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Mary Ann Stevenson

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OXATHIOLANE NUCLEOSIDE ANALOG RESISTANCE IN A PATHOGENIC MOLECULAR CLONE OF FELINE IMMUNODEFICIENCY VIRUS

AND

MAPPING IN VIVO REPLICATION AND PATHOGENESIS DETERMINANTS OF ALEUTIAN MINK DISEASE PARVOVIRUS

by

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A Dissertation
Presented to the Faculty of Biochemistry/Microbiology
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Part I

Antiretroviral drug resistance develops during treatment of human immunodeficiency virus type 1 (HIV-1) infection, but the consequences of drug resistance on HIV-1 pathogenesis and the clinical relevance of these consequences have not been thoroughly studied. These questions can be addressed in an animal model of natural lentivirus infection. The present study examined site-directed mutants of a pathogenic molecular clone of feline immunodeficiency virus (FIV), FIV-pPPR, as potential models for studying the effects of (-)-L-2',3'-dideoxy-3'-thiacytidine (3TC, lamivudine) resistance on lentiviral pathogenesis. A methionine-to-valine (M183V) mutation at codon 183 of FTV, located in the highly conserved YMDD motif of the RT-encoding region of the pol gene, conferred high-level phenotypic resistance to 3TC and cross-resistance to the related compound, (-)-β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine [(−)-FTC]. 3TC resistance was similar in magnitude to HIV-1 with a homologous mutation at codon 184 (M184V) that characteristically develops in 3TC-treated HIV-1-infected patients. This FIV-pPPR M183V mutant will be useful in studying the effects of 3TC resistance on lentiviral pathogenesis.

Part II

The ADV-Utah 1 biological isolate of Aleutian mink disease parvovirus (ADV) replicates efficiently and is highly pathogenic in mink but cannot be propagated in vitro. The ADV-G molecular clone derived from cell culture-adapted ADV-Utah 1 does not replicate or cause disease in adult mink but can be propagated to high titers in vitro. Recently, the first chimeric viruses of ADV-Utah 1 and ADV-G that could replicate both in vitro and in vivo and cause pathogenesis in mink were described. ADV-G/U-8 infection was associated with higher antibody titers and more severe histopathological lesions than ADV-G/U-10. These chimeras, however, differed by only one capsid and three nonstructural protein amino acid changes. The purpose of the current studies was to determine whether differences in in vivo replication and pathogenesis between G/U-8 and G/U-10 were attributable to capsid or nonstructural protein changes. In order to answer this question, additional chimeric viruses were constructed, propagated in vitro, and used to infect mink. Antiviral antibody responses, viremia, hypergammaglobulinemia, and histopathological lesions indicated that codon 352 of the capsid protein influenced in vivo replication and pathogenesis, but the effects of the nonstructural protein likely played an interactive role as well.
PREFACE

My experiences as a graduate student held many surprises and lessons for me. The biggest surprise was that the most important things I learned during the past four years were about life, not about science. There were times when the obstacles seemed so arbitrary and unfair and insurmountable that I felt certain they could not be overcome. Other moments were filled with the most extreme loneliness, fear, and self-doubt I have ever experienced. So many things seemed to be completely beyond my control. And, yet, through the toughest of times, there were those champions of moral decency and integrity who, by their own living examples, supported and encouraged me to be honest and fair, yet unwavering in my convictions, despite the consequences. They continued to believe with convincing sincerity that strength of personal character was more important than scientific or career accomplishments. And that, in fact, sooner or later, a life lived pursuing truth could overshadow the temporary power of politics. I am indebted to these advisors and role models who not only helped me weather difficult times, but who also gave me hope for the future: Drs. Don Christian, Jim Gannon, Walt Hill, and Bill McBroom, and my parents, Windell and Johann McCrackin. The influence of these people helped me turn a situation ripe for prolonged, destructive anger and bitterness into an opportunity for unexpected positive personal and professional growth and a great lesson in forgiveness.

I am also grateful for the wonderfully rewarding friendships that were a result of being in the right place at the right time. Drs. Jim Battisti, Angelika Longacre, Rachel
LaCasse, Scott Knight, Jim Fox, Klaus Jensen, Heidi Super, and Karin Peterson were all a help to me personally and professionally. Doug McBroom, Joan Strange, Deb Nycz, Betsy Kimmel, Kathy Pollis, Jim Wolfinbarger, Cynthia Favara, Kathy Wehrly, Don Dale, Dirk Whitsitt, Bob Evans, and Gary Hetrick taught me technical aspects of my work as well as to love what I do every day. A special thanks is due Doug McBroom for being the embodiment of true, lasting friendship. I am also thankful to my graduate committee for their time, understanding, and standards: Drs. Michael F. Minnick, Marshall E. Bloom, Rich Bridges, George L. Card, Walt E. Hill, and D. Scott Samuels. And to one of my biggest supporters and one of the finest gentlemen and veterinary pathologists I will ever know, Dr. William J. Hadlow, thank you very much for histopathological interpretations, clinical mink advice, stimulating discussions, and for being enthusiastic about my work.

The time I was able to spend at the Rocky Mountain Laboratories was an especially productive, rewarding, and healing time for me. I will always be indebted to Drs. Marshall Bloom and Bruce Chesbro for making this opportunity happen for me. Their personal support and encouragement of me and my career efforts has given me renewed confidence in myself and the scientific community. They, as well as other members of the Laboratory of Persistent Viral Diseases (special mention to Drs. Don Lodmell and John Portis), pushed my scientific thinking beyond the limits of the restrictive mental “box” I had created for myself in clinical veterinary practice. They opened up new possibilities and new ways of thinking on a broader scale than I had allowed myself previously. They also convinced me that I was ready for the next step
into independence as a biomedical researcher. Marshall, in particular, not only put up with but also cooperated with my demands to do certain things certain ways when it came to animal research. I know I was a pain and at times irritationally exacting about details concerning things that may have seemed unimportant at the time. Thanks for indulging me, Marshall. I have already made plans to start saving for your future therapy as a result of my year and a half in your lab. You deserve it.

And, finally, because there are just not enough appropriate words for describing the amazing and selfless contributions of my husband, Mike, thank you. I will always love you and could not have done this without you.

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CHAPTER 1
INTRODUCTION

DRUG RESISTANCE

Introduction. The emergence of drug-resistant pathogens, including bacteria, fungi, parasites, and viruses, represents the newest frontier in healthcare challenges facing the developed world. Methicillin-resistant *Staphylococcus aureus*, multidrug-resistant Gram negative enteric bacteria, and isoniazid-resistant *Mycobacterium tuberculosis* are currently responsible for significant morbidity and mortality in the United States. Fluconazole-resistant *Candida albicans* (1) and flucytosine-resistant *Cryptococcus neoformans* (103) are fungal opportunists particularly causing problems for immunocompromised patients. Parasites such as *Leishmania amazonensis* and *Trypanosoma cruzi* carrying genes encoding multidrug resistance (41) andazole resistance (8). Many viruses have also developed important drug resistance, including influenza A, herpes simplex virus (HSV), poxviruses, poliovirus, and human immunodeficiency virus type 1 (HIV-1) (86).

Antiviral drug resistance is not a new phenomenon. Reports of viruses resistant to antiviral agents first appeared in the 1960s and included influenza A (amantadine) (11),
HSV (iododeoxyuridine) (40), poxviruses (thiosemicarbazone) (3), and poliovirus (guanidine) (61). Widespread awareness of and interest in antiviral drug resistance did not occur until the 1980s when the difficulties of treating HIV-1-infected patients were well-publicized in the popular press. Advances in molecular biology during the 1980s also provided sophisticated new research tools for studying the small genomes of many of the pathogenic viruses.

At least four factors are believed to work together to make the development of drug resistance likely for any given virus. These factors are viral mutation frequency, magnitude and rate of virus replication, mutability of the viral target, and selective pressure of the antiviral drug (86). Retroviruses fit these criteria particularly well for being likely candidates to develop drug resistance. The in vivo mutation frequency of lentiviruses, such as HIV-1, is approximately $10^{-4}$ to $10^{-5}$ per nucleotide per round of replication (54). This means that approximately one mistake occurs in a 10 kilobase retroviral genome every time it is replicated. This relatively high mutation frequency, combined with a high rate and magnitude of viral replication ($10^{10}$ new HIV-1 virions per day per infected individual) (34), creates a diverse viral population capable of having almost any mutation pre-existing at any time (86) before drug selection pressure is present. The viral target of many antiretroviral drugs, the reverse transcriptase (RT) enzyme, has been shown to mutate rapidly in the presence of natural substrate analogs containing distinctive sugar moieties, such as 3'-azido-3'-deoxythymidine (AZT) and (-)-β-L-2',3'-dideoxy-3'-thiacytidine (3TC) (86). Drug selection pressure is dose-dependent. At suboptimum drug doses, drug-resistant quasispecies replicate well, while wild-type
virus is inhibited. Once the drug dose surpasses a critical concentration, even drug-resistant virus replication is inhibited, slowing the overall rate and magnitude of viral replication (87).

The importance of trying to reduce retroviral replication rate to the lowest possible level in order to successfully control lentiviral disease cannot be overstated. The replication capabilities and persistence of HIV-1 mean that even low levels of virus replication will eventually be detrimental to the host. Treatment attempts that have been the most successful at significantly reducing virus replication are combination drug therapies directed against more than one viral target. The current approach, called highly active antiretroviral therapy (HAART), combines two RT inhibitors and a protease inhibitor. While several other novel targets are currently being investigated, including virus binding and entry (43), integrase (44), rev (20), and RNA secondary structure (20), nucleoside analog RT inhibitors continue to be the basic foundation of current antiretroviral therapy.

**Reverse transcriptase.** RT is a viral-encoded RNA-dependent DNA polymerase that plays a crucial role in the life cycle of retroviruses. Double-stranded proviral DNA that can be integrated into the host cell genome must be made from single-stranded genomic viral RNA. The process of reverse transcription by RT involves first making a minus-sense strand of DNA complementary to the plus-sense genomic RNA. The viral RNA template is then degraded by the RNase H activity of RT as a complementary plus-sense DNA strand is synthesized based on the new minus-sense DNA template. Blocking this step of the retrovirus life cycle inhibits viral replication because to persist inside the host
cell and to be transcribed by the host cell machinery, RNA polymerase II, proviral DNA must be produced and integrated into the host cell genome (99).

RT is a heterodimer composed of two subunits, p66 and p51. The larger subunit contains the active sites of polymerase activity and RNase H activity. The smaller subunit is identical in primary amino acid sequence to the larger subunit but lacks the C-terminal portion that makes up the RNase H active site. The tertiary structure of RT has been compared to a human hand in shape, exhibiting palm, fingers, thumb, and connection domains (99). The palm domain of the large subunit contains the polymerase active site, consisting of three aspartic acid residues (residues 110, 185, 186) (6,49). Two of these aspartic acid residues are part of the YXDD motif, the most highly conserved portion of RT across species (107).

The ability to easily measure RT function and its inhibition on the enzyme level in vitro has greatly added to the understanding of RT function and the interactions of some inhibitors. RT is not posttranslationally modified, so recombinant RT has the same enzymatic properties as virion RT (99). p51 subunits can be produced by either treating recombinant RT (rRT) with species-specific protease or allowing autodigestion of bacterial lysates containing rRT at 4°C overnight (D. G. McBroom, unpublished data). While data describing RT kinetics, inhibition, processivity, and fidelity can be generated in vitro in convenient test tube reactions, their meanings can easily be misleading if not interpreted in light of more physiologically relevant data. For instance, Mansky and Temin showed that the in vivo mutation rate of HIV-1 is lower than that predicted from the in vitro measured fidelity of purified HIV-1 RT (54). Also, it is well known that the
phenotypic resistance of AZT-resistant patient isolates often does not correlate with in vitro data generated using purified RT (23,47,56).

**RT inhibitors.** The two classes of antiviral drugs that have been used clinically to inhibit RT function are the nucleoside analog inhibitors and the non-nucleoside RT inhibitors (NNRTI) (20). NNRTIs are noncompetitive RT inhibitors that exhibit very specific binding, so NNRTIs effective against HIV-1 RT are not useful for RT of other species. Resistance to NNRTIs develops very rapidly in both cell culture and in treated patients, so their usefulness has been limited. However, of the antiretroviral agents currently available, the relationship between the development of drug resistance and treatment failure is most straightforward for the NNRTIs (88,89).

Nucleoside analog inhibitors are the most commonly used antiretroviral drugs to treat lentivirus infections. Several of them, such as AZT, ddI, and ddC, were used for cancer treatment prior to their use as antiretrovirals because of their ability to inhibit cellular polymerases (20). In fact, this may be partly responsible for the toxicity associated with high doses of some of the nucleoside analogs. Nucleoside analogs function as molecular mimics of the natural substrates of RT, the nucleoside triphosphates. The marketed drugs must be metabolized intracellularly to their active 5'-triphosphate forms. Their mechanisms of action include competitive inhibition and chain termination (28,62).

**Transmission of drug-resistant virus.** Drug resistant isolates of HIV-1 cause problems not only in infected patients, but also as infectious pathogens (13). Initially, it was suspected that drug-resistant isolates may not be present in high enough numbers or
replicate well enough to survive in vivo transfer from one individual to another (13). However, transmission of AZT-resistant virus from one person to another has been documented for a fetus from an infected mother (93), by parenteral inoculation (2,24), and by sexual intercourse (12,22,104). It has not yet been determined if infection with AZT-resistant HIV-1 follows a different clinical course or a different rate of virological or immunological disease progression (13). The number of reports of natural transmission of AZT-resistant HIV-1 suggests that the incidence of newly-diagnosed HIV-1-infected patients carrying drug-resistant virus populations will continue to increase. It will be important to understand how the biology of initial drug-resistant viral infection may vary from infection with wild-type virus so that appropriate treatments can be devised.

FELINE IMMUNODEFICIENCY VIRUS: ANIMAL MODEL FOR HIV-1

Clinical Syndrome. Feline immunodeficiency virus (FIV) is a natural disease of the domestic cat population, as well as wild, giant cats of North America and Africa (68). Clinical disease caused by FIV in domestic cats is almost identical to that of HIV-1 in humans (21,71,72,76,109). Cats experience an acute phase of disease characterized by lymphadenopathy, fever, malaise, viremia, and a decrease in CD4+ T cells (71). This is followed by a long latent period of several years identified by high antibody and low viral antigen levels in the peripheral blood. Clinical decline is marked by a resurgence of viremia, anorexia, poor haircoat, weight loss, gingivitis, neurological signs, and opportunistic infections (21,71,72,76,109).

Use of specific pathogen free (SPF) cats in experimental infection consistently
produces acute phase disease, including immunological and neurological abnormalities and behavioral disorders (33). In addition to classical alterations in the CD4+ T cell count, functional immune suppression as evidenced by susceptibility to a normally avirulent strain of *Toxoplasma gondii* was clearly demonstrated in cats infected with FIV-NCSU (15). Recent experimental work on the neurovirulence of FIV and its mechanisms showed that neonatal kittens infected with a cerebrospinal fluid isolate of FIV were neurodevelopmentally delayed compared to control kittens and kittens infected with a peripheral blood mononuclear cell (PBMC) isolate of FIV (78).

Neurodevelopment was assessed by determining the age at which infected and control kittens could playfully interact, walk, run, air right, walk along a plank, and acquire a blink reflex (78). In addition to virus strain, immune suppression likely played a role in neurovirulence. Kittens infected with a PBMC isolate of FIV, FIV-Petaluma, and treated with cyclosporin A, a potent T cell inhibitor, showed delay of neurodevelopment and reduced *N*-acetyl aspartate/creatine ratios in the brain (78). Another group studying the neurovirulent FIV-MD biological strain of FIV recently showed that infected SPF cats exhibited higher basal activity levels and higher distractibility during a spatial learning task compared to control cats (96). Field observations, locomotion tests, traversing planks of varying widths for a food reward, and a spatial learning task were used to measure behavior and cognition in adult cats that were infected with FIV-MD as kittens (96). Two investigative groups have also shown that neuronal loss, a hallmark of neuropathogenesis in HIV-1 infection, occurs with experimental FIV infection (7,79). The target cells of FIV infection in the central nervous system are likely astrocytes and
microglia (17,18), with neuronal injury occurring secondarily (26).

Microscopic neuropathologic changes have been documented in both naturally infected (18) and experimentally infected cats (19,72,76). Lesions included mild histiocytic and lymphocytic infiltration of vessel walls in cats infected with the minimally neuropathogenic FIV-Petaluma (19). The characteristically neuropathogenic FIV-MD biological isolate was associated with cortical (72) and subcortical (72,76) perivascular lymphocytic or monocytic infiltrates. In addition, neuronal satellitosis was evident in the deep cerebrocortical lamina of the parietal/occipital region of the brain (76), and glial nodules were seen in the parahippocampal gyrus (72). These histopathological abnormalities are similar to those seen in HIV-1 infection (18) of the central nervous system with the exception of the low incidence of multinucleated giant cells (26).

**Pathogenic molecular clones of FIV.** Pathogenesis studies in the FIV model in the United States were initially done using various clinical isolates, or swarm populations (21,71,72,76,109). Lack of a completely sequenced pathogenic molecular clone limited drug resistance studies based on single point mutations. Phillips, et. al., developed the FIV-pPPR molecular clone in 1990 (74). The complete sequence is registered in the GenBank database (accession no. M36968) and is carried in a pUC119 background along with 150 - 300 bases of feline genomic DNA at the 5' and 3' ends of the virus (74). The immuno- and neuropathogenesis of FIV-pPPR have been extensively characterized in SPF cats (73). Immunological abnormalities in acute phase disease include plasma antigenemia, plasma viremia, inversion of the CD4+/CD8+ T cell ratio, and decreased total CD4+ T cell counts. Later, a rise in antibody titers and decrease in viral load are
seen (73). Neurological abnormalities observed include delayed pupillary reflex, delayed righting reflex, and anisocoria. Approximately 50% of SPF cats infected with wild-type FIV-pPPR develop posterior paresis within 6 months of infection (73). Measurable neurological dysfunctions include delayed brainstem auditory evoked responses (BAER), visual evoked potentials (VEP), and peripheral nerve conduction velocities (33, 72, 73). These electrophysiological changes all occur during the first six months following infection and can be detected even in the face of clinical normalcy. Sleep disturbances and electroencephalographic (EEG) alterations have been measured one year postinfection with the FIV-MD biological strain of FIV (80). These findings parallel early neuropsychiatric symptoms seen in HIV-1-infected patients, often heralding the onset of AIDS.

A primarily immunopathogenic molecular clone of FIV, FIV-NCSU₁-JSY3, was developed in the laboratories of Wayne A. F. and Mary B. Tompkins in 1996 (110). A lambda clone containing the full-length FIV genome was determined infectious for cats (110). The cloned viral genome has since been transferred into a plasmid vector, and this entire plasmid is currently being sequenced (Tedd Childers, personal communication, March, 1999). At the time the present work was started, this clone was not characterized sufficiently to be used for molecular virology studies; however, it will provide another alternative to FIV-pPPR for a pathogenic molecular clone of FIV in the future.

In comparison to FIV-pPPR, the JSY3 molecular clone of FIV-NCSU₁ was not reported to have primarily neurologic manifestations in experimentally infected cats but rather signs of immunologic impairment very similar to the biological parent virus. The
JSY3 molecular clone maintained tropism for primary cells of cats, induced a significant inversion of the CD4+/CD8+ ratio by six weeks postinfection, provoked a strong antibody response to gag and env antigens, and stimulated a heavy burden of FIV provirus in PBMC during acute-stage infection (110). Most convincingly, SPF cats experimentally infected with the JSY3 clone of FIV-NCSU were susceptible to a normally avirulent strain of Toxoplasma gondii by 29 weeks postinfection (110). These results demonstrated severe functional immunosuppression associated with FIV-NCSU-JSY3 infection.

**Drug resistance of nucleoside analogs to FIV in vitro.** Nucleoside analog drug resistance in the FIV model has been studied both at the enzyme level as well as the phenotypic level using a nonpathogenic molecular clone of FIV in an immortalized cell line. The work presented in this thesis represents the first attempts to study nucleoside analog drug resistance using a pathogenic molecular clone in primary cell culture of PBMC.

FIV RT of the nonpathogenic molecular clone, FIV-p34TF10 (97), is similar to HIV-1 RT in physical properties, catalytic activities, and sensitivity to the active forms of nucleoside analogs (63,64,66). FIV RT has also been expressed in a recombinant protein expression system and used extensively to study RT kinetics (65).

The first reported AZT-resistant lentivirus selected in cell culture was characterized in Crandell feline kidney cells (CrFK) using the biological isolate, FIV-Petaluma (82). Subsequently, resistant mutants of FIV-p34TF10 to ddI (27), ddC (59), d4T (113), FTC (94), 3TC (95), AZT and ddI (27), and AZT and 3TC (95) were selected in vitro. Single point mutations have proven to be responsible for the observed drug
resistance of these mutants. Limiting the study of these mutants in vivo is the inability of FIV-p34TF10 to cause clinical disease in experimentally infected cats. Limiting the direct application of in vitro-generated drug resistance information from the FIV system has also been the nonhomologous nature of the selected FIV mutations to HIV-1, with the exception of the M183T mutation selected with FTC (94). This mutation has only been seen during in vitro selection procedures in HIV-1 (42); it has never been reported from an oxathioline nucleoside-treated HIV-1-infected patient. Subsequently, its clinical relevance remains unclear.

**Drug resistance of nucleoside analogs to FIV in vivo.** Previous in vivo work in cats using nucleoside analogs targeted feline leukemia virus (FeLV) and primarily involved the drugs, AZT (57,58,98,111) and ddC (77,98,112). Limited work has been reported treating FIV infection with AZT (32) and ddC (53). Interest in the phosphonoformates, pyrophosphate analogs that do not require intracellular activation (13), also began in the FeLV model (35). Experiments evaluating the effectiveness of phosphonoformates in treating FIV-infected cats used the drug PMEA (30,31,75), which was limited by toxicity, and other phosphonoformate derivatives, [(R)-PMPDAP] (101) and FPMPA (31), which showed promise for clinical use. Current therapy in the United States for naturally FIV-infected cats includes AZT and 3TC (Julie Levy, personal communication, 1999). Drug resistance mutations have not yet been reported in naturally infected, nucleoside analog-treated cats. However, AZT resistance mutations have been studied to a limited degree in macaques using simian immunodeficiency virus (SIV) (102).

As mentioned above, the work reported in this thesis represents the first attempts
to study drug resistance mutations in a pathogenic molecular clone of FIV in primary cell culture of PBMC. The ultimate goal of transferring the technology of studying drug resistance into primary cells was to be able to study \textit{in vitro}-engineered and characterized mutants in the \textit{in vivo} cat model. This would allow the comparison of pathogenicity between wild-type and specific drug-resistant mutants in experimentally infected SPF cats. These comparisons may be helpful in unraveling the question of whether drug-resistant mutants are more or less pathogenic than wild-type virus. The answer to this question may significantly impact the therapy of FIV-infected cats and HIV-1-infected people.
CHAPTER 2
MATERIALS AND METHODS

ANIMALS

Uninfected specific pathogen free (SPF) cats were used as blood donors in order to harvest peripheral blood mononuclear cells (PBMC) for primary cell culture. Cats were neutered adults, three females and one male, on loan from the laboratory of Niels C. Pedersen (University of California, Davis, CA). Housing and husbandry practices were in strict conformance with federal guidelines. All animal procedures were approved by the Institutional Animal Care and Use Committee at The University of Montana.

Cats were tranquilized with 0.25 mg acepromazine maleate and 20 mg ketamine hydrochloride intravenously in the saphenous vein. Whole blood was collected by aseptic jugular venipuncture and transferred to sodium heparin vacuum tubes (Becton-Dickinson, Franklin Lakes, NJ). Heart rate and respiratory rate were monitored until the cats were sternally recumbent.

CELLS

Crandell feline kidney cells. Crandell feline kidney cells (CrFK) (American Type Culture Collection, Rockville, MD) were maintained in cell culture as previously described (66).

Peripheral blood mononuclear cells. SPF PBMC were separated over a Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) from heparinized whole blood diluted with an
equal volume of Hank’s balanced salt solution. PBMC were maintained in RPMI-1640 medium containing human recombinant IL-2 (100 u/ml) (gift of Niels C. Pedersen), penicillin (50 IU/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer (10 mM), β-2-mercaptoethanol (5 x 10^-5 M), sodium pyruvate (1 mM), and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) after an initial 48-h mitogenic stimulation with conconavalin A (con A) (1 μg/ml).

VIRUS

Pathogenic molecular clone. First passage virus stocks of the pathogenic molecular clone, FIV-pPPR, were prepared by transfecting CrFK cells with proviral plasmid DNA (74) purified from the J5 strain of *Escherichia coli* JM109 using a Wizard Plus miniprep kit (Promega, Madison, WI). *E. coli* JM109-J5 containing the FIV-pPPR plasmid was a generous gift of Tom R. Phillips (The Scripps Research Institute, San Diego, CA). Transfection was done using DOTAP liposomal transfection agent (Boehringer Mannheim, Indianapolis, IN) as recommended by the company, then transfected CrFK were cocultured for 48 h with SPF feline PBMC. Supernatants containing nonadherent PBMC were then transferred to new flasks and cultured separately. Supernatants from infected PBMC cultures were monitored for rising RT activity measured on a poly(rA)-oligo(dT)\textsubscript{10} template as previously reported (66). Cell-free virus was harvested on the 3 to 5 days of maximal RT activity, clarified by centrifugation for 10 min at 500 x g, pooled, and filtered through a 0.45 μm filter into aliquots for -80°C storage.
**Natural virus population.** The propagation of a natural virus population, named FIV-Maxam, was done by culturing PBMC from an untreated, naturally infected cat with equal numbers of uninfected SPF feline PBMC as previously published (71). Heparinized whole blood was a kind gift of Alan B. Applebury (Valley Pet Clinic, Hamilton, MT). Plasma screening was positive for FIV antibody and negative for feline leukemia virus (FeLV) antigen (IDEXX, Portland, ME). Supernatant RT activity was measured and cell-free virus harvested on 5 days of peak RT activity. Cell-free virus was processed as for FIV-pPPR stocks.

Three parameters used to verify the identity of the cultured virus included successful PCR amplification of the RT-encoding region of the *pol* gene, maximal RT activity in the presence of Mg$^{2+}$ rather than Mn$^{2+}$ cations to distinguish from FeLV, and the ability to infect uninfected PBMC. First, genomic DNA was harvested from infected PBMC using a QIAamp tissue kit (Qiagen, Inc., Valencia, CA) and was used as template for PCR amplification using FIV-p34TF10 RT primers four (5'-GTAATGTTTGTGTCTTAGAAGGATAACTC-3') and six (5'-ATCATATCCTGACATCTTTGACCT-3'). A 25-min hot start at 94°C followed by 30 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 2 min produced a 2-kb product identical in electrophoretic mobility to the FIV-pPPR positive control (data not shown). Next, RT analysis of crude lysates of FIV-pPPR and FIV-Maxam on a poly(rA)-oligo(dT)$_{10}$ template in the presence of varying concentrations of either Mg$^{2+}$ or Mn$^{2+}$ ions was done as previously described (71), demonstrating maximal Mg$^{2+}$-dependent RT activity at 5 mM for both viruses (Fig. 2.1). Finally, stored viral stocks were thawed and

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Figure 2.1. Reverse transcriptase (RT) activity of FIV-pPPR and FIV-Maxam in the presence of varying concentrations of Mg$^{2+}$ and Mn$^{2+}$. FIV-pPPR is a pathogenic molecular clone; and FIV-Maxam is a clinical isolate. Viruses were propagated in peripheral blood mononuclear cells (PBMC) of specific pathogen free (SPF) cats. Crude lysates of cell culture supernatants were tested for RT activity on a poly(rA)-oligo(dT)$_{10}$ template in the presence of various concentrations of the cations, magnesium and manganese. Magnesium-dependent RT activity is characteristic of feline immunodeficiency virus.
incubated with uninfected SPF feline PBMC. Rising RT activity confirmed viral stock infectivity (data not shown).

ASSAYS

Introduction. While titering and drug susceptibility testing for HIV-1 biological isolates and infectious molecular clones are standardized in human PBMC (39), these types of assays were not routine nor standardized for feline PBMC when this work was begun. These assay conditions were maximized for feline PBMC in 24-well plates for an assay endpoint of 7 days postinfection (data not shown). This timing was similar to that used for HIV-1 testing as recommended by the AIDS Clinical Trials Group (39,55).

Virus titering. Virus stocks were titered in two 24-well plates by culturing quadruplicate replicas of 2 x 10^5 infected SPF feline PBMC per well (0.7 ml medium per well). Infections were 5-fold serial dilutions of virus starting with either a 1:5 dilution or 1:10 dilution (one plate for each) (modification of original protocol provided by Lawrence E. Mathes, The Ohio State University, Columbus, OH). Fresh medium was added 72 h postinfection (0.5 ml/well), and 0.3 ml supernatant was harvested from each well 6.5 days postinfection and stored at -80°C.

Samples were tested for p24 antigen expression using an antigen capture ELISA assay (antibodies kindly provided by Niels C. Pedersen) (14). Wells designated positive were those with detectable levels of antigen more than 2 standard deviations greater than the mean of the negative control wells. Titers expressed as tissue culture infective doses for 50% of the cultures (TCID₅₀) were calculated using the Spearman-Karber equation.
Drug susceptibility. To assess nucleoside analog susceptibility, dose response assays for PBMC were designed to parallel current CrFK assays used in the laboratory and methods used by the AIDS Clinical Trials Group (39). Five million \((5 \times 10^6)\) con A-stimulated SPF feline PBMC cultured for 6 days were infected \textit{en masse} at a multiplicity of infection (MOI) of 0.001 in a total volume of 2 ml medium for 2-4 h. Quadruplicate replicas of sterile distilled water or one of five dilutions of drug were made in 2 ml of medium per well in a 24-well plate. Two hundred thousand \((2 \times 10^5)\) infected cells were then added to each well of the pretreated plate and incubated at 37°C in 5% CO\(_2\) for 7 days. One ml of medium was removed from each well 3.5 days postinfection and was replaced with fresh medium and drug. Supernatant samples were harvested on day 7 postinfection, tested for p24 antigen expression, replicas averaged, and data plotted as inhibitor concentration versus p24 gag protein production expressed as a percentage of the control value (Fig. 3.2). Drug concentration needed for 50% inhibition (IC\(_{50}\)) of p24 gag protein production was determined from the linear portion of the curve using computer-generated linear regression as previously reported for CrFK assays (83). Experiments evaluating AZT (gift of Burroughs Wellcome Co., Research Triangle Park, NC), 3TC (kind gift of Raymond F. Schinazi, Emory University, Atlanta, GA), and 2',3'-dideoxycytidine (ddC) (Sigma Chemical Co., St. Louis, MO) were done at least twice with each virus. Mean IC\(_{50}\) values of FIV-Maxam and FIV-pPPR mutants were compared to wild-type FIV-pPPR data using a Student’s t-test with a significance level of \(p \leq 0.05\) (SPSS, Chicago, IL).
**Replication kinetics.** In order to compare replication kinetics between different virus stocks, 5 x 10⁶ SPF PBMC were adsorbed with virus at an MOI of 0.001 in 2 ml medium for 2 - 4 h. Three ml of medium were added for a final volume of 5 ml and a final cell concentration of 1 x 10⁶ cells ml⁻¹ in an upright T-25 culture flask. Cells were incubated at 37°C in 5% CO₂. One-ml samples for RT activity determination were collected on days 3, 6, 8, 10, 13, and 15, and 0.3-ml samples for p24 antigen measurement were collected on days 1, 2, 3, 4, 5, 6, 8, 10, 13, and 15. RT samples were centrifuged at 41,000 x g for 60 min at 4°C, the resulting virion pellets lysed with 15 µl detergent buffer as described previously (66), and samples stored along with p24 antigen samples at -80°C until assayed.

**Reverse transcriptase.** Virion RT from FIV-pPPR and FIV-p34TF10 was harvested from cell culture supernatants and purified using DEAE-cellulose and phosphocellulose (Whatman, Hillsboro, OR) column chromatography as previously described (64). Assays for RT activity using poly(rA)-oligo(dT)₁₀ or poly(rI)-oligo(dC) template-primers (Pharmacia LKB, Piscataway, NJ) were done as previously reported (66). Assays were repeated at least twice with 3 determinations per experiment. Double reciprocal plots were used to determine kinetic constants (Kₘ and Kᵢ) as previously published (63). Means were compared using a Student’s t-test with a significance level of p ≤ 0.05 (SPSS, Chicago, IL).

**MOLECULAR CLONING OF MUTANT VIRUSES**

An 877-bp Nco₁-Bgl₂ fragment, corresponding to nucleotides 2499 to 3376 of the...
parental FIV-pPPR plasmid, was cloned into Litmus-29 (New England Biolabs, Inc., Beverly, MA) and mutagenized with the Muta-Gene Phagemid kit (Bio-Rad Laboratories, Hercules, CA). Mutagenesis primers 5'-TCAATATGTGGATGACA-3' and 5'-TCAATATAACGGATGACA-3' were used to introduce the M183V and M183T mutations, respectively (mutations underlined). After sequence analysis to verify the presence of the desired mutations, the 877-bp NcoI-BglII fragment was ligated into NcoI-BglII-digested FIV-pPPR and transformed into SURE-2 Supercompetent E. coli (Stratagene) and incubated at 32°C. The complete insert was sequenced to confirm the presence of desired mutations and the integrity of the remainder of the fragment. All sequencing was done at the Murdock Molecular Biology Center using a Taq DyeDeoxy Terminator sequencing kit and a model 373A automated DNA sequencer (Applied Biosystems). Full-length mutated clones were transformed into the J5 strain of E. coli JM109 to prevent bacterial degradation of the plasmid during long-term storage and plasmid propagation.

Proviral mutant DNA was transfected into CrFK cells and cocultured with SPF feline PBMC as described for FIV-pPPR. Mutant viruses were cultured in the presence of 5 μM 3TC to maintain selection pressure for the introduced mutations. Viral stocks were titered as described for FIV-pPPR and FIV-MAXAM.

REVERSION STUDIES

The FIV-pPPR codon 183 mutants were passaged for three rounds of infection in SPF feline PBMC in the absence of 3TC. Viral stocks and infected PBMC were
harvested from each passage. Viral stocks from each new round of infection were titered so that the subsequent round of infection was started with an MOI of 0.001. For consistency throughout the experiment, virus was always passaged in cells from the same donor, and titers were always measured in cells from the same donor. Drug susceptibility assays were all done in parallel in cells from one donor after virus passaging was completed.

Genomic DNA was isolated and purified from infected PBMC of each round of infection using a QIAamp tissue kit (Qiagen, Inc., Valencia, CA). Purified genomic DNA was used as template for PCR amplification using FIV-specific primers as described for FIV-MAXAM. PCR amplification products were sequenced to determine if the introduced mutations were present after each round of infection.
CHAPTER 3

MUTANTS OF PATHOGENIC FELINE IMMUNODEFICIENCY VIRUS
RESEMBLE HOMOLOGOUS MUTANTS OF HUMAN IMMUNODEFICIENCY
VIRUS TYPE 1 IN 3TC RESISTANCE

INTRODUCTION

Antiretroviral drug resistance develops during treatment of patients infected with human immunodeficiency virus type 1 (HIV-1) (46,60,85). The effects of drug resistance on the pathogenesis of HIV-1 are not known and cannot be thoroughly studied in humans (46,85). There is currently a need for studies in appropriate animal models to answer basic questions about the effects of drug resistance on lentiviral pathogenesis (85). Data from such studies may provide valuable information for making better therapeutic decisions in the treatment of HIV-1-infected patients.

Understanding the effects of oxathiolane nucleoside analog resistance on the pathogenesis of lentiviruses is especially critical since (-)-β-L-2',3'-dideoxy-3'-thiacytidine (3TC, lamivudine) has become an important part of highly active antiretroviral therapy (HAART) for HIV-1 infection (9,29). A mutant virus population carrying a methionine-to-valine mutation at codon 184 (M184V) of the reverse transcriptase (RT)-encoding region of the pol gene characteristically arises in HIV-1-infected patients within weeks of starting treatment with 3TC (92). This mutation is interesting because it also changes pre-existing phenotypic 3'-azido-3'-deoxythymidine

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(AZT) resistance to AZT susceptibility (48,100), enhances RT in vitro insertion fidelity (36,69,70,105,106), and may decrease viral replication rate (4,42,108). These changes could be favorable for HIV-1-infected patients if they decreased the pathogenicity of the mutant virus compared to wild-type virus. Another mutation of methionine-to-threonine (M184T) at codon 184 of HIV-1 has been selected in vitro and also confers resistance to 3TC (42). HIV-1 codon 184 is located in the highly conserved YMDD motif of the RT-encoding region of the pol gene (38,45), increasing the probability that genetically similar mutants of other lentiviral species may show functional homology and comparable drug resistance.

FIV is a lentivirus that causes an immunodeficiency syndrome in cats similar to that seen in HIV-1-infected humans (21,71,76,109). FIV has been used as an in vivo animal model for studying HIV-1 pathogenesis issues such as immunological and neurological abnormalities (21,33,72,76,80,109,110), vertical transmission (67), opportunistic infections (15,84), and cytokine dysregulation (50,52). The cell culture-adapted molecular clone of FIV-Petaluma, FIV-p34TF10 (97), has been used extensively for in vitro selection of nucleoside analog-resistant mutants (27,59,83,94,95,113). In fact, a FIV-p34TF10 M183T mutant, homologous to the HIV-1 M184T mutant, was selected in cell culture in the presence of the 3TC-related oxathioline nucleoside analog, (-)-β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine [(-)-FTC] (94). However, in vivo pathogenesis studies using drug-resistant mutants have been hindered by the inability of FIV-p34TF10 to produce clinical disease in experimentally infected cats. The development and characterization of an immuno- and neuropathogenic molecular clone, FIV-pPPR (74),

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has provided a tool for studying pathogenesis and drug resistance in cats.

The purpose of the present studies was threefold. First, we compared in vitro replication kinetics and nucleoside analog susceptibilities of FIV-pPPR with those of a clinical FIV isolate to determine if the molecular clone accurately reflected the properties of natural FIV. Next, we characterized the nucleoside analog susceptibility and enzyme kinetics of purified FIV-pPPR virion RT. Finally, we engineered site-directed mutations homologous to codon 184 mutations of HIV-1 into the RT-encoding region of the pol gene of FIV-pPPR and determined the susceptibility of the resulting mutants to 3TC and other selected nucleoside analogs.

**EXPERIMENTAL RESULTS**

**Virus Titering.** It was necessary to propagate and store first passage viral stocks of FIV-pPPR, a biological FIV isolate (FIV-Maxam), and FIV-pPPR mutants in order to do replication studies and nucleoside analog susceptibility testing on virus allowed minimal time in culture for acquiring new mutations. Viral growth and titer assays using FIV-pPPR and FIV-MAXAM were done in the absence of drug. Titers were $8.5 \times 10^3$ TCID$_{50}$/ml for FIV-pPPR and $5.5 \times 10^3$ TCID$_{50}$/ml for FIV-Maxam.

First passage viral stocks of FIV-pPPR M183V and M183T mutants were propagated in the presence of 5 μM 3TC in order to maintain drug selection pressure and to assure maintenance of the alleged drug resistance mutations. This drug concentration was chosen because it was about 10-fold higher than the measured IC$_{50}$ of wild-type virus,
and both mutants were predicted to be about 100-fold resistant to 3TC based on data in the HIV-1 literature (4,42,90,91,100). Titers were $7 \times 10^3$ TCID$_{50}$/ml for M183V and $8.1 \times 10^2$ TCID$_{50}$/ml for M183T. Drug susceptibility assays done later would show that the IC$_{50}$ of 3TC for the M183T mutant was actually slightly less than 5 μM. Subsequently, the titer of the virus stock should have been suppressed by at least 50% as a consequence of the relatively high drug concentration present. The corrected titer would be about $2 \times 10^3$ TCID$_{50}$/ml.

**Molecular clone compared to natural virus isolate.** Next, in order to ensure that results of future studies would be applicable to natural FIV infection, we determined if the replication kinetics and nucleoside analog drug susceptibilities of the pathogenic molecular clone, FIV-pPPR, accurately reflected properties of a natural FIV isolate. Curves representing RT activity of FIV-pPPR and FIV-Maxam were similar, with standard error bars overlapping on days 6 and 8 postinfection during the log phase of virus replication (Fig. 3.1). p24 antigen levels gave similar results (data not shown). These experiments indicated that FIV-pPPR and FIV-Maxam replicated at similar rates and that day 7 postinfection was an appropriate endpoint for drug susceptibility assays since this time point occurred during the log phase of viral replication. FIV-pPPR and FIV-Maxam IC$_{50}$ values for AZT and ddC were similar, but the IC$_{50}$ for 3TC for FIV-Maxam was significantly less than that of FIV-pPPR (Table 3.1). These results confirmed that FIV-pPPR closely reflects the *in vitro* behavior of a natural FIV isolate in replication kinetics. Nucleoside analog susceptibilities were similar between FIV-pPPR and FIV-Maxam. The increased susceptibility of FIV-Maxam to 3TC may have reflected
Figure 3.1. Replication kinetics of wild-type feline immunodeficiency virus (FIV) and site-directed mutants. Replication kinetics were measured by reverse transcriptase (RT) analysis of supernatants from specific pathogen free (SPF) feline peripheral blood mononuclear cells (PBMC) infected with uninfected tissue culture supernatant (sham) (■), wild-type pathogenic molecular clone (FIV-pPPR) (●), natural virus isolate (FTV-Maxam) (▼), and codon 183 mutants [FIV-pPPR M183V (○) and FIV-pPPR M183T (▼)]. Data from two or more experiments are graphed as mean values with error bars representing SEM.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IC₅₀ (µM) +/- SEM&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FIV-Maxam&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>AZT</td>
<td>0.07 +/- 0.02</td>
</tr>
<tr>
<td>3TC</td>
<td>0.14 +/- 0.005*</td>
</tr>
<tr>
<td>(-)-FTC</td>
<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>ddC</td>
<td>0.86 +/- 0.07</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values were determined by p24 gag protein production inhibition assays in feline peripheral blood mononuclear cells and represent two or more experiments with four determinations per experiment.

<sup>b</sup> FIV-Maxam: natural isolate

<sup>c</sup> FIV-pPPR: wild-type pathogenic molecular clone

<sup>d</sup> M183V and M183T are site-directed mutants of FIV-pPPR. The wild-type methionine (ATG) at codon 183 of the RT-encoding region of the pol gene was changed to valine (GTG) and threonine (ACG), respectively.

<sup>e</sup> ND: not done

* Statistical significance was defined as $p \leq 0.05$ using a 2-tailed t-test (SPSS, Chicago, IL) comparing values for FIV-pPPR to other viruses.
the heterogeneity of the quasispecies of the biological isolate. The dominant quasispecies in the population may have been more susceptible to initial 3TC treatment than the homogeneous FIV-pPPR viral clone. In spite of this difference, FIV-pPPR appears to be suitable for modeling nucleoside analog susceptibility issues of natural FIV infection in PBMC.

**FIV-pPPR virion RT analysis.** Since RT is the target of nucleoside analog inhibitors, the interaction of the 5'-phosphorylated form of these drugs with the purified, physiologic form of the enzyme is important. Also, in order to use pathogenic FIV as a model for mechanisms of nucleoside analog resistance in HIV-1, it was important to determine how similar the interactions of activated drug with RT were between HIV-1 and FIV-pPPR. Previous work showed that the RT of a nonpathogenic molecular clone of FIV, FIV-p34TF10, was similar to HIV-1 RT biochemically and in nucleoside analog susceptibility (63). Therefore, we used purified virion RT of FIV-p34TF10 to compare to purified virion RT of FIV-pPPR. Kinetic values ($K_m$ and $K_i$) for FIV-pPPR RT were like FIV-p34TF10 with two exceptions (Table 3.2). The $K_m$ of dTTP was significantly less for FIV-pPPR compared to FIV-p34TF10. In addition, the $K_i$ for ddCTP was significantly higher for FIV-pPPR than for FIV-p34TF10. These kinetic data suggest that FIV-pPPR RT processes the substrate, dTTP, more efficiently than the RT of FIV-p34TF10. Also, the RT of FIV-pPPR requires a higher concentration of ddCTP to effect enzyme inhibition compared to the RT of FIV-p34TF10. FIV-pPPR kinetic measurements, however, were similar to values previously reported for HIV-1 (63,81).

**FIV-pPPR codon 183 mutants.** In order to determine if FIV-pPPR codon 183 mutants
TABLE 3.2. Inhibition of FIV-pPPR and FIV-p34TF10 purified virion RT by analogs of dTTP and dCTP

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>$K_m$ (μM) +/- SEM$^b$</th>
<th>$K_i$ (nM) +/- SEM$^b$</th>
<th>$K_i/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FIV-pPPR</td>
<td>FIV-p34TF10</td>
<td>FIV-pPPR</td>
</tr>
<tr>
<td>AZTTP</td>
<td>dTTP</td>
<td>2.7 +/- 0.7*</td>
<td>7.1 +/- 0.9*</td>
<td>7.0 +/- 1.2</td>
</tr>
<tr>
<td>D4TTP</td>
<td>dTTP</td>
<td>2.7 +/- 0.7*</td>
<td>7.1 +/- 0.9*</td>
<td>9.0 +/- 3.0</td>
</tr>
<tr>
<td>3TCTP</td>
<td>dCTP</td>
<td>6.2 +/- 0.3</td>
<td>5.2 +/- 1.2</td>
<td>251 +/- 48</td>
</tr>
<tr>
<td>(-)-FTCTP</td>
<td>dCTP</td>
<td>6.2 +/- 0.3</td>
<td>5.2 +/- 1.2</td>
<td>178 +/- 31</td>
</tr>
<tr>
<td>ddCTP</td>
<td>dCTP</td>
<td>6.2 +/- 0.3</td>
<td>5.2 +/- 1.2</td>
<td>370 +/- 79*</td>
</tr>
</tbody>
</table>

$^a$ RT assays were done using poly(rA)-oligo(dT)$_{10}$ template-primer for dTTP and its analogs and poly(rl)-oligo(dC)$_{10}$ for dCTP and its analogs.

$^b$ Values represent at least two experiments with three determinations per experiment. The mode of inhibition for all nucleoside analogs tested was competitive with respect to substrate.

*Statistical significance was defined as $p \leq 0.05$ for a 2-tailed t-test comparing FIV-pPPR with FIV-p34TF10 (SPSS, Chicago, IL).
exhibit drug resistance similar to their HIV-1 counterparts, FIV-pPPR mutants homologous to 3TC-resistant HIV-1 M184V and M184T mutants were cloned and characterized in vitro. The replication rates during the log phase of replication for FIV-pPPR M183V and M183T were similar to FIV-pPPR and FIV-Maxam (Fig. 3.1). While the magnitude of replication was slightly higher for wild-type FIV-pPPR than the other viruses, the initial rates were not detectably different from each other.

FIV-pPPR M183V was highly resistant to 3TC while M183T was intermediate in resistance to 3TC (Fig. 3.2, Table 3.1). Both mutant viruses appeared cross-resistant to the related oxathiolane nucleoside, (-)-β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine [(-)-FTC], to similar magnitudes over wild-type levels as they were to 3TC, but there was not a statistically significant difference between FIV-pPPR and FIV-pPPR M183V. Variation of the data for FIV-pPPR M183V likely affected the statistical results. More experimental repetitions might overcome this problem. Cross-resistance to AZT or ddC was not detected (Table 3.1). These data showed that mutations in the conserved YMDD motif of FIV-pPPR RT homologous to codon 184 mutations of HIV-1 RT confer drug resistance to 3TC.

**Mutant reversion studies.** Finally, in order to study the genetic stability of the FIV-pPPR M183V and M183T mutations in the absence of drug selection pressure, mutants were passaged for three rounds of infection in SPF feline PBMC without 3TC present. Both mutants remained phenotypically resistant to 3TC (30- and 4-fold for M183V and M183T, respectively), but their IC$_{50}$ values decreased from 38.3 to 12.2 μM for M183V and 4.8 to 1.6 μM for M183T. Sequence analysis of PCR-amplified proviral DNA from
Figure 3.2. 3TC dose response curve of wild-type feline immunodeficiency virus (FIV) and site-directed mutants. 3TC inhibition of p24 gag protein production in specific pathogen free (SPF) feline peripheral blood mononuclear cells (PBMC) infected with wild-type pathogenic molecular clone (FIV-pPPR) (○), natural virus isolate (FIV-Maxam) (▼), and codon 183 mutants [FIV-pPPR M183V (○) and FIV-pPPR M183T(▼)] was measured by ELISA. Results are from two or more experiments with four determinations per experiment. Error bars represent SEM.
genomic DNA extracts of infected PBMC confirmed the presence of the M183V and M183T mutations after three rounds and two rounds of infection, respectively. No evidence of emergence of the wild-type nucleotide sequence was demonstrated on sequencing chromatographs. These data suggested that FIV codon 183 mutations are genetically stable, but that some factor(s) outside the YMDD motif may be arising in the absence of selection pressure and suppressing the resistance effects of these mutations.

**DISCUSSION**

In summary, the FIV-pPPR M183V mutant was comparable to its HIV-1 homologue in magnitude of drug resistance (~80-fold). HIV-1 M184V mutants showed 100-fold or greater resistance to oxathiolane nucleosides when obtained from clinical patients treated with 3TC (92) or from in vitro selection using 3TC (25,91,100) or (-)-FTC (91). FIV-pPPR M183V also showed no cross-resistance to AZT or ddC, similar to results reported previously for HIV-1 M184V (91,100) and simian immunodeficiency virus (SIV) (10). The functional homologies we have shown between HIV-1 M184V and FIV-pPPR M183V and the clinical importance of the HIV-1 M184V mutant make the FIV-pPPR M183V mutant a logical first choice for pathogenesis studies in experimentally infected cats. The FIV-pPPR M183T mutant was somewhat less resistant (~10-fold) to 3TC than its in vitro-selected HIV-1 homologue (~100-fold). This transversion mutation (ATG to ACG) has only been found as a result of certain in vitro selection protocols in both FIV (94) and HIV-1 (42); its clinical significance is not
known.

Experiments evaluating replication kinetics showed no differences between wild-type FIV-pPPR and the codon 183 mutants. These findings contrasted with reported replication impairment and decreased viral fitness of the homologous HIV-1 mutants, HIV-1 M184V (4,42,108) and M184T (4,42). These observations may be explained by differences between the HIV-1 and FIV viruses.

Reversion studies allow multiple rounds of viral replication in the absence of drug. This mimics the discontinuation of drug therapy in drug-treated HIV-1-infected patients. Phenotypic 3TC resistance of FIV-pPPR M183V and M183T mutants decreased from 80-fold to 30-fold and from 10-fold to 4-fold, respectively, after 3 rounds of infection in the absence of 3TC. However, there was no sequencing chromatographic evidence of wild-type virus emergence. This suggested that factor(s) outside the YMDD motif affecting phenotypic drug resistance developed during the absence of drug selection pressure. These factors could be the development of mutations in other viral genes or changes in host cell factors. Previous work on rapidly reverting AZT-resistant mutants of FIV-p34TF10 (83) suggested a similar explanation involving areas outside the RT-encoding region of the pol gene. Determining and understanding these mechanisms may help clarify if scheduled drug rotation therapy is warranted or reasonable.

Our present work describes a new direction for using a pathogenic molecular clone of FIV, FIV-pPPR, as a model for studying nucleoside analog resistance of lentiviruses with direct application to HIV-1. Mutations responsible for drug resistance in conserved sites of HIV-1 RT can be identified and homologous mutations introduced
into FIV-pPPR. In vitro screening of the FIV mutants for drug resistance confirms whether functional homology exists. These mutants can then be studied in vivo to address questions of drug resistance effects on pathogenesis that cannot be evaluated in humans infected with HIV-1. Using highly conserved sites for initial mutation studies maximizes the likelihood that results of FIV pathogenesis studies will be applicable to HIV-1 treatment.
CHAPTER 4
FUTURE DIRECTIONS

In spite of the advances made in the treatment of human immunodeficiency virus type 1 (HIV-1) infection, there is still no cure. As drug resistance continues to develop against each new therapeutic agent, the focus of the drug industry is on developing novel approaches to inhibit more crucial steps in the life cycle of lentiviruses. Multiple drug therapy, for those who can afford it, will continue to be the foundation of treatment in the near future. As more and more different classes of drugs targeting everything from viral-encoded enzymes to viral regulatory proteins to RNA secondary structure are used simultaneously, the opportunity for drug interactions and toxicity increase. In addition, many questions about the basic biological mechanisms causing the observed pathogenesis associated with HIV-1 infection remain unanswered. Many of these issues can best be addressed in an animal model of natural lentiviral infection. Until there is a cure for HIV-1 infection, it is likely the feline immunodeficiency virus (FIV) model will continue to play an important role in lentiviral research.

The obvious first step after the work presented in this dissertation is to test the FIV-pPPR M183V mutant in specific pathogen free (SPF) cats. Cats infected with the 3TC-resistant mutant and treated with drug mimic HIV-1-infected patients on drug therapy who have developed resistant virus. Cats infected with FIV-pPPR M183V and not treated with 3TC mirror patients who have acquired drug resistance before
discontinuing drug therapy. Controls would include sham-infected and parental (FIV-pPPR) virus-infected groups with and without drug treatment. The purpose of this type of initial study is quite broad. From the sham-infected control groups, one would expect to learn the responses of healthy cats to testing procedures and the side effects of 3TC therapy in normal cats. Next, the untreated group infected with wild-type virus demonstrates the immunological and neurological consequences of viral infection, a positive control. In comparison, the wild-type virus-infected group treated with 3TC shows the efficacy of the drug treatment on the progression of disease and allows for the natural outgrowth of drug-resistant virus. Finally, the disease progression, host antiviral responses, viral replication, and tissue distribution of the mutant-infected groups would be compared to those of the wild-type virus-infected groups. Differences between these groups may help answer questions concerning the effects of drug resistance on viral replication and pathogenesis.

If pathogenic differences between wild-type and drug-resistant viruses are demonstrable, then continuing this line of research would be interesting and reasonable. The possibilities are that the mutant virus would be more pathogenic, less pathogenic, or the same as the wild-type parent. Studies would focus on the question of how the difference in virus genetics could account for those differences seen at the animal level. Tissue tropism, level of virus sequestration in tissue, viral replication, plasma viral load, functional immune capacity, and neurologic parameters may all be helpful in deciphering how variation occurs between parental and mutant viruses (5,15,16,73). In addition, the pharmacology of 3TC in the cat would need to be completely understood in order to
understand the drug selection pressure being applied to virus in various tissue types and how this may influence the development of drug resistance or reversion to a drug-susceptible state. On the other hand, if no detectable differences between FIV-pPPR- and FIV-pPPR M183V-infected cats were present, then the primary experimental question would return to how to more effectively and protractedly suppress viral replication, regardless of drug-resistance phenotype.

Another important question of drug resistance that should be answered in the FTV system is that of whether the M183V mutation introduced into an AZT-resistant clone can reverse AZT resistance to AZT sensitivity. This is one of the intriguing aspects of the M184V mutation in HIV-1. This experimental question could be approached in two different ways. It is possible that AZT-resistant FIV could be constructed by introducing the point mutation at codon 202 previously reported to confer AZT resistance on the nonpathogenic FIV molecular clone, FIV-p34TF10 (83). It is presently not known if this mutation confers AZT resistance in a pathogenic molecular clone of FIV. An alternative approach that mirrors HIV-1 more closely would be to introduce point mutations homologous to those seen in AZT-resistant HIV-1 in a step-wise fashion into a pathogenic FIV clone. In either case, once an AZT-resistant mutant was developed, the M183V mutation could be introduced into this (or these) clone(s) and the phenotypic drug susceptibility profile determined. Suppression of AZT resistance would further support FIV as an animal model for studying HIV-1 drug resistance issues.

In the area of new drug development, the FTV model may be useful for initial studies in animals. While cats have a clinical reputation for unusual drug metabolism,
drugs that are nontoxic for cats are generally also nontoxic for humans. This rule of thumb, however, does not always work in the opposite direction, so some drugs available for treatment of humans may not be workable in the FIV model. With new protease inhibitors effective against FIV, HIV, and simian immunodeficiency virus (SIV) in vitro (51), the possibility for studying highly active antiretroviral therapy (HAART) in the feline model now exists. Other new therapies being developed include integrase inhibitors, cell binding and entry inhibitors, rev and tat inhibitors, ribozymes, antisense oligonucleotides, and intracellular inhibitory antibodies (20). Any of these modalities could be studied for effectiveness in the FIV model. Cats are particularly manageable for these types of studies because of their small size, and yet they are large enough for easy placement of central venous or arterial catheters needed occasionally for monitoring, sampling, or drug administration procedures.

In addition to drug studies, the FIV model can be used to answer questions about the basic biology of lentiviral pathogenesis. Cats are of sufficient size to allow for sophisticated longitudinal measurements of neurological disease by electrodiagnostic methods as well as neurologic metabolic testing. Cats can also be behaviorally tested (78;96) and trained for sleep (80) and cognitive testing (96). Serial cerebrospinal fluid (CSF) and blood samples can be reasonably obtained, and brain regions can be identified electrodiagnostically as well as histopathologically. This provides ample measurements for following disease progression long-term. With the availability of two pathogenic molecular clones of FIV in the United States that appear to have different tropisms and clinical manifestations (immunopathogenesis versus neuropathogenesis), molecular
virology studies evaluating the genetic basis of these differences are now possible. Specialized studies of viral distribution in the brain, presence or absence of neuronal injury, antiretroviral drug distribution in the nervous system, and the effects of viral infection on excitatory neurotransmitter metabolism can all be studied as well.

In conclusion, the work presented in this dissertation is the basis for beginning molecular virology studies of nucleoside analog drug resistance using the FIV model in cats. This is only a very limited aspect, however, of what is possible to do with this experimental system. Basic pathophysiology, neuropharmacology, toxicology, viral replication, and tissue tropism are all candidates for future experiments using FIV in cats.
PART II

MAPPING IN VIVO REPLICATION AND PATHOGENESIS DETERMINANTS OF ALEUTIAN MINK DISEASE PARVOVIRUS

CHAPTER 5

INTRODUCTION

PARVOVIRUSES

Genomic organization. The autonomous parvoviruses are recognized as the etiologic agents of a variety of important diseases affecting many vertebrate animals, including mice, rats, cats, mink, dogs, pigs, cows, and man (126,133,158,181,212). Parvoviruses are small, nonenveloped icosahedral viruses with linear, single-stranded deoxyribonucleotide (DNA) genomes approximately 5000 nucleotides in length (158). All contain palindromic sequences at the 5′ and 3′ termini of the genome that form secondary hairpin structures necessary for parvoviral replication (127,153,190,208-211). These small genomes contain two major open reading frames (ORFs). The left ORF is controlled by a promoter at ~ 4 map units (MU) and specifies at least one nonstructural (NS) protein necessary for viral replication and gene regulation (132,145,157,176,202,203,209,214,215). The promoter for the right ORF is located at ~ 38 to 40 MU. The right ORF encodes overlapping sequences of at least two structural, or capsid (VP), proteins (132,140,144,158,183,193,203).

Nonstructural proteins. Parvovirus progeny do not bud from the host cell surface like
retroviruses. Instead, host cell lysis releases newly generated virions into the surrounding environment. Although the exact effectors remain obscure, evidence suggests that the nonstructural proteins are integrally involved in this presumably cytotoxic mechanism (147,184,186,192,201). Subsequently, the nonstructural proteins may be important in determining the efficiency of viral spread through the infected host as well as in cell culture systems.

The largest of the nonstructural proteins, NS1, has several biochemical properties necessary for replication and gene regulation. NS1 shows ATP-binding, ATPase, helicase, site-specific endonuclease, and covalent and non-covalent DNA-binding properties (156,222). The carboxy-terminus of the NS1 protein contains a transcription-activating domain (182,185) and is thought to trans-activate the P38 promoter that controls transcription of the capsid genes (158,162,182,185). Therefore, NS1 may be able to influence the efficiency of viral replication and new virion formation as well as virion release from cells.

**Structural (capsid) proteins.** Structural proteins of parvoviruses are the building blocks of the capsid that encloses the viral genome. VP1 and VP2 are formed by alternate splicing of the messenger RNA transcribed from the right ORF (176). VP1 contains the complete sequence of VP2 and an additional amino-terminal domain that varies in size among parvoviruses (116). VP3, in parvoviruses that have a third capsid protein, is a proteolytic product of VP2 formed after virion assembly (116).

The number of capsid protein species per virion is a distinguishing feature of each parvovirus. These polypeptides all have a similar eight-stranded anti-parallel β-barrel
motif that contains about one-third of the amino acid content of each protein. The β-barrel motif lies below the capsid surface while the remaining two-thirds of the polypeptide structure is made up of surface loops, inserted between the β strands (116). These surface loops have the amino acid residues comprising cell receptors and antigenic epitopes (116). Small numbers of amino acid changes in these surface loops can dramatically alter biological properties of the paroviruses, such as the host ranges of canine parovirus (CPV) and feline panleukopenia (FPV). CPV and FPV are 98% identical. However, three amino acid differences in the capsid protein allow CPV to replicate in canine cells whereas FPV cannot (115).

**Clinical disease.** The clinical signs associated with acute paroviral infection are usually related to the site of viral replication. Target cells are typically those whose biology requires rapid cell replication and turnover. For instance, FPV and CPV both infect small intestinal crypt cells, the cell population responsible for replenishing the epithelium of intestinal villi (169). Clinical signs generally include severe, and often bloody, diarrhea. Human parovirus B19 selectively infects red blood cell progenitors in the bone marrow (161). Severe aplastic crises can be a clinical result.

The S phase of the cell cycle appears to be required for efficient paroviral replication (158). Host cell replication and the availability of host cell machinery is necessary for the intracellular replication of paroviruses that have so few viral-encoded accessory proteins. Subsequently, paroviral infection is common in young animals and children. The strict cellular requirements for replication limit the spectrum of target cells for paroviruses, particularly in adult animals (219).
ALEUTIAN MINK DISEASE PARVOVIRUS

Host factors. Aleutian mink disease was first described in mutant mink homozygous for a recessive dilute coat color gene (Aleutian gene) in the 1940s (167). The fur of these mink, called sapphire color-phase, was gun-metal gray in contrast to the darker and richer fur colors typical of mink of other color phases, such as pastel. Aleutian mink were noted to be highly susceptible to a chronic wasting disease later determined to be a parvovirus (143). In addition to the unusual coloration, immune dysfunction was mapped to the Aleutian gene. The genetic immune disorder is referred to as Chediak-Higashi syndrome, characterized by oculocutaneous albinism, recurrent pyogenic infections, platelet dysfunction, and enlarged granules in granulocytic blood cells and tissue mast cells (130). More specifically, immune dysfunction may include decreased fusion of lysosomes with phagosomes, decreased bactericidal capacity, decreased chemotaxis, defective natural killer cell function (148), nucleotide pool deficits in platelets (130,131), and serotonin storage dysfunction of platelets (130). Therefore, host factors are certainly important in the study of Aleutian mink disease parvovirus (ADV). Aleutian genotype mink are often used for experimental study since they develop disease rapidly and consistently when infected with pathogenic strains of virus (164).

Clinical disease–adults. Classical Aleutian disease (AD) of adult mink is characterized by chronic, progressive wasting, widespread proliferation of plasma cells and lymphocytes, hypergammaglobulinemia, glomerulonephritis (164), and necrotizing arteritis, particularly of the bladder and stomach walls (Dr. William J. Hadlow, personal communication) and brain. These abnormalities are a result of circulating antigen-
antibody immune complexes and their deposition in small capillary beds (205).

Plasmacytic, lymphocytic infiltrates of the liver can be severe enough to destroy the normal architecture of the liver lobules (unpublished data), and the medullary cords of the mesenteric lymph node can be dramatically thickened, primarily by plasma cells.

Aleutian genotype mink infected with highly pathogenic virus strains, such as ADV-Utah 1, are usually viremic by 10 days postinfection and remain persistently infected (138,166). Infection of adults is believed to be a restricted infection of mononuclear immune cells (118,135,142,204) and may be enhanced, rather than neutralized, by antiviral antibodies (114,163,177-179). Disease usually progresses quickly over 60 to 90 days (138,166), and clinical illness appears to be the result of renal failure, likely a result of severe glomerulonephritis (164) and possibly additional destructive renal tubular lesions (194). Mink of other color phases, such as royal pastel, respond to pathogenic virus strains erratically, even when inoculated with large numbers of virus (164). Sometimes only half of royal pastels become clinically affected by experimental infection, and many of these mink have a more protracted course than sapphire (Aleutian) mink (164).

**Clinical disease--kits.** Aleutian mink kits born to seronegative dams and infected perinatally with ADV-Utah 1 succumb rapidly to a fulminant interstitial pneumonia that causes severe respiratory distress and death by two to six weeks of age (118,119,121). ADV replicates permissively in type II pneumocytes of the lung and destroys surfactant production by these cells (118,119,121). A detectable antibody response is not generated by neonatal mink kits (117).
**Disease variability.** While highly pathogenic strains of ADV cause a predictably severe disease, milder forms of the adult and neonatal diseases have been described (122,139,171). In addition, inapparent, or subclinical, forms of ADV have been reported (123-125). ADV-G, a molecular clone derived from a cell culture-adapted isolate of ADV-Utah 1 (134), is nonpathogenic for adult sapphire mink and only mildly pathogenic for mink kits (122,143,197). Other ADV isolates are intermediate in pathogenicity between ADV-G and ADV-Utah 1 (171,172). Subsequently, a broad range of disease severity for which host factors do not appear to account is seen among various ADV isolates.

**Viral factors.** In addition to the differences in disease severity between ADV-G and ADV-Utah 1, there are also variations in cell tropism. ADV-G replicates permissively and to high titers in Crandell feline kidney (CrFK) cells *in vitro* even though it does not replicate *in vivo* (121). In contrast, ADV-Utah 1 cannot be propagated in CrFK cells but replicates efficiently *in vivo* (121,173). These replication and pathogenesis differences suggest regulation by viral determinants (138,139,143,197).

Previous work by Bloom, et.al., involved dissecting the ADV genome in order to identify possible viral determinants of *in vitro* and *in vivo* replication and pathogenesis (136-138,166). A region of the capsid gene was identified as encoding a minimal *in vivo* replication determinant (138). The contributions of four individual amino acids within that region and one additional upstream amino acid were also studied in mink (166). Two amino acids that differ between ADV-G and ADV-Utah 1, encoded by codons 352 and 534 of the VP2 gene, were determined to likely participate interactively in governing *in*
in vivo replication (166). The nonstructural gene of ADV does not appear to affect in vivo replication and pathogenesis alone (137), but its interactive relationship with crucial capsid amino acids has not been determined.
MOLECULAR CLONING OF CHIMERIC VIRUSES

Plasmid vectors. Standard molecular biology techniques were used for all cloning (134, 136, 197). The full-length parent ADV molecular clones ADV-G (XXXI-K-11-15) (137), ADV-G/U-8 (XXIX-A-12), and ADV-G/U-10 (XXX-J-8-10) (138) were reported previously (Figure 7.1). In order to construct the new clones ADV-G/U-13 (29STG) and ADV-G/U-14 (29STJ8) (Figure 7.1, Table 7.1), the previously described pIC4-2 plasmid (166) was modified by replacing its 1.3 kb BamHIXhoI ADV-G sequence fragment with the corresponding fragment from ADV-G/U-8 that contained three coding (Table 7.1) and one non-coding (T to C at nucleotide 820 of NS1) ADV-Utah 1 nucleotide changes in the NS1 gene. This new plasmid was designated 29ST. The presence of the correct insert was verified by restriction enzyme digestion with ScaI. Plasmids receiving the ADV-Utah 1 NS1 segment contained two ScaI restriction sites, whereas parental pIC4-2 plasmids contained three ScaI sites. Digestion of these plasmids resulted in two and three fragments, respectively, when visualized on 0.7% agarose gels. The plasmids pIC4-2 and 29ST were maintained in E. coli JM109.

Construction of full-length chimeras. The downstream EcoRV/HindIII 156-bp cassette from pBR322 (previously engineered into the pIC4-2 plasmid) was removed from 29ST by restriction enzyme digestion and replaced with a similarly digested, but approximately 1-kb in size, fragment from ADV-G to construct ADV-G/U-13 and from ADV-G/U-10 to
construct ADV-G/U-14 (Figure 7.1). Full-length clones containing the correct inserts were larger than the unaltered acceptor plasmid by about 0.9 kb. This was easily demonstrated on 0.7% agarose gels after EcoRV/HindIII digestion of ligation reaction transformants. ADV-G/U-15 (Figure 7.1) was made by replacing the EcoRV/HindIII fragment of pIC4-2 with that of ADV-G/U-8. Screening for plasmids carrying the correct inserts was done as described for ADV-G/U-13 and -14.

Full-length molecular clones were transformed by electroporation and were amplified in *Escherichia coli* JC8111 (recBCsbcrecF) (134,146). This bacterial strain was required to reduce the degradation of the unstable parvoviral genomic right-hand hairpin that is necessary for parvovirus replication. Plasmid DNA was purified with a Wizard Maxi-prep kit (Promega, Madison, WI) and screened by restriction enzyme digestion for the presence of the unstable right-hand hairpin. Digestion with *EcoRl/BglII* resulted in the removal of a 2-kb fragment when the hairpin was present.

**CELLS AND VIRUSES**

**Cells.** Crandell feline kidney (CrFK) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) as previously described (134,137).

**Viruses.** CrFK cells in 6-well trays were transfected with 400 ng plasmid DNA of ADV-G or chimeric viruses per well using Effectene (Qiagen, Santa Clarita, CA) and were cultured at 31.8°C for 5 days. Virus propagation through three additional cell culture passages was done as previously described (137). Final passage supernatants were collected after cell disruption and centrifugation as previously reported (137,166).
containing supernatants were filtered through 0.45 μm filters, aliquoted, and stored at -20°C. Virus titers were determined by immunofluorescent assay (IFA) using fluorescein-conjugated polyclonal mink antiserum (137).

In vivo propagation of ADV-Utah 1 in mink kits and titer assays in adult mink have been reported previously (197).

ANIMALS AND INFECTION

Animals. The Rocky Mountain Laboratory animal research facilities were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures involving animals were approved by the Rocky Mountain Laboratory Animal Care and Use Committee.

Mink used in these experiments were 8-month-old female mink (Mustela vison) of the Aleutian phenotype obtained from an ADV-free mink ranch. Mink were housed in stainless steel modified primate cages in open-sided sheds as previously described (197). Blood samples were collected by aseptic jugular venipuncture under ketamine-acepromazine anesthesia at 0, 10, 30, 60, 90, and 120 days post infection (dpi). Serum was separated from blood cells by centrifugation and stored at -20°C.

Infection. 2 x 10^4 fluorescence-forming units (FFU) of chimeric viruses in a volume of 1 ml were injected intraperitoneally (IP) into groups of four mink each. A mock-infected group was injected with 1 ml of supernatant collected from CrFK cells that were mock-transfected and passaged three additional times in cell culture. A positive control group received 2 x 10^4 mink infectious doses of ADV-Utah 1.
IN VIVO INFECTIVITY AND PATHOGENESIS

Antibody and viremia. Antibody responses and viremia were determined at each blood collection time point. The antiviral antibody response to ADV infection was measured by CIEP using a commercial ADV antigen (United Vaccines, Madison, WI) (141,142). Viremia was detected using serum-based PCR using ADV-specific primers designed to amplify a 692-bp fragment in the VP2 region of the ADV-G genome (141,197). Prior to infection, sera from all mink tested negative for anti-ADV antibodies and for ADV-specific viral DNA amplification by PCR.

Gamma globulin measurement. At the experimental endpoint (clinical illness for ADV-Utah 1-infected mink and 120 dpi for all other groups), blood samples were collected and tissues for histopathological examination were harvested. Gamma globulin levels were measured in baseline and final serum samples by serum protein electrophoresis using a horizontal thin-layer agarose gel system (Helena Laboratories, Beaumont, TX). The percent contribution of gamma globulins to total serum protein was determined by laser densitometry and integration of the area under the curve (142,164). The differences between the percentage of gamma globulins in baseline and final samples were calculated.

Histopathology. At necropsy, tissue samples from the left lateral lobe of the liver, tail of the spleen, left kidney, and mesenteric lymph node were processed in a standard manner for hematoxylin and eosin-stained paraffin-embedded sections (164,170). Sections were reviewed by a board-certified veterinary pathologist (WJH) without knowledge of their origin.
CLINICAL MONITORING

Physical examination parameters. All mink were monitored for body weight, rectal temperature, heart rate, respiratory rate, hydration status estimate, coat condition, and clinically evident disease at 0, 10, 30, 60, 90, and 120 dpi.

Bloodwork. Complete blood counts (CBC), platelet counts, and serum biochemistries were measured on 0, 60, and 120 dpi. Whole blood samples for CBC and platelet counts were collected into ethylenediaminetetraacetic acid-treated (EDTA) microtainer tubes (Becton-Dickinson, Franklin Lakes, NJ). Blood smears for cytologic examination were made on clean glass microscope slides from EDTA-treated blood samples within four hours of blood collection. Serum for biochemical analysis was separated from blood cells in a clot activator vacuum tube (Becton-Dickinson, Franklin Lakes, NJ) with a gelatin separator. Samples were shipped directly to a commercial laboratory (Pathology Associates Medical Laboratory, Spokane, WA) the day of collection.

Urine specific gravity and DNA isolation from urine. Urine samples were collected at 0, 60, and 120 dpi for measurement of urine specific gravity (USPG). USPG was evaluated using a manual clinical refractometer.

Urine samples were also collected at 0, 10, 30, and 60 dpi for screening for the presence of viral DNA. In a second unrelated study, urine was collected 14, 28, and 43 dpi from two mink from each of three control groups, including ADV-Utah 1, ADV-G, and mock. DNA extraction from urine samples was done by 30% polyethylene glycol in 3 M sodium chloride precipitation as reported by Behzadbehbahani, et. al. (129), and by using a Viral RNA Mini Kit (Qiagen, Valencia, CA) as directed by the company to
remove PCR inhibitors commonly found in urine (129,165,175). Purified DNA samples were used as template (2.5 µl) in an ADV-specific PCR amplification reaction using primers to amplify a 692-bp fragment of ADV-G VP2. PCR reaction products were analyzed on a 2.5% agarose gel and compared to dilutions of ADV virion standards that had also been processed through the Viral RNA Mini-kit.

**Mink infection with filtered urine.** Four sapphire mink were injected IP with 1.5 ml of 0.45 µm-filtered urine from 30 or 60 dpi samples from mink 5716 or 5717 (ADV-Utah 1-infected mink). Two additional mink were injected IP with similarly processed samples from mock-infected mink collected at 60 dpi. These mink were monitored for the production of antiviral antibody titers and viremia as described above. The experimental endpoint was determined to be the onset of clinical signs at which time serum electrophoresis for gamma globulin evaluation and gross and microscopic pathological examinations will be done.
CHAPTER 7
INTERACTION OF CAPSID AND NONSTRUCTURAL PROTEINS IN IN VIVO REPLICATION AND PATHOGENESIS OF ADV

INTRODUCTION

Aleutian disease virus (ADV) is an autonomous parvovirus that causes disease in mink (Mustela vison) quite different from that caused by parvoviruses in other species. Infection of mink kits with virulent biological isolates such as ADV-Utah 1 results in fatal interstitial pneumonia in almost 100% of affected kits by two to six weeks of age (118,119,121). Adult infection is characterized by a protracted clinical course of viremia, hypergammaglobulinemia, immune complex disease, and plasmacytic, lymphocytic infiltration of lymphoid tissue, spleen, liver, and kidney (164). ADV-G, a molecular clone derived from cell culture-adapted ADV-Utah 1, is nonpathogenic for adult mink and only mildly pathogenic for mink kits of the susceptible Aleutian phenotype (134). Interestingly, sequence alignment of approximately 75% of the total genome of these two ADV isolates shows 97.5% identity at the nucleotide level (137,168).

Evidence from other parvovirus systems supports the ability of seemingly minor nucleotide sequence differences to be associated with profound biological variances. For instance, small differences in the capsid genes are responsible for host range variants of the minute virus of mice (MVM). MVM has two serologically indistinguishable types, MVM(p) and MVM(i), that are 97% identical in nucleotide sequence (128). However,
two capsid amino acid differences allow MVM(p) to productively infect mouse fibroblasts and MVM(i) to infect mouse T lymphocytes (128). In addition, no more than three amino acid changes in the capsid gene are necessary for changing the host ranges of canine parvovirus (CPV) and feline panleukopenia virus (FPV) although these viruses are 98% identical (151,217,218). Alternatively, small changes in the nonstructural proteins may also correlate with significant biological differences (149,195,220). Porcine parvovirus (PPV) host range is affected by one amino acid residue in both the nonstructural and capsid genes (220). Also, at least one change in the nonstructural gene of MVM is associated with an increase in replication efficiency in fibroblasts (195). Therefore, precedents in other paroviral systems suggest that either capsid or nonstructural proteins, or both, may be involved in controlling in vivo replication and pathogenesis of ADV.

Determinants of tropism of ADV for growth in Crandell feline kidney (CrFK) cells and for replication of ADV in mink have been mapped to the capsid gene (137). It is possible that determinants of pathogenesis may reside there as well. Alternatively, changes in the nonstructural gene may affect pathogenesis. Supporting this possibility is the high degree of variability between the amino acid sequences of the NS1 proteins of several ADV isolates. More variability is seen between ADV isolates than is seen between the NS1 proteins of FPV, CPV, and mink enteritis virus (MEV) (168). In fact, the ratio of synonymous to non-synonymous substitutions between ADV-G and ADV-Utah 1 was 0.42, suggesting a positive selection for variation in the NS1 protein may be occurring between these two isolates (168). It is possible that some of these changes may...
be responsible for the differences in \textit{in vivo} replication and pathogenicity between these two types of ADV.

Recent studies in our laboratory have been defining the molecular basis of \textit{in vivo} replication and pathogenesis of ADV by comparing chimeric viruses of ADV-Utah 1 and ADV-G. Two chimeras capable of replication in both cell culture and adult Aleutian mink and competent to cause varying degrees of disease in infected mink were recently reported (138). ADV-G/U-8 induced higher antiviral antibody titers and more severe histopathologic lesions in tissues of infected mink than ADV-G/U-10 (138). These chimeras differed by only four amino acids. ADV-G/U-8 contained one additional capsid and three additional nonstructural protein amino acid changes compared to ADV-G/U-10 (Figure 7.1, Table 7.1). The purpose of the current study was to determine whether the ADV-Utah 1 amino acid changes in the capsid protein or the nonstructural protein in ADV-G/U-8 were responsible for its increased \textit{in vivo} replication and pathogenicity compared to ADV-G/U-10.

\textbf{RESULTS}

\textbf{Development of ADV chimeras.} In order to compare chimeras with identical capsids differing only by a short segment of the nonstructural protein and vice versa, three ADV clones were constructed complimentary to ADV-G (ADV-G/U-13), ADV-G/U-10 (ADV-G/U-14), and ADV-G/U-8 (ADV-G/U-15) (Figure 7.1). This set of three pairs of viruses allowed comparison of the contributions of both ADV-Utah 1 NS1 and VP2 changes in \textit{in
Figure 7.1. Genetic structure of Aleutian mink disease virus (ADV) chimeras and *in vitro* titers. Genomic organization of chimeras between nonpathogenic ADV-G and highly virulent ADV-Utah 1 is shown. Sections with white background represent ADV-G sequence, and black sections illustrate segments of ADV-Utah 1 sequence. White numbers represent amino acid differences between ADV-Utah 1 and ADV-G. See Table 7.1 for nucleotide substitutions and coding amino acid changes in NS1 (upstream) and VP2 (downstream) genes and proteins, respectively. Virus titers were determined by immunofluorescent assay (IFA) in Crandell feline kidney (CrFK) cells.
ADV-G NUCLEOTIDE

ADV MAP UNIT

ADV-G OPEN READING FRAMES

CONSTRUCTION

CRFK VIRUS TITER (FFU/mL)

ADV-Utah

ADV-G

G/U-13

G/U-10

G/U-14

G/U-15

G/U-8

<45

4.0 x 10^6

2.5 x 10^6

8.0 x 10^4

1.1 x 10^4

1.0 x 10^4

4.2 x 10^3

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vivo replication and pathogenesis. ADV-G and ADV-G/U-13 both contained a complete ADV-G VP2 gene without and with three ADV-Utah 1 NS1 amino acid changes, respectively (Figure 7.1, Table 7.1). ADV-G/U-10 and ADV-G/U-14 both encoded four ADV-Utah 1 VP2 amino acid changes previously shown to be the minimal replication determinant for in vivo replication (138) without and with the short ADV-Utah 1 NS1 segment, respectively (Figure 7.1, Table 7.1). Finally, ADV-G/U-15 and ADV-G/U-8 capsids both included a fifth ADV-Utah 1 amino acid change, an isoleucine to valine at codon 352 of VP2, without and with the ADV-Utah 1 NS1 amino acid changes, respectively (Figure 7.1, Table 7.1).

Full-length ADV chimeras were transfected into CrFK cells as described in materials and methods. All clones replicated through four passages in cell culture, but there was a trend toward lower titers as more ADV-Utah 1 amino acid changes were present (Figure 7.1).

To assess the ability of ADV-Utah 1 to replicate in vitro, ADV-Utah 1 virus was used to infect CrFK cells and was passaged four times in cell culture. ADV-Utah 1 virus was used since a full-length ADV-Utah 1 molecular clone has not yet been developed. There were no positive cells detectable by IFA (Figure 7.1). These data confirmed previous data from our laboratory that highly pathogenic ADV-Utah 1 cannot be serially propagated in CrFK cells in vitro. This finding correlated with the trend toward lower in vitro titers as chimeras gained more ADV-Utah 1 amino acids. In fact, it suggested that there may be a limit to which molecular virology will be able to define in vivo replication and pathogenesis determinants of ADV until a cell line capable of propagating fully
TABLE 7.1. Nucleotide and encoded amino acid differences between ADV-G and ADV-Utah 1 in the NS1 and VP2 gene regions involved in chimera constructions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>#</th>
<th>Nucleotide</th>
<th>ADV-G</th>
<th>ADV-Utah 1</th>
<th>Codon</th>
<th>ADV-G</th>
<th>ADV-Utah 1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>758</td>
<td>A</td>
<td>G</td>
<td>185</td>
<td>Ile</td>
<td>Val</td>
</tr>
<tr>
<td></td>
<td>nc</td>
<td>820</td>
<td>T</td>
<td>C</td>
<td>205</td>
<td>Asn</td>
<td>Asn</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1182</td>
<td>G</td>
<td>A</td>
<td>326</td>
<td>Ser</td>
<td>Asn</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1339</td>
<td>T</td>
<td>G</td>
<td>378</td>
<td>Ile</td>
<td>Met</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>3459</td>
<td>A</td>
<td>G</td>
<td>352</td>
<td>Ile</td>
<td>Val</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3590</td>
<td>C</td>
<td>G</td>
<td>395</td>
<td>His</td>
<td>Gln</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3705</td>
<td>A</td>
<td>C</td>
<td>434</td>
<td>Asn</td>
<td>His</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3876, 3878</td>
<td>A, C</td>
<td>G, G</td>
<td>491</td>
<td>Asn</td>
<td>Glu</td>
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<tr>
<td></td>
<td>5</td>
<td>4005</td>
<td>C</td>
<td>G</td>
<td>534</td>
<td>His</td>
<td>Asp</td>
</tr>
</tbody>
</table>

\(^a\) Numbers in each gene region correlate with the numbers indicating amino acid differences in Figure 1.

\(^b\) nc represents a non-coding change that deletes a polyadenylation site from ADV-G (136)
In order to evaluate the immune response of mink to the ADV chimeras and control preparations, anti-ADV antibody was assayed on 0, 10, 30, 60, 90, and 120 dpi by CIEP. The model of consistent response was ADV-Utah 1 (Figure 7.2). All ADV-Utah 1-infected mink surviving to 60 dpi developed titers of 1:4096. Mock-infected mink were negative at all time points. Mink infected with ADV-G and ADV-G/U-13 were all 1:16 or lower throughout the study (Figure 7.2), indicating that the three amino acid changes in NS1 alone cannot alter in vivo ADV antibody responses. All other chimeras were associated with moderate to high antibody levels (approximately 1:64 to 1:4096) (Figure 7.2). When evaluated using a nonparametric Wilcoxon t-test, a significant difference (p ≤ 0.05) was detected between the antibody titers of the G/U-8 and G/U-15 groups compared to G/U-14 at 120 dpi. This data demonstrated a clear difference between the chimeras having five capsid amino acid changes and one having only four. It is possible that the antibody titers of the G/U-14-infected mink were declining more rapidly than those of the five capsid change chimera-infected mink. Graphs of the raw data for antibody titers showed a trend toward less variation between animals within groups when the ADV-Utah 1 NS1 changes were present in the clone (Figure 7.2, ADV-G/U-14 and ADV-G/U-8). This suggested that NS1 may play a role in making host responses to the virus more predictable and consistent, essentially making the virus more ADV-Utah 1-like.

These antiviral antibody titers were reevaluated by calculating the geometric mean titers (GMT) for each group at each time point and the reciprocal means graphed (Figure
Figure 7.2. ADV-specific antibody titers and viremia in control and ADV chimera-infected mink. Each circle represents one mink at each time point indicated. There were four mink in each group. One mink in the ADV-G group died of causes unrelated to Aleutian disease, and one mink in the ADV-Utah 1 group died of undetermined causes between days 30 and 60 postinfection. Open circles indicate reciprocal antiviral antibody titers of non-viremic mink as measured by counterimmunoelectrophoresis (CIEP). Solid circles show the reciprocal antibody titers of mink that were concurrently viremic as determined by an ADV-specific PCR reaction using serum as template. Viremia was detected in four of four (ADV-Utah 1), three of four (ADV-G/U-8), two of four (ADV-G/U-10 and ADV-G/U-14), and one of four (ADV-G/U-13) mink in groups infected with pathogenic viruses.
The highest final reciprocal GMT was 4074 for the ADV-Utah 1 group. A trend toward higher titers when five VP2 changes were present [ADV-G/U-15 (1445.4) and ADV-G/U-8 (1023.3)] rather than four [ADV-G/U-10 (255.6) and ADV-G/U-14 (128.8)] was seen. These data were similar to those found for raw antiviral antibody titers.

**Viremia.** To determine if viral replication was occurring at sufficiently high levels to be detected systemically, serum samples were used as template for an ADV-specific PCR reaction on 0, 10, 30, 60, 90, and 120 dpi. Solid circles (Figure 7.2) indicated mink whose serum tested positive by PCR. All ADV-Utah 1-infected mink were persistently viremic from 10 dpi throughout the study. There were no positive samples at any time point for mink infected with mock, ADV-G, or ADV-G/U-13 (Figure 7.2). Two of four mink infected with ADV-G/U-10 were detectably viremic until 60 and 90 dpi, respectively. One of four mink infected with ADV-G/U-14, differing from ADV-G/U-10 by three NS1 amino acid changes, was transiently viremic at 10 dpi. For viral constructs containing five capsid amino acid changes, two of four mink infected with ADV-G/U-15 were persistently viremic throughout the study. The addition of the three ADV-Utah 1 NS1 amino acid changes to the five capsid changes, ADV-G/U-8, was associated with detectable viremia in three of four mink in the group. Two of these mink were transiently viremic at 10 dpi while one became viremic at 90 dpi and remained viremic until the end of the experiment. These data showed that the addition of the ADV-Utah 1 NS1 segment alone was not sufficient for *in vivo* replication. However, the new chimeras containing four and five capsid amino acid changes, ADV-G/U-14 and ADV-G/U-15, were capable of replicating in mink. A clear difference between chimeras ADV-G/U-8,
Figure 7.3. Geometric mean antibody titers (GMT) in control and ADV chimera-infected mink. The mean GMT for each group was calculated and its reciprocal graphed. Error bars represent standard error of the mean (SEM). Symbols represent chimeras as indicated in the upper left corner of the graph. A statistically significant difference (p = 0.01) between ADV-G/U-8 and ADV-G/U-14 (differ by only a valine at codon 352 of VP2 in ADV-G/U-8) at 90 days postinfection was detected using a paired Student’s t-test. Final reciprocal mean GMT titers were 4074 (ADV-Utah 1), 1445.4 (ADV-G/U-15), 1023.3 (ADV-G/U-8), 255.6 (ADV-G/U-10), 128.8 (ADV-G/U-14), 1.6 (ADV-G), and 1.4 (ADV-G/U-13).
Geometric Mean Titer

ADV-Utah
ADV-G
ADV-G/U-13
ADV-G/U-10
ADV-G/U-14
ADV-G/U-15
ADV-G/U-8

Days Postinfection

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10, and -15 could not be established based on the incidence of viremia. However, the ADV-G/U-14-infected group had only one viremic mink while the ADV-G/U-8-infected group had three. These data correlated with the differences seen between these two groups in antiviral antibody production and supported the conclusion that V352 of VP2 may influence *in vivo* replication.

**Hypergammaglobulinemia.** The development of serum hypergammaglobulinemia is a classic sign of progressive Aleutian disease (AD) and usually correlates with the presence of histopathologic lesions in tissue sections (164). The ADV-Utah 1-infected group showed the greatest increase in gamma globulins with the smallest error (23.5 +/- 1.1), indicating a highly consistent response among mink (Table 7.2). Mink infected with either clone containing five ADV-Utah 1 VP2 amino acid changes (ADV-G/U-8 or ADV-G/U-15) also showed the large increases in gamma globulins (16.4 and 19.2, respectively) (Table 7.2). ADV-G/U-10-infected mink showed a moderate increase of 9.8. These data showed that when valine is present at codon 352 of VP2 in conjunction with the other four ADV-Utah 1 capsid amino acid changes, a marked hypergammaglobulinemia occurred. Three ADV-Utah 1 amino acid changes in the NS1 gene did not enhance this hypergammaglobulinemia.

**Histopathology.** Paraffin-embedded tissue sections of the liver, spleen, kidney, and mesenteric lymph node were evaluated for lesions consistent with Aleutian disease (AD) so that subclinical disease would be detected. ADV-Utah 1-infected mink (3/3) displayed widespread, moderate to marked plasmacytic, lymphocytic cellular infiltration in at least two of the four organs examined. In addition, glomerulonephritis or mesangial
TABLE 7.2. Induction of hypergammaglobulinemia by Aleutian mink disease parvovirus chimeras

<table>
<thead>
<tr>
<th>Virus</th>
<th>Baseline mean (%) +/- SEM</th>
<th>Final mean (%) +/- SEM</th>
<th>Difference mean +/- SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>16.1 +/- 3.3</td>
<td>17.3 +/- 2.5</td>
<td>1.2 +/- 1.2</td>
</tr>
<tr>
<td>ADV-Utah 1abc</td>
<td>13.7 +/- 1.0</td>
<td>37.3 +/- 0.2</td>
<td>23.5 +/- 1.1</td>
</tr>
<tr>
<td>ADV-Gb</td>
<td>15.4 +/- 2.3</td>
<td>17.0 +/- 1.3</td>
<td>1.6 +/- 2.5</td>
</tr>
<tr>
<td>ADV-G/U-13</td>
<td>15.7 +/- 3.2</td>
<td>18.3 +/- 2.7</td>
<td>2.6 +/- 1.1</td>
</tr>
<tr>
<td>ADV-G/U-10</td>
<td>11.8 +/- 0.7</td>
<td>21.6 +/- 5.4</td>
<td>9.8 +/- 5.5</td>
</tr>
<tr>
<td>ADV-G/U-14</td>
<td>13.5 +/- 1.5</td>
<td>15.8 +/- 1.9</td>
<td>2.3 +/- 0.9</td>
</tr>
<tr>
<td>ADV-G/U-15</td>
<td>13.9 +/- 0.1</td>
<td>33.1 +/- 7.5</td>
<td>19.2 +/- 7.5</td>
</tr>
<tr>
<td>ADV-G/U-8</td>
<td>12.5 +/- 0.9</td>
<td>28.9 +/- 6.3</td>
<td>16.4 +/- 5.7</td>
</tr>
</tbody>
</table>

a Electrophoretograms of mink sera were analyzed by laser densitometry and integration of the area under the curve. Numbers represent the percent contribution of gamma globulins to total serum protein.

b Three mink per group. One mink from the ADV-Utah 1 group died of unknown causes, and one mink from the ADV-G group died from causes unrelated to Aleutian disease prior to the experimental endpoint. All other groups contained four mink.

c Final samples were collected approximately 60 days post infection (dpi) from mink infected with ADV-Utah 1. All other groups were sampled at 120 dpi.
thickening in the glomerular basement membrane was seen, indicating severe renal disease. These are lesions associated with classical AD (164). Tissue specimens from mock-, ADV-G-, and ADV-G/U-13-infected mink did not show evidence of AD. Mink infected with ADV-G/U-8 (4/4) had histopathologic lesions consistent with AD varying from mild to severe (Table 7.3). One of these mink had lesions suggestive of progressive renal disease. The ADV-G/U-15-infected group had similar findings (3/4) with two mink exhibiting severe glomerular lesions. The groups infected with chimeras containing four capsid changes, ADV-G/U-14 and ADV-G/U-10, showed slightly milder histopathologic lesions compared to ADV-G/U-8 and -15. ADV-G/U-14 mink (3/4) had mild, focal lesions in the mesenteric lymph node and occasionally the liver characterized by plasmacytic, lymphocytic infiltrates. Affected ADV-G/U-10 mink (2/4) had widespread, moderate infiltrative lesions of the mesenteric lymph node and liver. There were no mink infected with ADV-G/U-14 or -10 that showed renal lesions typical of classical, progressive AD. These microscopic data confirmed that the chimeras ADV-G/U-8, -15, -14, and -10 were all capable of inducing histopathologic lesions consistent with AD in sapphire mink. However, only mink infected with ADV-G/U-8 or -15 had lesions severe enough to suggest progressive disease. The clinical significance of mild to moderate histopathologic lesions is difficult to determine since histopathology alone cannot predict whether mink will recover from or succumb to ADV infection. The only chimera associated with AD-consistent tissue lesions in all mink infected was ADV-G/U-8, the clone containing five capsid and three nonstructural amino acid changes.
TABLE 7.3. Incidence and severity of histopathologic lesions in mink infected with Aleutian mink disease parvovirus (ADV)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Virus</th>
<th>Histopathologic lesions\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Utah 1</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>4</td>
</tr>
<tr>
<td>ADV-G</td>
<td>3</td>
</tr>
<tr>
<td>G/U-13</td>
<td>4</td>
</tr>
<tr>
<td>G/U-10</td>
<td>2</td>
</tr>
<tr>
<td>G/U-14</td>
<td>1</td>
</tr>
<tr>
<td>G/U-15</td>
<td>1</td>
</tr>
<tr>
<td>G/U-8</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Tissue samples from the left lateral lobe of the liver, tail of the spleen, left kidney, and mesenteric lymph node were obtained at 60 days postinfection (dpi) for ADV-Utah 1-infected mink and 120 dpi for all others. Tissues were embedded in paraffin, stained with hematoxylin-eosin, and evaluated by light microscopy.

\textsuperscript{b} Histopathologic lesions were graded by a board-certified veterinary pathologist (WJH). In general, mild lesions were focal infiltrations of plasma cells and lymphocytes into one or more tissues. Moderate lesions were more widespread than mild ones and contained more abundant cellular infiltrates. Severe lesions included hepatic or renal arteritis and massive, diffuse plasmacytic, lymphocytic infiltrates.
DISCUSSION

This study examined the differences in *in vivo* replication and pathogenesis between ADV-G/U-8 and ADV-G/U-10, two previously reported viruses chimeric between nonpathogenic ADV-G and highly virulent ADV-Utah 1 (138). ADV-G/U-8 was associated with higher antiviral antibody titers and more severe histopathologic lesions even though it contained only one amino acid difference in VP2 and three in NS1 compared to ADV-G/U-10 (138). Antibody responses and incidences of hypergammaglobulinemia and histopathologic lesions in our present experiments showed that the valine residue of ADV-G/U-8 at codon 352 (V352) of the VP2 protein substituted for the isoleucine of ADV-G/U-10 is primarily responsible for the differences in antibody responses and pathogenesis between these two viruses. Previous studies in our laboratory demonstrated that the presence of V352 alone in an ADV-G background produced a low, but persistent, antibody response but was not associated with histopathologic lesions (166). The current experiments, however, strongly indicated that V352 had a cooperative effect when present with the other four ADV-Utah 1 VP2 amino acid changes previously determined to comprise the minimal capsid region determining *in vivo* replication (138). Not only did V352 appear to enhance *in vivo* replication, but it may also have contributed to the severities of both hypergammaglobulinemia and disease at the tissue level.

The mechanisms by which V352 may influence *in vivo* replication, antibody responses, and pathogenesis are not known. We speculate that it may be due to changes in capsid surface structure induced by the V352 substitution for isoleucine. ADV VP2
residue 352 is thought to lie just below the capsid surface near the three-fold axis based on cryo-electron microscopy-based structural modeling (189). Parvovirus surface structure determines antigenic epitopes, cell receptor binding, and virus particle stability (116). The surface conformational changes generated by V352 may increase viral antigenicity and virus binding and entry into target cells. These effects would logically be associated with higher antibody responses and improved total viral replication as a result of more efficient target cell infection.

The effects of V352 on antibody responses, viremia, hypergammaglobulinemia, and histopathologic lesions were most clearly demonstrated when the three ADV-Utah 1 NS1 amino acid changes were present concurrently in the chimeras differing by only V352 (ADV-G/U-8 and ADV-G/U-14). Even though the three NS1 changes alone in this study (ADV-G/U-13) or larger segments of ADV-Utah 1 NS1 in a previous study (137) did not alter virus replication or pathogenesis, NS1 may play a role in replication and pathogenesis once a critical number of ADV-Utah 1 capsid changes are present. It is possible that virus capsid changes required for efficient cell binding, entry and replication must be present before the effects of NS1 are evident. The presence of the NS1 amino acid changes and non-coding nucleotide change may play a role in eliciting a more predictable and repeatable host response to the virus. This could occur either as a direct result of altered NS1 protein function or as an indirect effect of coding or noncoding changes on the downstream P36 promoter that controls transcription of the capsid gene. While none of the chimeras in this study showed the reproducibility of ADV-Utah 1 virus infection, the most ADV-Utah 1-like chimera, ADV-G/U-8, approached it most closely.
More studies involving larger regions of the nonstructural gene concurrent with multiple capsid changes are necessary to clarify the role of NS1 in *in vivo* replication and pathogenesis.

It was interesting that the histopathologic lesions associated with infection by the currently studied pathogenic chimeras were consistent with AD. These data were in contrast to previous findings of an unusual diffuse hepatic microvesicular steatosis seen with infection of a point mutant of ADV-G (166). This mutant contained a histidine to aspartic acid change at codon 534 of VP2 in an ADV-G background (166). The H534D mutant was also able to elicit a persistent, moderate antibody response and transient viremia (166). These data and results from the present study confirmed the importance of both the H534D and I352V capsid amino acid changes in *in vivo* replication and pathogenesis. Although neither change alone caused classical AD, it was apparent that the interactions of these two residues with other amino acid(s) was necessary for the induction of antibody responses and histopathologic lesions characteristic of AD. Since AD-like lesions were associated with chimeras containing the four amino acid changes in the minimal VP2 determinant region for *in vivo* replication (H395Q, N434H, N491E, H534D), at least one of the other three amino acid changes must have interacted with H534D to alter its unusual pathogenesis to signs more typical of AD. Further molecular experiments are necessary to define the minimum number of capsid changes necessary for interaction sufficient to precipitate AD in mink. These experiments may be limited by the ability of chimeras to replicate in CrFK, a necessary step for propagating chimeric virus stocks *in vitro* until an alternative system is found in which ADV-Utah 1-like viruses can
be serially passaged.

Studying viral replication and pathogenesis in an out-bred animal species can be challenging when data are highly variable. However, there was excellent agreement between different measured parameters in this study. The presence of histopathologic changes correlated well with other data in this study. Hypergammaglobulinemic (eight) and viremic (seven) chimera-infected mink all showed evidence of histopathologic changes consistent with AD. Of the chimera-infected mink, eight had gamma globulin changes of greater than 10 between baseline and final sample percentages. All eight of these mink were diagnosed with AD histopathologically. Seven chimera-infected mink were viremic at one or more time points during the study. All of these animals were positive for histopathologic lesions of AD. The only mink diagnosed with AD by histopathology that was neither hypergammaglobulinemic nor viremic had a persistently robust anti-ADV antibody response with a positive titer at a dilution of 1:1024. The correlation of these data strengthen the individual findings and support the diagnoses of AD in affected mink.

In conclusion, our current studies established the importance of V352 of the ADV VP2 protein in contributing to in vivo replication and pathogenesis in mink. While definite roles for V185, N326, and M378 of the NS1 protein were not determined, it is likely that one or more of these residues or a noncoding change may have influenced the predictability of the host response to the virus. Our conclusion is that there may be an interactive relationship between the effects of capsid and nonstructural proteins or their gene sequences on replication and pathogenesis of ADV in mink.
CHAPTER 8

SHEDDING OF ALEUTIAN MINK DISEASE PARVOVIRUS IN URINE MAY BE A ROUTE OF VIRUS TRANSMISSION AND ENVIRONMENTAL CONTAMINATION

INTRODUCTION

The successful prevention of viral disease is based on understanding the biology and transmission of the virus. The virus can then be avoided or immunization used to prevent productive infection. An effective commercial vaccine for Aleutian mink disease parvovirus (ADV) has not been developed. In fact, experimental vaccination of mink with ADV capsid proteins enhances rather than prevents disease (114). While body fluids have long been suspected of shedding ADV (personal communication, Dr. William J. Hadlow, 1999), no definitive evidence has proven a mode of transmission for infectious virus particles.

Evidence from canine parvovirus (CPV) demonstrates how important the spread of infectious virus particles in body fluids can be. During the acute phase of CPV-induced gastroenteritis, infectious CPV particles can be found in the saliva and vomitus of infected dogs (169). The major distribution of virus particles, however, is in the feces. Up to $10^9$ median tissue culture infectious doses (TCID$_{50}$) per gram of feces can be detected during acute disease, and only $10^3$ TCID$_{50}$ are necessary for infection of susceptible dogs (169). In addition, paroviruses are known to be highly stable virus
particles, resistant to environmental conditions that would rapidly kill other viruses, such as retroviruses. ADV particles are resistant to heating at 37°C for 8 hours, 56°C for 30 minutes, ether treatment for 17.5 hours, and 1:300 formalin treatment for 4 hours (164). Canine parvovirus is believed to survive in soil for up to 12 months or longer (169). Therefore, the release of infectious ADV particles into the environment through body fluids could be a major source of disease transmission and ranch contamination that could be particularly difficult to disinfect completely.

The dispersal of ADV particles in the urine was suspected based on the known pathophysiology of Aleutian disease (AD). Glomerulonephritis resulting from immune complex deposition has long been suspected as the immediate cause of fulminant renal failure and death of infected mink (206,207). We suspected that as glomerular lesions progressed, the small ADV particles (25 nm diameter) might be able to leak through damaged glomeruli into the urine. Alternatively, low-level ADV replication has been documented in renal tubular cells (194). Lysis of these cells at the end-stage of infection might allow release of virus particles directly into the renal tubules and, subsequently, the urine. Detection of viruses in clinical urine samples can, however, be challenging since many viruses are often found in only small amounts in cell-free body fluids (175,196). In addition, urine samples often contain non-proteinaceous substances that can inhibit standard molecular biology reactions, such as PCR (129,165,175).

The purpose of the current experiments was to determine if viral DNA of ADV could be detected by PCR in the urine of mink experimentally infected with a highly pathogenic isolate, ADV-Utah 1.
RESULTS

In order to isolate any viral DNA away from PCR inhibitors present in mink urine, all positive controls were processed for DNA purification using either a polyethylene glycol precipitation protocol (129) or a commercial kit as described in materials and methods (see Chapter 6). Positive control samples processed by polyethylene glycol precipitation did not produce a PCR product. However, positive controls purified using the commercial kit generated appropriately sized PCR products. We determined that the commercial was easier to use and was reliable for isolating parvovirus DNA from mink urine samples.

All three mink from the first study were negative by PCR at 0 and 10 days postinfection (dpi) with ADV-Utah 1. Three of three mink were positive by PCR at 56 dpi. Of two mink for whom there were sufficient 30 dpi samples, one was negative and one was positive (Table 8.1). None of these mink were displaying clinical signs of AD at 30 dpi, but all were clinically sick by 56 dpi. One ADV-Utah 1-infected mink from the second study showed results similar to those from the first study. This mink did not have viral DNA detected until 43 dpi. However, the other mink in the second study was positive beginning at 14 dpi and remained positive at both 28 and 43 dpi (Table 8.1). These data showed that viral DNA may be present in the urine of ADV-Utah 1-infected mink as early as 14 dpi, while ADV infection is still subclinical.

Estimates of the amount of viral DNA present in two of the 60 dpi samples (mink 5716 and 5717) were made by comparing PCR-amplified dilutions of these samples with...
### TABLE 8.1 Detection of ADV DNA in urine samples from sapphire mink by PCR

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mink</th>
<th>Days Postinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>5719</td>
<td>neg neg neg neg neg</td>
</tr>
<tr>
<td>ADV-Utah 1</td>
<td>5714</td>
<td>neg neg neg POS</td>
</tr>
<tr>
<td></td>
<td>5716</td>
<td>neg neg POS POS</td>
</tr>
<tr>
<td></td>
<td>5717</td>
<td>neg neg neg POS</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>5748</td>
<td>neg neg neg neg</td>
</tr>
<tr>
<td>ADV-G</td>
<td>5758</td>
<td>neg neg neg neg</td>
</tr>
<tr>
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<td>5784</td>
<td>POS POS POS POS</td>
</tr>
<tr>
<td></td>
<td>5785</td>
<td>neg neg POS</td>
</tr>
</tbody>
</table>

*a DNA was extracted from urine samples (140 µl) using a Viral RNA Mini Kit (Qiagen, Valencia, CA). 2.5 µl of purified DNA (final volume after elution, 60 µl) was used as template for a PCR reaction amplifying a 692-bp fragment of the VP2 gene of ADV-G.*
dilutions of a known standard. Approximate virion DNA amounts were $1 \times 10^5$ (5716) and $2 \times 10^3$ (5717) viral genomes/ml of urine. These results confirmed that while varying amounts of viral DNA were present in different animals, there was ample viral DNA present to substantially contaminate the environment and to infect other animals if the viral DNA was present in infectious virus particles.

In order to determine if the viral DNA detected by PCR was associated with infectious virus particles, filtered urine from mink 5716 and 5717 was used for IP injection of healthy mink. These animals developed antiviral antibody titers and viremia by 30 dpi (Table 8.2). These mink will be monitored until signs of clinical illness are evident, at which time assays for hypergammaglobulinemia and gross and microscopic examinations will be done to confirm the presence of classical AD. Verification of the development of AD will prove that infectious parvovirus particles were present in urine samples of ADV-Utah 1-infected mink.

**DISCUSSION**

In these experiments, we proved that ADV DNA can be found in the urine of ADV-Utah 1-infected mink. This is an important step toward identifying a mode of transmission for ADV. While the presence of viral DNA does not assure the presence of infectious virus particles, it is suggestive that urine may be a route for the shedding of ADV.

Also of interest is the concentration of viral DNA detected. One mink had
Table 8.2 Serological responses of sapphire mink injected with filtered urine from ADV-Utah 1-infected mink

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mink</th>
<th>Antiviral Antibody Titers$^a$</th>
<th>Serum Viremia$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Days Postinfection</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Mock</td>
<td>5786</td>
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<td>neg</td>
</tr>
<tr>
<td>ADV-Utah 1</td>
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<td>neg</td>
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<tr>
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<td>5789</td>
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<td>neg</td>
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<tr>
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<tr>
<td></td>
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</table>

$^a$ Antiviral antibody titers were measured by counterimmunoelectrophoresis using serial four-fold dilutions of serum.

$^b$ Serum viremia was measured by using serum as template for an ADV-specific PCR reaction amplifying a 692-bp fragment of the VP2 gene of ADV-G.
approximately $1 \times 10^5$ viral genomes/ml. Mink can be productively infected with ADV-Utah 1 with as little as three orders of magnitude less virus than this. All three mink tested were confirmed in renal failure based on inappropriate urine concentration in the face of azotemia or clinical dehydration. Subsequently, all three had abnormally dilute urine samples. These mink were also assessed to be in polyuric renal failure, so they were urinating more frequently and in greater volumes than normal. These data suggest that if the viral DNA we detected is present in infectious virus particles, large numbers of infectious virus particles could be shed by these mink that are urinating increased amounts of dilute urine that already contains a substantial amount of virus. This could be the source of extensive environmental contamination.

The next step in determining if urine is the source of infectious ADV particles is to demonstrate infectivity in these samples. Experiments are currently underway in which sapphire (Aleutian genotype) mink have been injected IP with filtered urine samples from the mink of this report. They are being examined and tested for signs of classical AD. Signs of disease consistent with AD will be convincing evidence that infectious virus particles are shed in the urine of ADV-Utah 1-infected mink.

The spread of infectious ADV in mink urine has far-reaching implications. The most important impact is on mink ranchers. Knowing the mode of transmission of ADV may help them target environmental and property disinfection after outbreaks. It also indicates the importance of possible fomite transmission, so ranchers can be more careful about buying equipment or caging from ranches that have recently had outbreaks of ADV. In addition, ADV can infect not only mink, but also ferrets, weasels, fishers, martens,
striped skunks, and raccoons (180,197). Environmental contamination with a virus that is resistant to heat, cold, and many chemicals creates a situation in which neighboring wildlife may be at increased risk for infection and spreading the disease themselves. Domestic ferrets (Mustela putorius) are closely related to mink (Mustela vison) and are popular as household pets as well as being raised commercially. ADV in pet ferrets usually presents as coughing or other respiratory signs and progressive posterior paresis several weeks later (221). The route of transmission of ADV between ferrets has not been established yet, but those raised on ferret ranches that have had ADV on the property may be at increased risk. The results of our final experiments will verify whether these concerns are valid.
CHAPTER 9

ISOLATION OF MINK ALVEOLAR TYPE II EPITHELIAL CELLS

INTRODUCTION

The productive infection of alveolar type II epithelial cells (or type II pneumocytes) with Aleutian mink disease parvovirus (ADV) in mink kits (121) causes severe interstitial pneumonia (117) and probably decreased surfactant production (118), which contributes to severe respiratory distress and death. The infection of alveolar type II epithelial cells is considered permissive since the virus replicates to very high copy numbers of both single stranded virion and double stranded replicative form DNA ($5 \times 10^4 - 1 \times 10^5$) (121). Large numbers of ADV-specific mRNA transcripts have also been demonstrated (120). The only immortalized cell line in which some isolates of ADV will replicate permissively is Crandell feline kidney (CrFK) cells (120,121). Although elaborate studies have been done using the CrFK system to analyze subcellular aspects of permissive ADV infection (198-200), it is not known if the same events occur in the natural host cell, alveolar type II epithelial cells.

Rat and human alveolar type II epithelial cells have been successfully isolated from lung tissue and cultured ex vivo (150,152,160,161,174,188,213,216). In addition to supporting viral replication (150), alveolar type II epithelial cells also show a variety of interesting immune functions, including complement production (216), arachidonic acid release and metabolism (152), and expression of major histocompatibility complex
(MHC) class II molecules (174). The major known functions of these cells, however, are
the production and release of surface active phospholipid materials (surfactant), the repair
of airway epithelium, and limitation of leakage of solutes and fluid into the alveolar air
spaces (213). These immunological, functional, and structural properties make type II
pneumocytes critical to normal airway health and their metabolism unique compared to
many immortalized cell lines.

The purpose of the current work was to determine if mink type II pneumocytes
could be isolated and cultured ex vivo and support ADV infection. Our goal was to define
a primary cell culture system that could be used to study the subcellular events of
permissive ADV infection.

METHODS

Alveolar type II epithelial cell isolation protocol. Methods used for alveolar type II
epithelial cell isolation were modified from previous work by Dobbs, et. al. (161), and
Castleman, et. al. (150). Rats were first used to learn the technique, and then isolations
were done using mink. Briefly, the isolation process involves flushing contaminating
cells from the vasculature and airways, digesting the lung tissue with enzymes, then
separating type II cells from alveolar macrophages. A detailed protocol for alveolar type
II epithelial cell isolation follows:

1) Rats were anesthetized with pentobarbital IP (80 mg/kg) and mink with
acepromazine and ketamine IM. They were then administered heparin sodium
(400 IU/kg) either IP (rats) or IV (mink). The length of the sternum and ventral cervical area were clipped and aseptically prepared with betadine solution and 70% ethanol.

2) A ventral midline thoracotomy incision was made, the thorax opened, and the animal exsanguinated by direct intracardiac puncture. The dorsal aorta was transected mid thorax, and the caudal vena cava, cranial vena cava, and azygous veins were clamped with hemostats to prevent retrograde flow of flush solutions. A 22 gauge IV catheter was passed into the right ventricle of the heart and threaded into the pulmonary artery. The stylet was removed, and the lung vasculature was flushed with Solution II [140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 2.0 mM CaCl₂, and 1.3 mM MgSO₄] until the lungs were blanched white. This usually required about 20 - 60 ml for rats and 120 - 240 ml for mink in 60-cc syringes. The lungs were carefully removed from the thorax as well as the length of the trachea and placed in phosphate buffered saline solution for transport to the laboratory.

3) The open end (oral) of the trachea was cannulated with a metal teat cannula (rats) or a soft rubber feeding tube (mink) and tied snugly with nonabsorbable suture to prevent slipping. Bronchoalveolar lavage (BAL) was done eight (8) times using Solution I [140 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 10 mM HEPES, 6 mM glucose, 0.2 mM EGTA (ethylene glycol-bis-(β-amino ethyl ether), N, N’-tetraacetic acid), pH 7.4] in volumes sufficient to fully inflate the lungs. Wash
fluid was saved in 50-ml conical centrifuge tubes for alveolar macrophage isolation. Two (2) final BAL washes were done using Solution II and discarded. For continuation of alveolar macrophage isolation, see the section immediately following type II cell isolation protocol.

4) The lungs were inflated with Solution II containing 30 orcein units/ml elastase (or 4.3 units elastase measured by succinyl-(L-alanine)₃-p-nitroanilide) (161). The cannula was sealed to maintain Solution II in the lung. The lung tissue was incubated in a large covered petri dish at 37°C for 20 min.

5) The large cartilaginous airways were excised and the lung tissue minced with sterile scissors, then with a sterile razor blade. The lung tissue was covered in 4 ml fetal calf serum, 5 ml DNAse solution (250 μg/ml), and adjusted to a final volume of approximately 40 ml with Solution II. The final suspension was shaken in a 50-ml conical centrifuge tube at 37°C for 30 min.

6) The suspension was filtered through 2 layers sterile cotton gauze, then 4 layers, then sterile nylon mesh (150 μm pores) into 50-ml conical centrifuge tubes. The filtered cells were washed 2 times with Solution II at 130 g for 8 min at 10°C.

7) The cell pellet was resuspended in 4 ml Dulbecco's modified Eagle medium (DMEM) with 0.1 mg/ml bovine serum albumin and 0.6 mg *Griffonia simplicifolia* lectin/4 ml and was incubated at 37°C for 30 min, mixing gently every 5 min. *Griffonia simplicifolia* lectin I agglutinates alveolar macrophages but not type II pneumocytes (213). A reddish-brown precipitate was evident after the incubation. The suspension was filtered through a 20 μm mesh into a 15-ml
conical centrifuge tube and centrifuged at 130 g for 10 min.

8) The resulting cell pellet was resuspended in 10 ml plain DMEM and panned onto a rat IgG-coated plastic bacterial culture plate prepared as described by Dobbs, et. al. (161). The plate was incubated at 37°C for 1 h. Loose cells were gently removed from the plate by rocking back and forth. The nonadherent cells were transferred to a 15-ml conical centrifuge tube and centrifuged at 130 g for 8 min.

9) The final cell pellet was resuspended in a volume of DMEM with 10% fetal calf serum appropriate for the experiments to be done. Cells were seeded at a density of $2 \times 10^5$ to $5 \times 10^5$ cells per well in basement membrane-coated (1:10 dilution in plain DMEM of Matrigel, Collaborative Research Inc., Bedford, MA) 24-well plates and incubated at 37°C for 5 days. Half of the medium was replaced every other day.

Alveolar macrophage isolation protocol. Alveolar macrophages were isolated from the BAL fluid collected in step 3 above. A detailed protocol for the remainder of alveolar macrophage isolation follows:

1) 50-ml conical centrifuge tubes containing BAL fluid were centrifuged at 130 g for 8 min at 10°C. Supernatant was decanted and the cell pellet washed twice more with phosphate buffered saline solution.

2) The final cell pellet was resuspended in DMEM with 10% fetal calf serum. Cells were seeded at a density of $5 \times 10^5$ cells per well in basement membrane-coated 24-well plates as for alveolar type II pneumocytes above. Half of the medium was replaced every other day.
Cell morphology and purity. Alveolar type II epithelial cells and alveolar macrophage cultures were examined daily using an inverted light microscope. Samples were collected immediately after isolation for cytocentrifuge to assess cell morphology and purity. Cells were fixed with tannic acid and stained using a polychrome stain as described by Mason, et. al. (187), and examined by light microscopy.

Ultrastructure. Cells were seeded on basement membrane-coated cell well inserts with a pore size of 3.0 µm. Inserts were fixed for transmission electron microscopy two to three days later. Standard fixation procedures including cacodylate-buffered glutaraldehyde and tannic acid were used in the Microscopy Branch of The Rocky Mountain Laboratories.

Virus infections. Experiments were done trying to infect alveolar type II pneumocytes and alveolar macrophages with 1:10 dilutions of either ADV-G or ADV-Utah kit virus. Cells were seeded into basement membrane-coated wells in a volume of 225 µl. 25 µl virus stock was added to create a 1:10 dilution of virus. Plates were incubated at 37°C for 90 min, then 1.75 ml of medium were added and plates returned to the incubator for the remainder of the experiment. Cells were trypsinized and cytocentrifuged for fluorescent antibody staining at 24, 48, 72, 96, and 120 h postinfection. Mock-infected controls were harvested at 48 and 120 h postinfection.

Fluorescent antibody staining. Cytocentrifuged specimens were fixed in acetone for 10 min, rinsed with 70% ethanol, and air-dried. Slides with fixed specimens were stored at -20°C until fluorescent staining was done. Slides were blocked for 30 min with 10% normal mink serum and then directly stained with fluorescein-conjugated polyclonal mink
serum that recognized capsid and nonstructural proteins. Differential staining was done using rabbit-generated anti-capsid or anti-nonstructural antibodies or a monoclonal anti-capsid antibody (Mab 165). Slides were mounted with anti-fade mounting medium and were viewed with a fluorescent microscope.

RESULTS

Isolation procedure. Alveolar type II epithelial cells were successfully isolated from the lungs of both rats and mink. The most difficult aspect of the isolation procedure was achieving adequate clearance of blood from the pulmonary vasculature. Transection of the aorta to allow an exit for the flush solution and clamping the large veins to prevent retrograde flow were the two most important steps necessary to attain complete clearance of the pulmonary vessels. These types of procedural details are best described by Mason, et. al. (188), and Simon, et. al. (213).

Cell morphology, purity, and yield. In order to assess the morphology and purity of isolated cells, cytocentrifuge samples were made immediately after type II pneumocyte and alveolar macrophage isolation. Type II pneumocytes were easily identified as they contained large round cytoplasmic inclusions, called lamellar bodies, that stained deeply brown with tannic acid alone (Figure 9.1, A) or bluish-brown with the addition of polychrome stain (Figure 9.1, B and C). Lamellar bodies contain phospholipids that contribute to surfactant. Immediately after isolation, type II pneumocytes accounted for approximately 65% to 85% of the cell population. These numbers increased to 85% to
Figure 9.1. Cytology of mink alveolar type II epithelial cells immediately after isolation. (A) shows type II pneumocytes fixed with tannic acid and magnified 20X. Mink type II pneumocytes have brown-staining cytoplasmic inclusions, or lamellar bodies. Lamellar bodies contain phospholipids that contribute to surfactant. (B) is a 20X magnification of mink type II pneumocytes fixed with tannic acid and stained with a polychrome stain (187). Macrophages are slightly larger than type II pneumocytes and do not contain lamellar bodies. Four macrophages are located in the center of the photograph. One macrophage contains phagocytosed particles in the cytoplasm. They can be distinguished from lamellar bodies based on their variable sizes and shapes. (C) shows a 40X magnification of mink type II pneumocytes stained as in (B). All cells seen are type II pneumocytes with the exception of one macrophage in the lower right corner of the photograph.
90% after two to three days in culture. Total type II pneumocyte cell yield from each mink varied from $4 \times 10^6$ to $7 \times 10^6$. Alveolar macrophage cultures were approximately 95% pure immediately after isolation. Macrophages were distinguished from type II pneumocytes by their peripheral cytoplasmic extensions (187) (Figure 9.2, A), multinucleated cells (Figure 9.2, B), and their general lack of cytoplasmic inclusions. When cytoplasmic inclusions were present (Figure 9.1, B), they were of unequal sizes and shapes and stained a deep red rather than bluish-brown color.

The organization of type II pneumocytes in cell culture changed over time similar to rat type II pneumocytes as described by Castleman, et. al. (150). Initially, cells were round and arranged individually in a monolayer covering the bottom of 24-well plate wells. Lamellar bodies were apparent as clear, refractile areas in the cytoplasm of type II pneumocytes by light microscopy. By 48 h postinfection, the type II pneumocytes were arranged in small circular groups with an internal lumen, and individual cells appeared more teardrop-shaped than round. Between two and five days postinfection, these cell aggregates appeared to expand upward towards the medium surface.

**Ultrastructure.** Transmission electron microscopy (TEM) confirmed the presence of lamellar bodies in the rat (not shown) and mink (Figure 9.3, A) alveolar type II epithelial cells. The “onion skin” appearance of the layers of phospholipids within these inclusions is very distinct, and surfactant can occasionally be seen as it extrudes from the cell membrane (Figure 9.3, B). Other contaminating cells seen by TEM included polymorphonuclear, monocytic, and ciliated bronchial epithelial cells.

**Virus infections.** Rat alveolar type II epithelial cells never showed evidence of infection.
Figure 9.2. Cytology of alveolar macrophages immediately after isolation. (A) shows alveolar macrophages fixed with tannic acid and stained with a polychrome stain (187). The cytoplasm of alveolar macrophages stains pale to medium pink compared to the deeper blue seen in type II pneumocytes (see Figure 9.1). A magnification of 40X reveals the peripheral cytoplasmic extensions that help distinguish macrophages from alveolar type II epithelial cells (187). In (B), a multinucleated macrophage is present. Multinucleation also distinguishes macrophages from type II pneumocytes.
Figure 9.3. Transmission electron microscopy of mink alveolar type II epithelial cells 48 hours after isolation. A type II pneumocyte is seen in (A) containing several lamellar bodies in its cytoplasm (17,400X). Inside the lamellar bodies is the characteristic “onion-skin” pattern created by the arrangement of the polar heads of the phospholipids contained within the lamellar bodies. (B) shows a portion of a type II pneumocyte at a magnification of 90,000X. A small lamellar body is being extruded from the cell. The ultrastructural features of lamellar bodies seen by transmission electron microscopy confirmed the identity of these cells as alveolar type II epithelial cells.
with either ADV-G or ADV-Utah 1 as determined by fluorescent antibody staining in two separate experiments per virus. Mink type II pneumocytes and alveolar macrophages never appeared infected with ADV-G in two separate experiments. However, mink type II pneumocytes incubated in the presence of ADV-Utah 1 showed both cytoplasmic and nuclear staining between three and five days postinfection reminiscent of ADV-G-infected CrFK (Figure 9.4) in three different experiments. When specific staining was done to identify capsid and nonstructural proteins, however, no staining was seen. The primary antibodies and secondary fluorescent antibodies worked well on control ADV-G-infected CrFK cells that were stained simultaneously with the type II pneumocytes.

**DISCUSSION**

Alveolar type II epithelial cells were successfully isolated from the lungs of sapphire mink. By gross examination, they appeared to survive well in culture for up to five days after isolation and displayed morphological characteristics described for rat type II pneumocytes (150,187,188). The purity of cultures was similar to that reported for rat type II pneumocytes (160,188,213) purified using similar steps. Increased initial purity may be possible by including density centrifugation or additional filtering steps, but the loss in yield may be prohibitive.

The total yield of type II pneumocytes per mink was disappointing compared to numbers reported for rats (161). However, earlier literature suggested that the supplier and the housing conditions substantially altered the yield of type II pneumocytes in rats.
Figure 9.4. Fluorescent antibody staining of mink alveolar type II epithelial cells incubated with ADV-Utah 1. All panels show cytocentrifuge specimens of mink alveolar type II epithelial cells stained with a 1:15 dilution of fluorescein-conjugated polyclonal anti-ADV mink serum. (A) illustrates mock-infected cells 48 hours after infection. No cellular staining is evident. (B) shows two positively staining cells. In the upper cell, nuclear and cytoplasmic staining is evident while the lower cell has nuclear staining only. (C) illustrates type II pneumocytes 72 hours postinfection with ADV-Utah 1. Nuclear and cytoplasmic staining is evident in one cell. (D) is an example of type II pneumocytes 120 hours postinfection. A small cell adjacent to a larger cell shows both nuclear and cytoplasmic staining. While these panels suggest that ADV replication is occurring in the nucleus, viral protein-specific stains did not demonstrate the presence of nonstructural proteins.
from $8 \times 10^6$ to $20 \times 10^6$ cells/rat (188). Our yields were comparable to those for non-specific pathogen free rats without special housing. However, increased yields would substantially improve the ability to set up adequate repetitions of experiments with cells isolated from one animal. This can be a difficult task as it is estimated that alveolar type II epithelial cells account for only 14% of total cells in the lungs of rats (159).

Although with polyclonal anti-ADV serum there appeared to be positive staining in mink type II pneumocytes incubated with ADV-Utah 1, we were discouraged that viral protein-specific staining did not yield positive results. We speculate that the viral proteins in the type II pneumocytes may be degraded. While it is common for ADV proteins to be degraded in infected mink, polyclonal antiserum should contain antibodies directed against epitopes of degraded viral proteins. This may not be the case with rabbit or mouse antibodies generated against specific protein preparations. It is also possible that the staining we saw with polyclonal antiserum is artifactual. The argument against this explanation is that the patterns of staining were identical to those seen in CrFK cells infected with ADV-G. Finally, only very few cells exhibited positive staining. It is possible that the incidence of infection was so low that specimens stained with protein-specific antibodies did not contain any infected cells. More experiments are necessary to determine if these cells were productively infected with ADV-Utah 1.

Additional experiments that remain to be done are alveolar type II epithelial cell isolations from mink kits with attempted infection. Evidence from humans and from ferrets suggest that permissivity for herpes simplex virus and influenza virus, respectively, is age dependent (154,155,191). This may be due to the permissivity of type
II pneumocytes, the lack of a mature pulmonary immune response, or both. Nevertheless, since there is a proven age dependency in ADV infection, it is possible that alveolar type II epithelial cells from mink kits may be productively infected \textit{ex vivo}. Further experiments must be done to prove this hypothesis.
CHAPTER 10

FUTURE DIRECTIONS

In vivo replication and pathogenesis. The work presented in this dissertation and previous work in our laboratory (138,166) show the importance of the Aleutian mink disease parvovirus (ADV) capsid amino acids, V352 and D534, in in vivo replication. Additionally, experiments in this dissertation work demonstrate that interactions between multiple capsid amino acids are necessary for the induction of classical Aleutian disease (AD) in sapphire mink. Finally, the nonstructural gene may be playing an interactive role as well in influencing the consistency of host immune responses and the occurrence of histopathologic lesions consistent with AD. However, none of the molecular constructs made to date reproduce the level of in vivo replication and pathogenesis seen with ADV-Utah 1. There are still pieces of the puzzle that are missing.

The mapping of in vivo replication and pathogenesis determinants can continue along several different lines of approach. Work remains to be done with the capsid gene, the nonstructural gene, and combinations of the two. In order to more specifically map replication determinants of the capsid gene, double and triple capsid mutants need to be evaluated. The first combination of amino acids that is obvious is V352 and D534. This chimera has been constructed and awaits in vitro propagation and in vivo studies (J. M. Fox, unpublished data). Additional combinations of three amino acid changes may help resolve which other single amino acid change in addition to V352 and D534 could contribute to in vivo replication and histopathologic lesions consistent with AD. These
mutants would be constructed of ADV-G background with V352 and D534 in the capsid gene. The additional point mutations, H395Q, N434H, and N491E, would be individually introduced into the capsid gene for a total of three new chimeras. H395Q and N434H, when present in ADV-G as single point mutations, abolish the ability of ADV-G to replicate in Crandell feline kidney (CrFK) cells (166). It is not known if these mutations in combination with V352 and D534 will inhibit replication in vitro. It is also possible that a minimum of four capsid changes may be necessary to induce AD-like histopathological changes. If necessary, additional mutants can be made after more information is gathered from the double and triple mutant studies.

Next, a larger portion of the nonstructural gene of ADV-Utah 1 should be studied in the context of the five capsid amino acid changes present in ADV-G/U-8 and -15 (Figure 7.1, Table 7.1). Additional amino acid differences between ADV-G and ADV-Utah 1 in the nonstructural gene include I378M and F481L as well as a noncoding change at nucleotide 1705 (codon 500) (136,168). All of these changes reside upstream of the P36 promoter that controls transcription of the capsid gene. These changes could have direct effects on the nonstructural proteins or have indirect effects on the downstream promoter or a combination of the two.

Finally, when the influence of capsid changes are mapped more narrowly and the contribution of the nonstructural gene is more evident, crucial changes from each region should be combined in the same chimera. If this chimera replicates in vitro, it can be tested in mink. The only portions of the genome left unstudied at this point will be a few individual residues at the left end of the capsid gene, a few changes in the mid open
reading frames, and noncoding regions in the 3' and 5' hairpin ends that may influence viral replication.

An additional project that could potentially improve the quality of the antiviral antibody measurements made in the aforementioned experiments would compare new capsid antigens for use in the counterimmunoelectrophoresis (CIEP) assay with the commercial standard currently used (United Vaccines, Madison, WI). The commercial antigen we currently use is derived from ADV-G. It is possible that some of the altered capsids created by site-directed mutagenesis have epitopes differing from ADV-G. Subsequently, in order to accurately measure antiviral antibody produced against these capsids, it may be necessary to test antiserum against virions made of these mutated capsid proteins. It is possible to do this by cloning the mutated capsid sequences into a baculovirus system, propagating the VP2 protein, and allowing self-assembly of empty virus particles. These virus particles could then replace the commercial antigen in the CIEP assay. If no differences between baculovirus-generated and commercial antigens were seen, the commercial antigen would be retained as the standard. Alternatively, if virus titers are changed by using the appropriate antigen for suspected antibody produced, it may be necessary to generate new antigen for each new capsid construct.

**Replication at the tissue level.** Although we evaluate viral replication at the systemic level by testing serum, we have been concerned that replication may be occurring at the tissue level and that virus remains sequestered from the peripheral vasculature. Alternatively, low level replication may be below the detectable level of our PCR assay. Subsequently, individual tissue samples must be screened for viral DNA in order to
determine if virus is present in various organs. Preliminary results from PCR amplification of purified genomic DNA isolated from liver, spleen, kidney, and mesenteric lymph node of chimera-infected mink in this dissertation study suggest that the V352 residue of VP2 may influence the ability of virus to infect the kidney (Table 10.1). Only chimeras with five capsid changes were found in the kidney. Since volume, rather than mass, of DNA solutions were equalized for the PCR reactions, there was a potential for error when comparing results among mink. Screening agarose gels showed that PCR positive reactions did not correlate with samples containing the highest DNA concentrations. PCR reactions using mass-equalized samples have yet to be done.

In order to determine whether viral replication was occurring in the tissues that tested positive by PCR, Southern blotting to probe for replicative form and virion DNA must be done. It is possible that the viral DNA detected by PCR is merely input virus that remained sequestered in organs for which the virus was most tropic. Also, target immune cells could traffic to these organs, carrying virus with them. It would be very interesting if Southern blot analysis demonstrated replicative form viral DNA in some tissues and not others. This kind of analysis may be helpful in learning more about the tropism and in vivo behavior of ADV chimeras.
TABLE 10.1. Detection of parvoviral DNA and histopathologic lesions in mink infected with ADV chimeras

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<sup>a</sup> DNA template samples were amplified using ADV-specific primers complementary to a region of the VP2 gene of ADV-G.

<sup>b</sup> Untreated serum was used as template for the PCR reaction.

<sup>c</sup> DNA template was isolated from 10 mg of spleen and 20 mg of other tissues using a Viral RNA Mini Kit (Qiagen, Valencia, CA).

<sup>d</sup> Histopathology was graded as described in table 7.3.
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