Selection and characterization of a 2',3'-dideoxycytidine-resistant mutant of feline immunodeficiency virus

Holly Kay Medlin

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SELECTION AND CHARACTERIZATION OF A
2',3'-DIDEOXYCYTIDINE-RESISTANT MUTANT
OF FELINE IMMUNODEFICIENCY VIRUS

by

Holly Kay Medlin
B.S., University of California-Berkeley, 1991

Presented in partial fulfillment of the requirements
for the degree of
Master of Science
The University of Montana
January, 1995

Approved by

[Signature]
Chairman, Board of Examiners

[Signature]
Dean, Graduate School

May 4, 1995
Date
A mutant of feline immunodeficiency virus (FIV), designated DCR-2c, was selected in cell culture in the continuous presence of 25 μM 2',3'-dideoxycytidine (ddC). This mutant displayed a 5-fold resistance to ddC, a 3-fold resistance to 2',3'-dideoxyinosine (ddl), a 4-fold resistance to phosphonoformic acid (PFA), slight resistance to 2',3'-dideoxy-3'-thiacytidine (3TC) and wild-type susceptibility to 3'-azido-3'-dideoxythymidine (AZT) and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) compared to the wild-type molecular clone of FIV, 34TF10. This mutant, designated DCR-5c, was plaque-purified by a modification of the procedure developed in this lab. This plaque-purified mutant retained the phenotype of the mutant population, DCR-2c. DCR-5c is similar to ddC-resistant human immunodeficiency virus type 1 (HIV-1) in ddl cross-resistance. It also has an RT resistant to ddCTP, like the HIV-1 mutant. The RT purified from DCR-5c demonstrated a 10-fold resistance to ddCTP compared to wild-type RT. In addition, the sequence of the RT-encoding region of DCR-5c was elucidated and compared to FIV 34TF10. A single point mutation was found in the ddC-resistant mutant that resulted in the amino acid substitution of His for Asp-3. No drug resistant mutation has previously been described in this area in either FIV or HIV-1. This mutation is in a region consistent with altered binding of template-primer contributing to resistance.
ACKNOWLEDGMENTS

I thank Dr. Tom North, my mentor, for teaching me that science is much more than learning facts; it is expanding one’s search into what is not known. I thank Drs. Michael Minnick and George Card for their advice and assistance in both academics and research. Without Dr. Kathy Remington and Rachel LaCasse to answer my endless questions, I could not have accomplished all that I set out to do. I thank Judy Gobert for training me in the lab and for helping me remember all the things in life that are important. YaQi Zhu is irreplaceable for her reverse transcriptase expertise and her humor. Joan Strange is a miracle-worker to me for all the assistance she provided during sequencing of my mutant. I thank Doug McBroom, my deskmate, for his comraderie during the writing of our theses. I must also thank the rest of the North lab who may think I’m crazy, but have been true friends throughout my stay. Lastly, I dedicate this to Alain Paquette and my family for their love, support and encouragement.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ABSTRACT</strong></td>
<td>ii</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGMENTS</strong></td>
<td>iii</td>
</tr>
<tr>
<td><strong>TABLE OF CONTENTS</strong></td>
<td>iv</td>
</tr>
<tr>
<td><strong>LIST OF TABLES</strong></td>
<td>vii</td>
</tr>
<tr>
<td><strong>LIST OF FIGURES</strong></td>
<td>viii</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td><strong>FELINE IMMUNODEFICIENCY VIRUS</strong></td>
<td>3</td>
</tr>
<tr>
<td>Life cycle</td>
<td>4</td>
</tr>
<tr>
<td>Genome structure and organization</td>
<td>6</td>
</tr>
<tr>
<td>Transmission and pathogenesis</td>
<td>9</td>
</tr>
<tr>
<td>Treatment</td>
<td>11</td>
</tr>
<tr>
<td>Reverse transcriptase biochemical features</td>
<td>13</td>
</tr>
<tr>
<td>Drug resistance</td>
<td>15</td>
</tr>
<tr>
<td><strong>SPECIFIC AIMS</strong></td>
<td>19</td>
</tr>
<tr>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>20</td>
</tr>
<tr>
<td>CHEMICALS</td>
<td>20</td>
</tr>
<tr>
<td>ISOTOPES</td>
<td>21</td>
</tr>
</tbody>
</table>
CELLS AND VIRUS

FOCAL INFECTIVITY ASSAY (FIA)

SELECTION OF ddC-RESISTANT MUTANT

PLAQUE-PURIFICATION OF ddC-RESISTANT MUTANT

ENZYMES

ENZYME ACTIVITY ASSAY

ENZYME INHIBITION ASSAY

NUCLEIC ACID PREPARATION

PCR AMPLIFICATION AND NUCLEOTIDE SEQUENCE ANALYSIS

RESULTS

SELECTION OF ddC-RESISTANT MUTANT

PLAQUE-PURIFICATION OF ddC-RESISTANT MUTANT

SUSCEPTIBILITY OF ddC-RESISTANT AND 34TF10 FIV TO ddC

CHARACTERIZATION OF ddC-RESISTANT MUTANT

Susceptibility to antiviral compounds

Reverse transcriptase

Nucleotide sequence analysis
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sensitivities of FIV 34TF10 and DCR-2c to antiviral compounds, as determined by FIA.</td>
<td>35</td>
</tr>
<tr>
<td>2</td>
<td>Sensitivities of FIV 34TF10 and DCR-5c to antiviral compounds, as determined by FIA.</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>Comparison of kinetic constants for RT from FIV 34TF10 and DCR-5c.</td>
<td>44</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Retrovirus life cycle from virus entry to release.</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Organization of the FIV genome.</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Nucleoside and pyrophosphate analogs with antiviral activities.</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>Inhibition of FIV 34TF10 and DCR-2c by ddC as determined by FIA.</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Focus of FIV-infected CrFK cells.</td>
<td>36</td>
</tr>
<tr>
<td>6</td>
<td>Inhibition of FIV 34TF10 and DCR-5c by various inhibitors as determined by FIA.</td>
<td>39</td>
</tr>
</tbody>
</table>

viii
Determination of $K_i$ values for inhibition of wild-type recombinant RT and DCR-5c RT.

Nucleotide sequence of RT-encoding region of FIV 34TF10 clone in pUC119, virus derived from this molecular clone, and DCR-5c.
INTRODUCTION

GENERAL INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus and the causative agent of acquired immunodeficiency syndrome (AIDS) (2,14). The virus targets cells of the immune system, specifically the helper T lymphocytes. Infected patients are susceptible to many opportunistic infections due to the weakened immune system. HIV-1 is currently the number two killer of people between the ages of 25 and 45 years. Over 13 million people are infected with the virus today and predictions for the future are frightening (33). Current research into control of the disease falls into two categories: protective vaccines and chemotherapy. Vaccine development has been slowed because of the antigenic diversity of HIV-1. Research into chemotherapy for AIDS has achieved some moderate success. Four nucleoside analog inhibitors have been approved by the Federal Drug Administration (FDA). However, resistance to these FDA-approved drugs has arisen in clinical treatment of AIDS patients. In addition, virus resistant to
experimental drugs have been selected in vitro. It is not known whether or not drug-resistant variants are pathogenic, and this question will significantly impact the course of therapy. In addition to these molecular problems which have arisen with respect to the virus, testing of new drugs and vaccines has presented ethical problems with respect to human use. Although many activists encourage experimental drug treatment in humans, the science community has sought alternatives, such as in vitro systems and animal models.

Due to the many obstacles stated above, an animal model is essential. Not only is an animal model safer to work with for the scientist, but in vivo pathogenesis studies can be performed. Many animal models have been evaluated for use in studying HIV-1, including Simian immunodeficiency virus (SIV), avian retroviruses, murine retroviruses, severe combined-deficiency (SCID) mouse models, lentiviruses in sheep, goats, horses and cows, HIV type 2 (HIV-2) infection in macaques, and feline immunodeficiency virus (FIV). Criteria that an animal model should meet include: 1) naturally occurring disease, 2) relatively inexpensive, 3) non-
infective to humans, and 4) easily manipulated in *in vitro* and *in vivo* studies (17). FIV has become recognized as a valuable animal model for AIDS for several reasons: FIV causes a natural infection in cats and a similar disease to human AIDS. The virus is not infectious to humans, and cats are much more practical and economical to work with than non-human primates. (17). FIV also offers both *in vitro* and *in vivo* systems in which to study drug resistance.

**FELINE IMMUNODEFICIENCY VIRUS**

Feline immunodeficiency virus (FIV), a lentivirus, is the etiological agent of a natural acquired immunodeficiency syndrome (AIDS) in cats (36,37). This disease occurs in domestic cats, and has also been produced by experimental infection of specific-pathogen-free cats (52,58). The virus is highly T lymphotrophic which results in an immunodeficient state in the animal. Infected cats are susceptible to many opportunistic diseases, as are humans infected with HIV-1. FIV was first described in 1987 following isolation from infected cats in a cattery. At first, many believed the disease was caused by feline leukemia virus (FeLV), however, all the animals in the cattery were seronegative for FeLV, therefore
ruling out FeLV as the cause of this immunosuppressive disease (37). Since the original identification of the disease, FIV has been shown to be a worldwide infection of domestic cats. FIV is morphologically, as well as pathologically, similar to HIV-1. Transmission of FIV to humans, however, has not been reported. With these similarities in mind, FIV offers a unique opportunity to study as an animal model for AIDS.

**Life Cycle.** FIV and HIV-1 share the prototypical life cycle of a retrovirus (figure 1). The virus is first adsorbed by a receptor on the host cell membrane. HIV-1 binds to the CD4 receptor on T cells. A CD4 homologue, CD9 has been implicated in FIV binding and infection (57). Once in the cell, the virus sheds its envelope and releases its RNA genome along with other proteins, including the enzyme reverse transcriptase (RT). This enzyme is unique to retroviruses. RT is the virally encoded RNA-dependent DNA polymerase responsible for synthesis of a DNA copy of the viral RNA genome. After synthesis of the complementary strand of DNA, the enzyme has a second function, that of RNase H activity. The original RNA strand is removed by this RNase H and a second strand of DNA is
Figure 1. Retrovirus life cycle from virus entry to release (drawn by David Hill).
synthesized by RT. Once the double-stranded DNA has been produced, it is integrated by the viral protein integrase into the host genome as a provirus (51). The provirus will remain dormant until some, as of yet unknown, factor(s) stimulates the provirus into active replication to produce infectious virus particles. The cell will then produce viral RNA and proteins. These progeny viruses assemble and are released from the cell surface by budding. The virus can then infect other cells.

**Genome structure and organization.** FIV and HIV-1, are enveloped, single-stranded RNA viruses. The FIV virion contains two copies of its RNA genome which are held together by a dimer linkage structure (DLS) near the 5' ends. The FIV genome consists of three large open reading frames (orfs) corresponding to the main structural genes, *gag*, *pol* and *env* (fig 2). The three structural genes are flanked by long terminal repeat (LTR) regions. There exists an overlapping *gag* and *pol* coding sequence which is characteristic of lentiviruses. In addition, the FIV genome also contains several small orfs that are multiply spliced to generate genes that regulate the expression of viral proteins (22,38,39,52). Coding regions of the
Figure 2. Organization of the FIV genome.
FIV genome have been identified that are responsible for determining whether or not a viral infection is productive or latent (22,38). These regions which encode the Rev protein and the Rev-responsive element, are also found in HIV-1. Another small orf contains a putative vif gene, that regulates viral infectivity and is similar structurally and functionally to the vif gene of HIV-1 (52). A putative tat orf has been described, (35,38,50) and may encode the FIV equivalent to the transactivating protein of virus which infect primates. No FIV counterpart to the HIV-1 nef gene has been reported.

The entire FIV genome is approximately 9500 bases. The gag gene is reportedly 450 residues long and the calculated molecular mass of the resulting polypeptide is 24 kDa. This agrees with estimated mass of the core protein (p24) of FIV. The gag gene also encodes for two small proteins (p10 and p17)(50). The pol gene encodes the enzymes reverse transcriptase, integrase, a protease, and a protease-like protein (49) with dUTPase function (12). The env gene of FIV, as with HIV-1, is located near the 3' end of the genome. At least four small orfs exist. Two can be found in
between the pol-env genes, and at least two are located in the env-3' long terminal repeat (LTR) regions. The env gene is approximately 750 base pairs long. Two env gene products of FIV are synthesized from a 145 kDa precursor glycoprotein. The precursor is quickly "trimmed" to a 130 kDa glycoprotein and is further processed to two glycoproteins, gp95 and gp40.

Transmission and Pathogenesis. Unlike HIV-1, sexual transmission does not appear to be a major factor in the spread of FIV. Transmission of FIV is generally through an infected cat biting or scratching an uninfected cat. Experimentally-infected female cats have been shown to transmit the virus to their offspring (56), although naturally-infected cats transmitting virus to offspring has not been reported. Vertical transmission is a significant mode of transmission in HIV-1.

FIV infection causes an AIDS-like disease in cats that can be broken down into five distinct stages. These stages correlate to the levels of infection seen with HIV-1. The five clinical stages are acute infection, asymptomatic carrier, persistent generalized
lymphadenopathy, AIDS related complex (ARC) and AIDS (21). With FIV the first signs of acute infection often include lymphadenopathy, transient neutropenia, acute diarrhea and mild upper respiratory signs (21). This correlates to the general lymphadenopathy and flu-like symptoms seen in early stages of HIV-1 infection (8). The acute phase of each virus is of varying time lengths, and is followed by a latent, asymptomatic phase. Virus has been detected during the asymptomatic phase, and these cats are classified as asymptomatic carriers. An extensive latent period of the FIV is seen in cats (up to 9 years) as is seen in humans infected with HIV-1 (average 6 years). Cats generally show general lymphadenopathy concurrently with clinical signs. Humans maintain a general lymphadenopathy while appearing otherwise healthy. In cats, it appears that the generalized lymphadenopathy stage is either extremely short or occurs at the same time that ARC-like symptoms are occurring. The ARC stage in cats and humans is characterized by chronic problems including lymphadenopathy, stomatitis/gingivitis, skin disease, upper respiratory disease, and/or enteric disease (5,21,36,). Neurological disorders can accompany either ARC or AIDS stages of both diseases (21). Cats progress to full-blown AIDS and die within eight months
of the beginning of the ARC stage. Due to the similarity in pathology of HIV-1 and FIV in their naturally occurring host, FIV is an obvious model for \textit{in vivo} pathogenesis studies. Additionally, drug-resistant isolates can be tested \textit{in vivo} with FIV.

\textbf{Treatment.} Due to the important role that RT plays in replication of both HIV-1 and FIV, it is an obvious choice as a target for antiviral chemotherapy. Currently there are two groups of drugs being studied for their inhibiting effect on RT: nucleoside analogs and non-nucleoside analogs (fig 3). The non-nucleoside analogs are noncompetitive inhibitors of RT and include phosphonoformic acid (PFA), nevirapine, tetrahydroimidazo[4,5,1-jk][1,4]benzodiazepin-2(1H)-one (TIBO). In addition, several protease inhibitors are also being studied.

To date, the FDA has only approved nucleoside analogs for treatment of humans with AIDS. The four RT inhibitors are the nucleoside analogs: 3'-azido-3'-dideoxythymidine (AZT), 2',3'-dideoxyinosine (ddl), 2',3'-dideoxycytidine (ddC), and 2',3'-dideoxy-2',3'-didehydrothymidine (d4T). These nucleoside analogs are
Figure 3. Nucleoside and pyrophosphate analogs with antiviral activities.
metabolized by the cell to the corresponding 5'-triphosphate. The triphosphates are competitive inhibitors of HIV-1 and FIV RTs. The metabolized triphosphates can also be incorporated into the elongating DNA strand, causing termination of the chain (10). Since both HIV-1 and FIV RT are susceptible to the nucleoside analog inhibitors, FIV is an excellent choice as an animal model for drug resistance studies.

**Reverse transcriptase biochemical features.** Reverse transcriptase is the main target for chemotherapy. Not only does RT play an essential role in virus replication, it is also unique to the virus. FIV and HIV-1 RTs are very similar in physical properties, catalytic activity and sensitivity to several RT inhibitors (9,29,30,32). The RT of FIV and HIV-1 are extremely similar in both template specificity and Mg^{2+} requirements (30). Our lab has purified the RT from FIV and characterized the enzyme with respect to its physical and catalytic properties (30). The purified FIV RT consists of a p54-p66 heterodimer that is very similar to a p51-p66 heterodimer that can be generated in HIV-1 RT under proteolytic conditions (11,27). Both HIV-1 and FIV RTs used similar amounts of
Mg\(^{2+}\) for a given template. Although the RTs of HIV-1 and FIV exhibited many similarities with respect to physical properties, it has been shown that the two enzymes differ substantially in primary sequence (30), as determined by peptide mapping and later confirmed by DNA sequence (35,50). When the predicted amino acid sequence of FIV RT was compared to that of HIV-1 RT, 60% identity was observed (35). The similarity between the two enzymes creates an opportunity to study RT inhibitors on a model lentivirus.

The susceptibilities of FIV RT and HIV-1 RT to various inhibitors were also very similar. Inhibition constants for both enzymes were determined for several inhibitors, and the two enzymes were indistinguishable with respect to the following inhibitors: 2',3'-dideoxythymidine 5'-triphosphate (ddTTP), N\(_3\)dTTP, 2',3'-dideoxycytidine 5'-triphosphate (ddCTP), 2',3'-dideoxy-2',3'-didohydrothymidine 5'-triphosphate (d4TTP), 3'-fluoro-3'-deoxythymidine 5'-triphosphate (3'-F-dTTP), 3'-amino-3'-deoxythymidine 5'-triphosphate (3'-NH\(_2\)-dTTP) and two noncompetitive inhibitors phosphonoformate (PFA) and phosphonoacetate (PAA) (29). Due to the biochemical similarities of
HIV-1 RT and FIV RT, FIV is also an excellent model for studying drug resistance, especially since RT is the target for all approved antivirals.

Drug Resistance. Drug resistance is the limiting factor in the treatment of AIDS. AZT was the first drug approved and used for treatment (28). Clinically, drug resistance to AZT (25), and subsequently ddl and ddC has been reported (13,47). Our lab was the first to successfully select an AZT-resistant mutant of a lentivirus \textit{in vitro} (41). This was followed by the selection of AZT-resistant mutants of HIV-1 \textit{in vitro} (16,24). The AZT-resistant mutants of FIV have similar phenotypic characteristics to the AZT-resistant clinical isolates of HIV-1 (41). Resistance to AZT, ddl and ddC have been traced to mutations within the RT-encoding region of the \textit{pol} gene of HIV-1 (19,20,26). The role of these mutations has been studied through site-directed mutagenesis and structure/function analysis of the RT.

Since the structure of HIV-1 RT was determined by
crystallization, domains and binding sites within RT have been identified. The crystal structure of FIV RT has not been determined, however, due to the 68% similarity of amino acid sequence of the two RTs, it is likely that structures are similar. The polymerase domains of p66 and p51 each contain the same four domains, which have been called finger, palm, thumb, and connection (23). Most of the mutations that confer resistance to nucleoside inhibitors have been shown to reside in either the fingers subdomain or the palm subdomain. Neither of these locations is in the immediate area of the polymerase active site (3). Boyer et al. have proposed that resistance to the dideoxynucleotide inhibitors results from a repositioning or change in the conformation of the template-primer binding to enzyme that alters the ability of the enzyme to select or reject an incoming dNTP (3).

ddC-resistance of HIV-1 has been traced to multiple sites in the RT encoding region of the pol gene. These include amino acids 65, 69, 74, 75, and 184(19,20,48,13). From analysis of the three-dimensional structure of HIV-1, these sites have been mapped to two places. Amino acids 65, 69, 74, and 75 all map within the
fingers subdomain of RT and have been implicated in template-primer interaction (3). Amino acid 184 is near the polymerase active site and may work by affecting the positioning of the template-primer or by changing residues that directly interact with incoming dNTPs.

ddC-resistant mutants of HIV-1 have arisen through selection with ddC, ddl, 3TC and AZT (13,16,19,20,24,47). The phenotypes of the ddC-resistant mutants of HIV-1 differ widely. ddC-resistant mutants of HIV-1 that were selected by ddC treatment are well described (15,16,19,20). One of these mutants was found to be cross-resistant to ddl and 3TC. This ddC-resistant mutant of HIV-1 contained a lesion within the RT-encoding region of the pol gene. In addition, Gu et al. have shown that the RT from the HIV-1 ddC-resistant mutant has reduced sensitivity to ddCTP, 3TCTP, ddATP, and AZTTP in vitro (19). This RT exhibited a ten-fold resistance to ddCTP compared to wild-type RT. This ten-fold resistance corresponds to the ten-fold resistance the virus had to ddC. Gu et al. have also selected a ddl-resistant mutant that was cross-resistant to ddC (20). Judy Gobert in our lab has selected a ddl-selected
mutant of FIV (classified as selected rather than resistant because the virus was <5 fold resistant relative to wild-type). In contrast to Wainberg's ddl-resistant mutant of HIV-1, the ddl-selected mutant of FIV is hyper-sensitive to ddC and also cross-resistant to PFA. In order to understand these data further we set out to select a ddC-resistant mutant of FIV.

In order to study drug resistance, it is important to have a defined system that is safe and easily manipulated. It is also useful to have a system which allows for timely selection of drug-resistant mutants. FIV has a well-defined cell culture system and drug-resistant mutants can be selected readily (43). In addition, there is a molecular clone of FIV, FIV 34TF10 with a known sequence (50). This molecular clone provides a defined genetic system in which to study mutations. Many of the questions raised by drug resistance can be best answered in an animal model. Not only can drug-resistant mutants be selected and characterized in a cell culture system, but the pathogenicity and infectivity of drug-resistant mutants can be determined in cats (1). Characterization
of drug-resistant mutants of FIV offers insight into the mechanism of drug-resistance in HIV-1 and can lead to the development of better chemotherapeutic agents for AIDS.

SPECIFIC AIMS

The goal of this study was to select and characterize a ddC-resistant mutant of FIV 34TF10. Specific objectives were to select variants of FIV that emerge when virus replication occurs in the presence of a high concentration of ddC, to determine whether virus resistant to ddC are cross-resistant to other antivirals, and to determine whether ddC-resistance is due to alterations in reverse transcriptase. In addition, the foundation will be laid for studies in cats, in which the pathogenicity of ddC-resistant FIV can be examined.
MATERIALS AND METHODS

Chemicals. The dCTP, Triton X-100, aminoethyl carbazole, PFA, -oligo(dC)<sub>10-15</sub>, and ddC were purchased from Sigma Chemical Co., St. Louis, Mo. AZT was provided by Philip A. Furman of Burroughs Wellcome Co., Research Triangle Park, N.C. ddI was provided by the Developmental Therapeutics Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases. 9-(2-phosphonylethoxymethyl)adenine (PMEA) was provided by H.-T. Ho of Bristol Myers-Squibb Co., Wallingford, Conn. 2',3'-dideoxy-3'-thiacytidine (3TC) was provided by Raymond Schinazi of Emory University, Atlanta, Georgia. Poly(rA)-oligo(dT)<sub>10</sub> and Poly(rl) were purchased from Pharmacia LKB, Piscataway, New Jersey. International Bio Technologies (IBI) phenol for DNA extractions was purchased from VWR Scientific. GeneAmp Polymerase Chain Reaction (PCR) Core Reagents were purchased from Perkin Elmer Cetus, Norwalk, CT. The Taq DyeDeoxy™ Terminator Cycle Sequencing Kit was purchased from Applied Biosystems, Foster City, CA. All other chemicals were reagent grade or better.
Isotopes. [5,-$^{3}$H]-dCTP and [methyl-$^{3}$H]dTTP were obtained from Dupont-New England Nuclear, Boston, MA.

Cells and Virus. Virus derived from a molecular clone of the Petaluma strain of FIV, 34TF10, was used in this study (50). Wild-type and mutant strains of FIV were grown and maintained in Crandell feline kidney (CrFK) cells. Uninfected and infected cells were grown in L & M medium which was composed of equal parts of Leibovitz L-15 and Dulbecco's Modified Eagle Medium, supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2.0 mM L-glutamine and 10% fetal bovine serum (FBS). FBS was heat inactivated for 30 min. at 56° C. All cultures were maintained at 37° C in a humidified 5% CO$_2$ atmosphere. ddC-resistant FIV was maintained in medium that contained 25 uM ddC, and the medium was replaced with fresh medium and the appropriate concentration of ddC every 2 days.

Virus stocks were prepared from medium containing extracellular virus released from CrFK cells that had been infected
with FIV for more than 21 days. Medium from confluent infected cells was centrifuged at 500 X g for 10 min to remove cells. Supernatants containing virus, were stored at -80° C in medium containing 10% dimethyl sulfoxide (DMSO). Aliquots of these stocks were thawed and used to infect CrFK cells for subsequent studies.

**Focal Infectivity Assay (FIA).** The FIA was developed in this lab by Remington et al. (41) and is a modification of the FIA developed by Chesebro and Wehrly to quantitate HIV-1 (6). In this assay, 24-well plates were seeded with uninfected CrFK cells at a density of 1.5 x 10^4 cells per well and incubated for one hour at 37° C in growth medium or growth medium containing appropriate concentration of drug. This incubation was to allow the cells to convert drugs to their active form. These cells were infected with FIV and allowed to grow to confluence (4-5 days). The medium was replaced with fresh medium and appropriate drug concentration every 2 days. When the cells reached confluency, the media was aspirated and the cells were fixed with methanol for five minutes. The cells were then washed twice with TNE (0.01 M Tris-HCl, pH 7.5 containing 0.15 M NaCl and 0.002 M EDTA), and once with TNE.
supplemented with 1% bovine serum. Immunostaining was done by incubating the cells for 30 minutes at room temperature with 0.2 ml of a 1/200 dilution of polyclonal cat anti-FIV that had been obtained from FIV-infected specific-pathogen-free cats. After the incubation, cells were washed twice with TNE containing bovine serum to remove excess anti-FIV. Next, the cells were incubated for 40 minutes with 0.2 ml of a 1/600 dilution of horseradish peroxidase-conjugated goat anti-cat immunoglobulin (Organon-Teknika, Durham, NC.). The cells were again washed with TNE and then stained with a solution of aminoethyl carbazole in sodium acetate buffer, pH 5.0 (1 part per 19 parts buffer). The cells were incubated with this substrate for 20 minutes in the dark. After incubation, the cells were rinsed three times with water and allowed to dry. Infected cells stained red against a clear background. A group of four or more infected cells is defined as a focus of infection. Foci were examined and counted under 10 to 40 X magnification. For visualization, light was reflected off a piece of ground glass to reduce the refractivity of the infected cells. For dose response curves with drugs, data were plotted as a percentage of control plaques (no drug) versus inhibitor concentration.
Concentrations required to inhibit focus formation by 50% (IC₅₀ values) were obtained directly from the linear portion of these plots, using a computer-generated regression line. Within an experiment, each point represents the mean of four determinations.

Selection of ddC-resistant mutant. Two 25 cm² flasks containing CrFK cells at 60% confluency were pretreated for one hour with 25 μM ddC to enable conversion of the drug to the active form. Cells were infected with 0.9 ml of FIV 34TF10 (approximately 500 foci of infection per ml). These two cultures were maintained by replacing medium and drug every two days and trypsinizing and reseeding as necessary. The cultures were monitored weekly for virus production by the FIA. When virus was detected in the cultures, a second round of infection was initiated using virus from the culture with the highest titer, designated DCR-2. Two new 25 cm² flasks containing CrFK cells at 60% confluency were pretreated for one hour with 25 μM ddC. After a one-hour incubation, the cells were infected with 0.5 ml of DCR-2 virus containing 400 foci of infection per ml. These cultures were maintained as described above and monitored weekly for virus production through the FIA.
When virus was detected and characterized, plaque-purification of the mutant virus was initiated.

**Plaque purification of ddC-resistant mutant.** Plaque purification of the mutant virus was carried out with a modification of the procedure described by Remington et al. (42). Virus was plaque purified from the second round of selection, using limiting dilutions of virus in the FIA. Limiting dilutions of virus were used to infect CrFK cells in 24-well plates in the presence of 25 $\mu$M ddC. After six days, each supernatant was transferred to a well of a new plate which had been seeded with uninfected CrFK cells in medium with 25 $\mu$M ddC. To enhance virus adsorption, just prior to the transfer of supernatants, cells of the new plate were treated with medium containing 8 $\mu$g/ml of DEAE dextran for 20 minutes (this medium did not contain fetal bovine serum). The original plate was stained and examined for those wells that contained a single focus. Corresponding wells of the subculture plate were incubated until the cells reached confluency. The cells and supernatant from each well (corresponding to a well that contained a single focus of infection) were transferred to flasks and maintained in medium containing 25
μM ddC. Cultures were monitored weekly for virus production by FIA. Only one culture yielded virus, and it was used for further characterization, sequence analysis, and RT assays.

Enzymes. RT was purified from virions of FIV 34TF10 and mutant FIV by a method developed by North et al. (30). Briefly, virions were disrupted with Triton X-100 and enzyme was purified by chromatography on DEAE-cellulose and phosphocellulose.

Enzyme activity assay. During purification, RT was assayed for activity as reported previously (29,30) with poly(rA)-oligo(dT)$_{10}$ as the template-primer. Reactions were typically carried out in a volume of 50 μl and contained 50 mM Tris-HCl, pH 8.5, 10 mM dithiothreitol, 0.05% Triton X-100, 250 μg of bovine serum albumin (nuclease-free) per ml, 6 mM MgCl$_2$, 80 mM KCl, 20 μM [methyl-$^3$H]dTTP (33 uCi/ml), 0.5 $A_{260}$ units (U) of poly(rA)-oligo(dT)$_{10}$ per ml, and appropriate amounts of RT. Samples containing 40 μl were taken at 1 hour and spotted onto filters (no. 3, 2.3-cm diameter; Whatman, Inc., Clifton, NJ) that had been presoaked with 5% trichloroacetic acid-1% sodium pyrophosphate. Filters were dried,
washed four times (at least 1 hour each) with 5% trichloroacetic acid-1% sodium pyrophosphate (4°C) and twice with 95% ethanol, dried and then counted in Liquifluor (Dupont-NEN Research Products, Boston MA). One unit of RT is defined as the amount of enzyme required to catalyze the incorporation of 1 nmol of dTMP per hour into poly(rA)-oligo(dT)_{10} at 37°C.

**Enzyme Inhibition assay.** Inhibition of RT from the ddC-resistant mutant by ddCTP was compared to inhibition of wild-type recombinant FIV RT. Double-reciprocal plots were used to determine kinetic constants (K_m and K_i) with the template-primer poly(rI)-oligo(dC)_{10-15} and ddCTP as the inhibitor (30). Reactions were typically carried out in a volume of 50 µl and contained 50mM Tris-HCl, pH 8.5, 10 mM dithiothreitol, 0.05% Triton X-100, 250 µg of bovine serum albumin (nuclease-free) per ml, 6 mM MgCl$_2$, 80 mM KCl, varying activity of [5,5'-3H]-dCTP (33, 16.5, 4.9, 3.3, 2.4, and 1.6 uCi/ml) corresponding to varying concentrations of dCTP (20, 10, 6, 4, 3, and 2 µM), 0.5 A$_{260}$ U of poly(rI)-oligo(dC)$_{10-15}$ per ml, varying concentrations of ddCTP (0, 100, 250, and 500 nM), and appropriate amounts of RT. Samples containing 40 µl were taken at
1 hour and spotted onto filters (no. 3, 2.3-cm diameter; Whatman, Inc., Clifton, NJ) that had been presoaked with 5% trichloroacetic acid-1% sodium pyrophosphate. Filters were dried, washed four times (at least 1 hour each) with 5% trichloroacetic acid-1% sodium pyrophosphate (4° C) and twice with 95% ethanol, dried and then counted in Liquifluor (Dupont-NEN Research Products, Boston MA).

Nucleic acid preparation. Total cellular DNA containing provirus was extracted from CrFK cells that were infected with ddC-resistant FIV. Confluent monolayers of infected cells were washed three times with TBS (25 mM Tris-HCl, pH 7.4 containing 5 mM KCl and 137 mM NaCl) and removed from flasks (cells peel from flask wall during the last wash). Cells were pelleted at 500 X g for 10 min. The cells were washed once with TE (10mM Tris-HCl, pH 7.6 containing 1 mM EDTA), then resuspended in this buffer. Cell suspensions were treated with 0.1 mg/ml Proteinase K and 0.5% SDS overnight at 37° C. The cell lysates were then extracted three times with phenol that had been equilibrated with 10 mM Tris-HCl, pH 7.5
containing 100 mM NaCl and 1 mM EDTA. The aqueous phase was collected and dialyzed against four changes of 10 mM Tris-HCl, pH 7.5 containing 100 mM NaCl and 1 mM EDTA until the A_{260} of the dialysate was less than 0.16. The dialyzed aqueous phase was treated with RNase A (0.1 mg/ml) at 37° C for 3 hours. The RNase A-treated aqueous phase was extracted with one-half volume equilibrated phenol and one-half chloroform:isoamyl alcohol (24:1). The aqueous layer was removed and then extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The salt concentration was adjusted by adding 1/30 volume of 3 M Sodium acetate, pH 5 to the aqueous phase. The DNA was precipitated by adding 3 volumes of cold 95% ethanol and incubating overnight at -20° C. The precipitated DNA was removed from the ethanol and allowed to dry. The DNA was resuspended in sterile water and used for amplification using the polymerase chain reaction (PCR).

**PCR amplification and nucleotide sequence analysis.**

Amplification of the RT-encoding region of the pol gene was performed using the Perkin-Elmer/Cetus GeneAmp PCR protocol. Each 100 μl reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl,
200 μM of each dNTP (dATP, dTTP, dCTP and dGTP), 4 mM MgCl₂, 0.2 μM of each primer, 2.5 Units of AmpliTaq® DNA polymerase, and 10-20 μg of target DNA. Reactions were overlaid with 50-100 ul of light mineral oil. The sense primer (5’-GTA ATG TTT GTG TCT TAG AAG ATA ACT C-3’) and the reverse complement primer (5’-ATC ATA TCC TGC ATC TTC TGA CCT-3’) were synthesized in the Murdock Molecular Biology Facility, University of Montana, Missoula, MT. The primers were chosen to amplify a 1763 base pair fragment that contained nucleotides 2268 through 4031. The PCR was run for 30 cycles, each cycle comprised of 30 seconds of denaturation at 94° C, 30 seconds of annealing at 62° C and two minutes of extension at 72° C. After PCR, the product was visualized on an agarose gel. For this, the mineral oil was separated from the PCR products and an aliquot of the mixture was run on a 0.8% low-melting agarose electrophoresis gel in 40 mM Tris acetate, pH 7.6 containing 1 mM EDTA (45). The agarose gel and running buffer contained 0.5 μg/ml ethidium bromide. A 1763 base pair band was identified by comparing the electrophoresed PCR reaction to a 1 Kb DNA ladder (Gibco BRL, Gaithersburg, MD). The remaining PCR product was purified using the QIAquick PCR Purification Kit, Qiagen Inc.,
Chatsworth, CA. Sequencing was performed in the forward and reverse directions with two or more primers covering each 250 base pair section of the RT-encoding region along with small flanking regions on either end of RT. Four different primers were used to sequence the 2342 region containing the point mutation. This DNA was directly sequenced by Joan Strange, The Murdock Molecular Biology Facility, The University of Montana, Missoula, MT, with a Taq DyeDeoxy™ Terminator sequencing kit and analyzed on a Model 373A automated DNA sequencer (Applied Biosystems, Foster City, CA).
RESULTS

SELECTION OF ddC-RESISTANT MUTANT

A ddC-resistant mutant of FIV was selected by infection of CrFK cells with FIV 34TF10 in the presence of inhibitory concentrations of ddC. For the initial round of selection, two 25 cm² flasks of cells were infected with a high multiplicity of infection (450 focal forming units/flask). The cells were pretreated with 25 μM ddC for one hour prior to infection to ensure that the drug had been converted to its active form by the cells. Cells were maintained with 25 μM ddC throughout the selection by replacing media and drug every two days. The drug concentration (25 μM) used in this experiment was chosen because it is five times the IC₅₀ value and should inhibit wild-type virus production completely. Cultures were monitored weekly for virus production by the FIA. No virus was detectable until 17 weeks post infection. It should be noted that virus production in other drug selections (AZT, ddI, PFA and FLT) usually takes between 3 and 8 weeks. One of the cultures, DCR-2c, was chosen to characterize and to continue selection. Once
it was established that the virus was resistant to ddC, a second
round of selection was initiated (Figure 4). In addition, DCR-2c was
characterized with respect to other antivirals (Table 1). Two or
more dose response curves were performed using ddC and ddl. One
dose response curve for each of the other antiviral drugs were
performed for preliminary screening of the ddC-resistant phenotype.

For the second round of selection, 200 focal forming units of
undiluted cell-free virus/flask from the first round were used to
infect two 25 cm² flasks of CrFK cells that had been pretreated with
25 μM ddC. Less virus was used in the second round of selection to
lower the chance of heterogeneity that might arise from mixed
infections. The second round cultures were maintained with 25 μM
ddC and monitored for virus production as before. When virus was
detected, the second round was characterized (Table 1) and ddC-
resistance was confirmed (Figure 4). The same dose response curve
protocol as the first round was repeated with this round. Plaque-
purification of the mutant virus was initiated.
ddC DOSE RESPONSE CURVE
FOR DCR-2c AND 34TF10

Figure 4. Inhibition of FIV 34TF10 and DCR-2c (Rounds 1 and 2 of selection) by ddC as determined by focal infectivity assay. Results are from two experiments and each point represents four determinations. Bars represent standard error of the mean, and are omitted where the standard error was too small to accurately show using error bars.
Table 1. Sensitivities of FIV34TF10 and DCR-2c to antiviral compounds as determined by FIA<sup>a</sup>.

<table>
<thead>
<tr>
<th>Compound</th>
<th>34TF10</th>
<th>DCR-2c Round 1</th>
<th>DCR-2c Round 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9±0.7</td>
<td>30±2</td>
<td>25±1</td>
</tr>
<tr>
<td>ddi&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5±0.8</td>
<td>21±1</td>
<td>23±2</td>
</tr>
<tr>
<td>AZT</td>
<td>0.34</td>
<td>0.62</td>
<td>0.50</td>
</tr>
<tr>
<td>PFA</td>
<td>113</td>
<td>&gt;400</td>
<td>&gt;400</td>
</tr>
<tr>
<td>PMEA</td>
<td>0.47</td>
<td>0.37</td>
<td>0.44</td>
</tr>
<tr>
<td>3TC</td>
<td>0.58</td>
<td>0.75</td>
<td>0.65</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent four determinations per experiment

<sup>b</sup> Values are from two or more experiments.

PLAQUE PURIFICATION OF ddC RESISTANT MUTANT

In an effort to minimize the degree of heterogeneity within the DCR-2c mutant population, the FIA was used to isolate a clone of virus that had descended from a single infectious particle. A focus of infection represents an infection by a single virion (Fig 5). CrFK
Figure 5. Focus of FIV-infected CrFK cells detected with polyclonal antiserum to FIV and peroxidase-conjugated goat anti-cat immunoglobulin.
cells were seeded at a density of 1.0 X 10^4 cells per well in TC24 plates and were infected with virus stocks that had been diluted to contain approximately one infectious particle per well. After six days, culture supernatants were transferred to uninfected cells in TC24 plates, and the original plates were immunostained to identify those wells that contained a single focus of infection. Cells infected with progeny from a single infection event were transferred along with supernatant to a flask and maintained with 25 μM ddC and monitored for virus production by the FIA. Plaque-purification of the ddC-resistant mutant proved to be difficult. The first three times plaque-purification of DCR-2c was attempted, virus was lost between the transfer of supernatant to the subculture plate and final transfer of supernatants and cells into flasks. Multiple wells containing single foci were identified. However, corresponding cells and supernatants did not yield infectious virus. To enhance virus adsorption to the cells in the subculture plate, the plaque-purification protocol developed by Remington et al. was modified (41). Before transferring the supernatants from the culture plate to the subculture plate, the CrFK cells on the subculture plate were treated with serum-free medium containing 8
μg/ml of DEAE dextran for 20 minutes. DEAE dextran treatment coats the cells with a positive charge, thereby making the cells more attractive to the negative charged virus particles. Using this modification, supernatants from eighteen isolates were transferred onto the DEAE dextran-treated CrFK cells of the subculture plate. When the cells and supernatants were transferred to flasks, virus was detected in one of the eighteen cultures. This virus, designated DCR-5c, was ddC-resistant (Figure 6) and was characterized against a battery of RT inhibitors.

**SUSCEPTIBILITY OF ddC-RESISTANT AND 34TF10 FIV TO ddC**

Susceptibility of virus from each round of selection to ddC was assayed with the FIA by determining the number of foci at varying concentrations of ddC. The data shown in Figure 5 verify that DCR-2c was considerably less sensitive to inhibition by ddC than the molecular clone. The IC₅₀ value for inhibition of DCR-2c by ddC was greater than 25 μM compared to 5 μM for FIV34TF10. These data confirmed that DCR-2c was at least 5-fold less susceptible to ddC than the molecular clone. DCR-5c, the plaque-purified mutant derived from DCR-2c, was also less sensitive to inhibition by ddC
Figure 6. Inhibition of FIV 34TF10 and DCR-5c by various antivirals as determined by focal infectivity assay. Results are from three experiments and each point represents four determinations. Bars represent standard error of the mean, and are omitted where the standard error was too small to accurately show using error bars.
than FIV 34TF10. The dose response curve shown in Figure 6 verified that the $IC_{50}$ value for inhibition of DCR-5c by ddC was greater than 12 $\mu$M compared to 3 $\mu$M for the molecular clone.

**CHARACTERIZATION OF ddC-RESISTANT MUTANT**

**Susceptibility to antiviral compounds.** The susceptibility of virus from each round of selection to a variety of other antiviral compounds was determined using the FIA and compared to the susceptibilities of wild-type virus (Table 1). These data showed that DCR-2c is cross-resistant to ddI and PFA. DCR-2c is also slightly less sensitive to 3TC than wild-type FIV 34TF10. The mutant FIV was similar to FIV 34TF10 in sensitivity to AZT and PMEA. Of the compounds tested, the mutant exhibited resistance to a similar dideoxynucleoside analog (ddI), and, interestingly, to a non-nucleoside analog (PFA). The $IC_{50}$ value for inhibition of DCR-2c by ddI was greater than 13 $\mu$M compared to 3 $\mu$M for FIV34TF10. DCR-2c demonstrated an $IC_{50}$ value for PFA of greater than 400 $\mu$M compared to 75 $\mu$M for FIV 34TF10. The $IC_{50}$ values obtained for inhibition of FIV 34TF10 and ddC-resistant FIV are summarized in Table 1.
The drug susceptibility of DCR-5c was determined to confirm that the phenotype of the mutant had not changed during plaque-purification. The sensitivities to ddC, ddl, AZT, PFA, PMEA, and 3TC of this plaque-purified mutant were compared to the molecular clone (Figure 6 and Table 2). Referring back to Table 1, it can be seen that the phenotype of the plaque-purified mutant (DCR-5c) remained similar to the mutant population (DCR-2c). The plaque-purified mutant demonstrated resistance to ddC, ddl and PFA, slight resistance to 3TC, and wild-type sensitivities to all other drugs tested.
Table 2. Sensitivities of FIV 34TF10 and DCR-5c to antiviral compounds, as determined by FIAa.

<table>
<thead>
<tr>
<th>Compound</th>
<th>FIV 34TF10 Mean IC50(μM)±SE</th>
<th>DCR-5c Mean IC50(μM)±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddC</td>
<td>3.5±0.6</td>
<td>13±0.6</td>
</tr>
<tr>
<td>ddl</td>
<td>3.0±0.4</td>
<td>9.8±0.5</td>
</tr>
<tr>
<td>AZT</td>
<td>0.3±0.2</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>PFA</td>
<td>78±9.0</td>
<td>&gt;600</td>
</tr>
<tr>
<td>PMEA</td>
<td>0.3±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>3TC</td>
<td>0.5±0.1</td>
<td>0.8±0.1</td>
</tr>
</tbody>
</table>

a Values are from three experiments, with four determinations per experiment.

Reverse transcriptase. Reverse transcriptase was purified from DCR-5c and the kinetic parameters of inhibition by ddCTP were determined and compared to the wild-type FIV RT (R-RT). As expected, ddCTP displayed competitive inhibition of the enzymes (Figure 7). The wild-type FIV RT was produced in *Escherichia coli*, and has been shown to be similar to virion-derived FIV RT in
Figure 7. Determination of $K_i$ values for inhibition of wild-type recombinant RT and DCR-5c RT. Values reported are averages from duplicate determinations.
susceptibility to ddCTP and other antiviral nucleotides (31). The two enzymes had comparable $K_m$ values for both dTTP and dCTP. However, the mutant enzyme was ten times less sensitive to ddCTP compared to the recombinant RT. Wild-type FIV RT had an $IC_{50}$ value of 49 nM compared to the mutant RT $IC_{50}$ value of 456 nM (Figure 7, Table 3). Future experiments should determine whether the mutant RT is susceptibility to ddATP (the active form of ddl), PFA, and AZTTP.

Table 3. Comparison of kinetic constants for RT from FIV 34TF10 and DCR-5c.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ for dCTP (μM)</th>
<th>$K_i$ for ddCTP (nM)</th>
<th>$K_i/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>34TF10</td>
<td>6.2±1.3</td>
<td>49±2.3</td>
<td>.008</td>
</tr>
<tr>
<td>DCR-5c</td>
<td>11±1.2</td>
<td>456±27</td>
<td>.04</td>
</tr>
</tbody>
</table>

$^{a}$ Values are means ± standard error from 2 or more determinations.

**Nucleotide sequence analysis.** Sequence analyses of the RT-encoding region of the pol gene from DCR-5c and FIV 34TF10 were performed in the forward and reverse directions. The results are shown in Fig. 8. FIV 34TF10 DNA had been subcloned into the
<table>
<thead>
<tr>
<th>lyAsnValCy</th>
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<th>eValLeuGly</th>
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<th>plasmid</th>
<th>FIV34TF10</th>
<th>DCR-5c</th>
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<tr>
<td>GTAAATTTGG</td>
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<th>ProValLeVL</th>
<th>ValValMetL</th>
<th>eLeuAspPro</th>
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<th>FIV34TF10</th>
<th>DCR-5c</th>
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<td>CTGAAAATTC</td>
<td>GGAGATCT</td>
<td>CAGGTATCC</td>
<td>GAGGATCTC</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

| GTAATC     | TCTCTTACAA | GATAACTCAT | TAATACAACC | ATTATTGGG  | AGAGATAATA | TGATTATT   |      |          |           |        |

| OCR-5c     | FIV34TF10  | DCR-5c     |            |            |            |            |      |          |           |        |

Figure 8. (cont on next page)
Figure 8. (cont. on next page)
Figure 8. Nucleotide sequence and deduced amino acid sequence of RT-encoding region of pol gene from 34TF10 clone of FIV in pUC119, virus derived from this molecular clone, and DCR-5c. “-” denotes identity.
plasmid pUC119 and was used as a sequencing wild-type control.

The sequence of DCR-5c was identical to the 34TF10 clone of FIV in pUC119 and virus derived from this molecular clone except for a single point mutation at position 2342 of a G to a C. This resulted in the replacement of aspartate with histidine at the third amino acid of RT, which produced a negative to positive change in the amino acid charge.
DISCUSSION

We report the first successful selection of a ddC-resistant mutant of FIV. This was the first ddC-resistant mutant from a molecular clone of either FIV or HIV-1. This mutant was selected by the method developed by Remington et al. (41) using a drug concentration that is known to completely inhibit replication of wild-type FIV in vitro. This ddC-resistant mutant of FIV proved to be unique in several ways. First, DCR-2c was not detectable in cell culture until 17 weeks post-infection. This is at least twice the length of time it takes for selection of mutants to AZT, ddl, PFA or 3’-fluoro-3’-deoxythymidine (FLT) (3-8 weeks). Although no direct correlations to the clinical setting can be drawn, at least within our well-defined cell culture system, it appears that ddC resistance arises more slowly than to other RT inhibitors.

The second unique quality of DCR-2c was the difficulty it posed in plaque-purification. Using the established plaque-purification protocol developed by our lab, I was unable to obtain virus production in four attempts starting with 30 possible single
foci. Even with the modified version, only one culture produced virus out of eighteen possible single foci. DCR-2c is the only drug-resistant mutant selected by our lab that was difficult to plaque-purify. The reason for this difficulty in plaque-purification is not known.

Thirdly, it is interesting that both DCR-2c and DCR-5c exhibited unusually high titers when virus was finally produced. The high titer displayed by the ddC-resistant mutants was in stark contrast to the other drug resistant mutants selected by our lab, most of which yielded titers 5 to 10-fold lower.

The plaque-purified ddC-resistant mutant of FIV, DCR-5c, demonstrated similar phenotypes when compared to the mutant population, DCR-2c. In addition, DCR-5c showed some similarities to the ddC-resistant mutant of HIV-1 selected by Gu et al. (20). Both DCR-5c and the HIV-1 mutant demonstrated cross-resistance to ddI and wild-type susceptibility to AZT (20). However, DCR-5c exhibited only a slight resistance to 3TC, whereas Gu et al. reported a 500-fold resistance of the HIV-1 mutant to 3TC (20). These
differences may be due to the different selection methods used to select HIV-1 drug resistant mutants. The HIV-1 mutants were selected by gradually increasing drug concentrations in a step-wise method during the selection process. This protocol has been used because researchers have had difficulty selecting mutants of HIV-1 with high drug concentrations, as used in FIV selections. Gu et al. did not test inhibition by PMEA or PFA, so we cannot compare these phenotypes. In addition to the similarities in the two mutants susceptibility to ddC and ddl, the RTs of the two mutants were also similar in that they were resistant to ddCTP.

Reverse transcriptases were purified from wild-type and the plaque-purified ddC-resistant mutant (DCR-5c) of FIV. The RT from the mutant virus was resistant to the active form of ddC, ddCTP, compared with wild-type RT. DCR-5c showed a ten-fold increase of resistance. This compares to the 20-fold increase of resistance shown by the ddC-resistant mutant of HIV-1 that Gu et al. described (19.). Assays of both RTs used poly(rI)-oligo(dC) as template-primer and dCTP as substrate. Although the RT appears to show a higher resistance to ddCTP than the virus replication did to ddC, the ratio
$K_i/K_m$ used to normalize for changes in substrate recognition by the enzyme was increased approximately 5-fold with the mutant virus. These findings are consistent with the 5-fold increase in IC$_{50}$ for inhibition of replication of DCR-5c by ddC. It appears that the mutation in the RT-encoding region confers resistance to ddC. Although it may seem intuitive that the RT of a drug-resistant mutant would be resistant to the ddNTP, this has not always been the case. Many studies have described isolation or selection of drug resistance FIV and HIV-1 that had RTs susceptible to the ddNTPs (26, 41, 55).

The resistance shown by the RT from DCR-5c can be correlated to sequence analysis. The ddC-resistant mutant that was derived from the 34TF10 clone of FIV enabled examination of the nucleotide sequence of ddC-resistant FIV. Sequence analysis of the RT-encoding region of the pol gene revealed a single base change in the mutant FIV, as compared to the FIV34TF10 wild-type. The nucleotide substitution at position 2342 in DCR-5c was the only divergence from the wild-type RT-encoding region. This substitution produced an asp-3 to a his amino acid change, resulting
in a negative to positive charge alteration. This is a significant change that would possibly result in a conformational modification of the RT. Asp-3 resides in an area analogous to where the template-primer binds during DNA elongation within the HIV-1 RT (23). This is based on HIV-1 structure and assumptions of similarities. Because of the RT data, it is likely that this mutation within RT is responsible for the ddC-resistance. No drug-resistance mutation has previously been mapped to this area in either FIV or HIV-1. This mutation in DCR-5c provides information regarding possible new sites for drug resistance.

Some of the mutations responsible for ddC-resistance of HIV-1 are also located within the finger-like domain of RT (15,16,19,20). Although the codon 3 of the ddC-resistant mutant is located close to the amino terminus end, it also is located within the finger-like domain, and therefore is in position to interact with the template-primer. Boyer et al. proposed that resistance to the dideoxynucleotide inhibitors results from a repositioning or change in the conformation of the template-primer that alters the ability of the enzyme to select or reject an incoming dNTP (3).
Conclusions and future directions. We have successfully selected a ddC-resistant mutant of FIV by culture of FIV in the presence of a high concentration of ddC (41). In order to further characterize ddC-resistant mutants with respect to the HIV-1 mutants, it would be useful to perform a step-wise ddC selection with FIV, under conditions similar to those used by Gao et al (16). Comparison of selection methods would answer whether selection methods play a role in the phenotype and genotype of drug resistant mutants. In addition, it would be extremely interesting to determine the susceptibility of RT from DCR-5c to ddATP, the active form of ddl, AZTTP, and PFA. The results from these experiments could confirm that the RT does in fact confer the phenotype of the whole virus.

Reversion and/or site-directed mutagenesis studies would provide additional confirmation whether the single point mutation is responsible for resistance of the RT to ddCTP and the virus to ddC. Reversion studies would also provide information on the stability of the drug-resistant population.
Lastly, *in vivo* studies would be especially useful for pathogenicity studies. Due to the similarities between FIV and HIV-1, *in vivo* studies would provide information on whether ddC-resistant mutants are pathogenic.
REFERENCES


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