RT activity had risen to levels that were above background, and a fourth round of infection was initiated. This cycle of infection was initiated at a higher input multiplicity in attempt to increase the amount of AZT-resistant virus that had been selected in previous rounds. Undiluted culture supernatant (4 ml) from the CrFK cells that had been infected the presence of AZT during the third round of selection was used to infect a 25-cm^2 flask. An estimate based on RT assay results indicated that the multiplicity was about 30-fold lower than that used in the initial selection. Detectable RT activity was observed after 6 weeks.

With this method, six potentially unique AZT-resistant mutants were selected, three in 10 μM AZT and three in 50 μM AZT. One mutant selected with 10 μM AZT was chosen for the initial characterization studies that are described in this article.

**FIA.** During the course of mutant selection, it became apparent that RT assays would not be adequate to determine the sensitivities of mutant FIV to a variety of antiviral compounds. In fact, the detection of mutant virus during the selection process may have been delayed because of the inability of the RT assay to detect low levels of infection (see Discussion). For these reasons, a focal infectivity assay was developed. This assay is a modification of the FIA developed by Chesebro and Wehrly to quantitate HIV (1).

For this assay, CrFK cells were infected with FIV, and foci of infected cells were detected by using FIV-specific antiserum and peroxidase-conjugated goat anti-cat immunoglobulin. These foci were visualized as areas of red cells against a background of unstained cells (Fig. 1). The assay responded to a dilution titration of FIV in a linear fashion (Fig. 2), suggesting that each focus represented infection by a single virion. The sensitivity of the assay was further demonstrated by the addition of various amounts of infected CrFK cells to a background of 1.5 × 10^4 uninfected target cells per 2-cm^2 well (Fig. 3). As few as two FIV-infected cells could be detected by this immunoassay, indicating that an excess of uninfected cells did not interfere with the ability of the assay to recognize infected cells.

**Sensitivities of mutant and wild-type FIV to antiviral com-
pounds. The AZT resistance of the mutant FIV was confirmed with the FIA by determining the number of foci at various concentrations of AZT (Fig. 4a). The dose-response curves shown in Fig. 4a verified that mutant FIV was considerably less sensitive to inhibition by AZT than the original FIV. The IC₅₀ of AZT for the mutant FIV was greater than 50 μM, compared with 1.4 μM for the wild-type FIV (Table 1). These data confirmed that the mutant FIV was at least 40-fold more resistant to AZT than the wild-type virus.

The susceptibility of the mutant FIV to a variety of other antiviral compounds was determined by the FIA and compared with the susceptibilities of the wild-type virus (Fig. 4).

These data showed that the AZT-resistant FIV was also resistant to AZdU and to a lesser extent to AZG. The mutant FIV was similar to wild-type FIV in sensitivity to D4T, ddI and PFA. Of the compounds tested, the mutant exhibited resistance only to nucleoside analogs that contained a 3′ azido group. The IC₅₀s obtained for inhibition of wild-type and AZT-resistant FIV are summarized in Table 1.

**FIG. 1.** Focus of FIV-infected CrFK cells detected with polyclonal antiserum to FIV and peroxidase-conjugated anti-cat immunoglobulin. Magnification, ×40.

**FIG. 2.** FIA with various concentrations of FIV. CrFK cells were infected with FIV by adding 50 μl of various dilutions of an FIV stock to the culture medium. Cells were fixed with methanol after 4 days, and foci of infection were detected by using antiserum from FIV-infected specific-pathogen-free cats and peroxidase-conjugated anti-cat immunoglobulin. Each point represents the mean of four determinations. Bars represent the standard error and are omitted when the standard error was too small to show accurately with error bars.

**FIG. 3.** Detection of FIV-infected CrFK by FIA. Various numbers of FIV-infected CrFK cells were added to 1.5 × 10⁶ uninfected CrFK cells. After 4 days, the cells were fixed with methanol, and infectious foci were detected by reacting the cells with antiserum from FIV-infected cats and peroxidase-conjugated anti-cat immunoglobulin. Each point represents the mean of four determinations. Bars represent the standard error and are omitted when the standard error was too small to show accurately with error bars.

**DISCUSSION**

We have developed an FIA that will reliably detect even low levels of infectious FIV, and this has enabled characterization of AZT-resistant mutants of FIV that were isolated in a cell culture system. The FIA is very similar to that developed by Chesbro and Wehrly for the detection of HIV (1). The use of a continuous cell line that is susceptible to FIV infection (Crandell feline kidney cells) eliminated the difficulties that were encountered in the HIV system, in which it was necessary to express the CD4 receptor on HeLa cells. This assay has many advantages over the conventional RT assay that is typically used to detect FIV. Infectious virions can be detected within 4 days of infection with the FIA, whereas the RT assay requires 2 to 4 weeks of culture before detectable levels are present. With this immunoassay, a single infectious virion can be detected in a small amount of virus culture supernatant, while the RT assay requires a much higher virus titer before RT activity can be detected. The FIA lends itself well to the assay of large numbers of samples, making it a valuable method for screening FIV for drug susceptibilities or for evaluating FIV infections in other tissue types (by cocultivation). It also lends itself to the detection of drug resistance in FIV.
AZT that are normally inhibitory. Attempts to confirm that these mutants were AZT resistant by measuring virion-associated RT yielded ambiguous results. However, results with the FIA confirmed that these were, in fact, mutant FIV. We initiated the selection process for AZT-resistant mutants before the FIA had been developed, and the initial data we obtained were based on RT assays. Therefore, it was not possible to know whether the four cycles of replication were actually required to obtain AZT-resistant populations of FIV or whether fewer replication cycles would have been sufficient. The FIA will facilitate monitoring of resistant populations of virus in future selection protocols.

The Petaluma strain of FIV that was used in the mutant selection process was obtained from a clinical isolate. Given the high mutation rate of these viruses, it certainly represents a mixed population, i.e., a viral "swarm." This closely mimics the in vivo situation, in which the prolonged presence of the drug selects for resistant members of a dynamic population. While drug-resistant virus may have been present in the viral swarm, the fact that many weeks were required before it was present in readily detectable amounts indicated that it did not represent a large proportion of the population. The presence of naturally resistant FIV in the wild-type population was suggested by the wild-type AZT and AZdU dose-response curves. With these drugs, infectious foci were not completely eliminated, even at very high drug concentrations (Fig. 4a and b). Variability in the wild-type parent sequence presents difficulties in characterizing the mutations that are responsible for AZT resistance. To address this problem, we have begun the selection process with FIV derived from infectious cloned DNA.

During this selection process, we isolated six AZT-resistant mutants of FIV. These mutants were not purified to individual clones and therefore may represent mixed populations of virus. We observed, however, that they replicated at different rates, and therefore it is possible that they represent six unique mutants. One mutant was chosen for further characterization. This mutant was cross-resistant to AZdU and AZG but remained sensitive to several other drugs (ddI, D4T, and PFA), suggesting that resistance was related to the presence of the 3'-azido group on the nucleoside. These patterns of resistance and cross-resistance were very similar to those of the AZT-resistant clinical isolates of HIV that were recently reported (4, 6).

Although the FIV mutant was resistant to AZT in culture, its purified RT was found to be sensitive to N3dTTP. Similar results were found when RT was purified from the AZT-resistant isolates of HIV (6). These results, which appear contradictory, may indicate that factors that are associated with RT in the intact virion, or components of the host cell, are necessary for resistance to be expressed.

This is the first report of the selection of an AZT-resistant lentivirus in which the first exposure to AZT occurred in a cell culture system. Attempts to select an AZT-resistant mutant of HIV in cell culture may have been thwarted by difficulties resulting from the cytopathic nature of HIV (15). Our success in selecting these FIV mutants was undoubtedly

### TABLE 2. Comparison of kinetic constants for RT from wild-type and AZT-resistant FIV

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<tr>
<th></th>
<th>$K_m$ (µM)</th>
<th>$K_i$ (µM)</th>
<th>$K/K_m$</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>4.4 ± 1.1</td>
<td>7.7 ± 4.1</td>
<td>0.0018 ± 0.009</td>
</tr>
<tr>
<td>Mutant</td>
<td>5.2 ± 0.9</td>
<td>8.1 ± 3.4</td>
<td>0.0015 ± 0.007</td>
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</table>

* Values are means ± standard deviation from four determinations.

### TABLE 1. Sensitivities of wild-type and mutant FIV to antiviral compounds as determined by FIA

<table>
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<tr>
<th>Compound</th>
<th>Mean IC$_{50}$ (µM) ± SD</th>
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<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>AZT</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>AZdU</td>
<td>71.6 ± 6.3</td>
</tr>
<tr>
<td>AZG</td>
<td>6.3 ± 1.4</td>
</tr>
<tr>
<td>ddI</td>
<td>2.1 ± 0.7</td>
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<tr>
<td>D4T</td>
<td>12.3 ± 3.5</td>
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<tr>
<td>PFA</td>
<td>60.1 ± 4.2</td>
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* Values are from two or more experiments, with four determinations per experiment.
influenced by the chronic type of infection produced by FIV, compared with the lytic infection seen with HIV. The only HIV mutants made in vitro were obtained by site-specific mutagenesis. Because the FIV mutants obtained in cell culture arise in response to selective pressure, they are more likely to mimic the HIV mutants that arise clinically.

The similarities we have observed between the FIV mutant that we have characterized and the AZT-resistant HIV from clinical isolates are striking. This mutant may be useful for further characterization of mechanisms of resistance to AZT and effects of mutations on the pathogenicity of the virus. FIV has been shown to be a promising model for AIDS chemotherapy (3, 10–12), and the pathogenesis of FIV has been well characterized (13, 14, 16). Experiments to determine whether AZT resistance alters the pathogenesis of FIV are under way.

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