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DISSERTATION

MUCOSAL TRANSMISSION AND EARLY PATHOGENESIS OF FELINE IMMUNODEFICIENY VIRUS SUBTYPE C INFECTION

Submitted by
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Department of Pathology

In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Spring 1999

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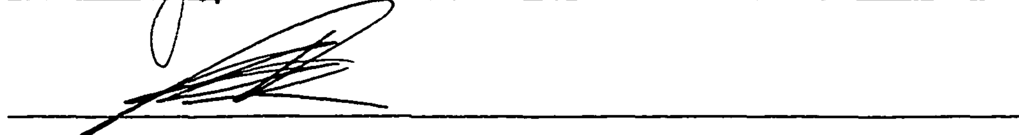
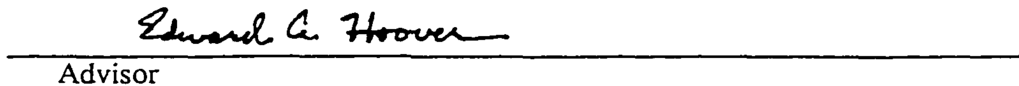
COLORADO STATE UNIVERSITY

April 2, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LESLIE ANN OBERT ENTITLED MUCOSAL TRANSMISSION AND EARLY PATHOGENESIS OF FELINE IMMUNODEFICIENY VIRUS SUBTYPE C INFECTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work





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ABSTRACT OF DISSERTATION

MUCOSAL TRANSMISSION AND EARLY PATHOGENESIS OF FELINE IMMUNODEFICIENY VIRUS SUBTYPE C INFECTION

Human immunodeficiency virus infection is initiated by mucosal exposure; however, the early events resulting in mucosal infection are unknown and difficult to study in humans. Likewise, such issues are unresolved for mucosal feline immunodeficiency virus (FIV) infection and could be examined utilizing the FIV-feline model. I have focused on the mucosal transmissibility and early pathogenesis of infection by a subgroup C FIV (FIV-C), because this virus is unique in inducing high viral burdens and rapidly progressive infection in cats infected by serial intravenous passage.

Neonatal and weanling cats were exposed to FIV-C via the oral, vaginal, or rectal routes. FIV-C proved to be transmissible by all three routes. Moreover, mucosal exposure of FIV-C resulted in a broader range of host-virus relationships than observed in the rapid parenteral serial passage studies. Similar to the latter studies, some of the vaginal inoculates developed a rapidly progressive course of FIV-C infection. However, rectal exposure was less efficient at inducing infection. A few animals developed regressive infections detectable rarely by virus isolation coculture (VI) or PCR and unaccompanied by hematologic abnormalities. The thymus was an early lymphoid system target organ in the FIV-C infections. The major thymic changes observed were depletion of dendritic, CD4+, and CD4+/CD8+ cells, and marked apoptosis. The magnitude of the thymic lesions correlated with disease severity.

To identify the early target tissues in transmucosal lentivirus infection, cats infected oral-nasally or vaginally were necropsied at specific time points within the first twelve days post

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infection (PI). Mucosal tissues, regional lymph nodes (LNs), peripheral blood mononuclear cells (PBMC), and other tissues were collected and examined by VI, PCR, and in situ hybridization (ISH) to identify the earliest FIV target tissues. FIV provirus was detected rarely within regional LNs and PBMC before eight days PI by PCR. However, rare FIV-bearing cells were identified in regional LNs by two days PI by ISH. The earliest FIV-bearing cells demonstrated by ISH had morphologic characteristic of dendritic cells and macrophages and were located primarily in the follicular germinal centers and parafollicular areas of the LNs. Rare FIV+ cells also were identified within the epithelium and submucosa of mucosal tissues as early as one day PI.

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DEDICATION

I dedicate this work to my parents, Richard and Barbara Obert. As far back as I can remember, they have always encouraged me to do my best and to never give up. With acceptance, patience, and confidence, they supported my ambitions and dreams. Regardless of success or failure, their love and devotion always abounded. No request was ever too big and no problem was ever too small; their attentiveness never abated. They provided me with the foundation to achieve success and mortar to endure life's adversities. I cannot thank them enough for their sacrifices, commitment, and unremitting love.

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INTRODUCTION

World-wide Problem:

The World Health Organization (WHO) estimated in 1995 that close to 2 million children were infected with human immunodeficiency virus (HIV), almost all of whom were infected by mother-to-infant transmission. [1] In the United States alone, over 7,000 HIV-infected women give birth to 1,000-2,000 HIV-infected children each year, making HIV the 7th leading cause of death in children 1 to 4 years of age. [2-4] HIV transmission from mother to child can occur by any of three routes: in utero, intrapartum, or postpartum via milk. The contribution each pathway makes to the total transmission rate is unknown. [5,6] Since HIV vertical transmission rates are halved by cesarean versus vaginal delivery, it has been inferred that virus exposure of the infant during delivery (intrapartum transmission) may be responsible for up to 50% of infant infections. [7]

The WHO estimated that greater than 90% of the global total of 38 million cumulative HIV infections that occurred in adults through 1997 were due to sexual transmission. [8] While a majority of the HIV-1 infections in industrialized Western nations are due to transmission among homosexual men and intravenous (iv) drug users, heterosexual transmission accounts for a very large proportion of infections in the developing countries of Africa, Asia, and Latin America where the HIV/acquired immunodeficiency virus syndrome (AIDS) epidemic is the most severe. [9] However, heterosexual HIV transmission in Western nations is increasing faster than transmission by other exposure routes. [10] Both male-to-female and female-to male transmission

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has been well reported. [11] In addition, the number of infected women is increasing in the new infected population. [12]

The early events in transmucosal HIV infection remain unknown and are at best difficult to study in humans. For this reason, the early pathogenesis of mucosal lentiviral infections can best be explored in an animal model, in which the earliest target cells and the sequence of cell and tissue infection in cats receiving a single mucosal virus exposure can be mapped. In addition, model studies could aid in identifying the stage in lentiviral infection progression at which antiviral therapy remains highly effective. Thus, my dissertation research utilized mucosal feline immunodeficiency virus (FIV) infection as an animal model for the study of early HIV mucosal transmission events.

FIV history and clinical course:

FIV, a lentivirus identified in 1986, [13] causes a disease syndrome similar to that caused by HIV.[14] The clinical phases of FIV infection parallel those staging HIV. The acute phase is characterized by a burst of viral replication, flu-like illness, lymphadenopathy, and an early progressive decline in CD4 T lymphocytes [15-18] which is followed by a prolonged asymptomatic phase featuring viral down-regulation and a continued gradual decline in CD4 cells. The terminal phase of FIV disease is characterized by immunological decompensation, wasting, hematologic suppression, recrudescence of plasma viremia, and clinical immunodeficiency with opportunistic infection. [14,19-23] FIV shares other features with HIV infection, including multiple hematologic disorders, neurologic abnormalities, and characteristic hyperplastic then depletive lymphoid tissue lesions. [14,19,23-30]

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FIV transmission:

Similar to HIV, FIV can be transmitted by mucosal exposure, blood transfer, and vertically via in utero, milk-borne, and intrapartum routes. [13,31-35] Virus can be isolated from vaginal washes of FIV-infected pregnant and postpartum queens providing evidence for potential intrapartum transmission. [33] FIV has also been isolated from saliva and semen of experimentally and naturally infected cats [36-38] suggesting the potential for natural horizontal (biting and sexual) modes of transmission. In addition, FIV infection has been experimentally transmitted by various mucosal routes (oral, rectal, vaginal) [34,39-41] utilizing either cell-free or cell-associated virus inocula. Infection via the oral mucosal route has been shown to be effective in newborn kittens foster nursed by FIV-infected queens [33] and has been reproduced by multiple oral exposures with FIV-infected cells. [34] However, oral transmission was less efficient than vaginal or rectal transmission when utilizing an inoculum of FIV-infected cells. [34] These data suggest that cell-free and cell-associated virus may utilized different mechanisms to cross different mucosal barriers.

FIV tropism:

FIV replication has been demonstrated in several target cells: T lymphocytes, [13,21,23,42-44] macrophages, [15,26,45-47] B cells, [19] megakaryocytes, [48] salivary gland ductular epithelium, [49] kupffer cells, [50] endothelial cells, [51,52] and astrocytes and microgia of the nervous system. [24,53] Both CD4+ and CD8+ T cells are susceptible to productive virus infection *in vitro*. [42,54] *In vivo* virus tropism studies have demonstrated viral replication in T cells during the early phase of infection (6 to 8 weeks) followed by macrophage localization later in infection. [15,45]

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Accelerated FIV model:

Development of the FIV model has been limited by its prolonged and variable disease course in infected cats. In recent experiments conducted in our laboratory, a virulent FIV strain which induces high virus burden and rapid immunodeficiency disease onset has been characterized. [55] By pooling plasma from cats acutely infected with an uncultured clinical isolate FIV-C-PGammar, fatal immunodeficiency was produced in greater than 50% of infected cats. [56] High FIV RNA titers were associated with rapid disease progression to a fatal outcome. [56] Conversely, lower plasma virus burdens correlated with survival and more long-term infection status. [56] Tissue viral burden determined by in situ hybridization (ISH) correlated with plasma virus load determined by quantitative competitive RNA PCR. Cats with high viral RNA titers had greater numbers FIV replicating cells in their tissues than did cats with low plasma viral burdens. [56,57] Ancillary studies demonstrate that FIV infection kinetics resembled those described for acute HIV infection. [56,58-60] Early peak viremia immediately preceded the onset of acute phase symptoms and coincided with the initial precipitous decline in circulating CD4 cells. Plasma virus titers remained highest throughout the symptomatic phase of infection (up to 10 weeks) and then declined as symptoms resolved. The accelerated FIV disease syndrome, therefore, could provide a rapid high virus expression model for mapping of the early events after mucosal exposure in cats.

Mechanisms of lentiviral mucosal infection:

Several theories have been proposed concerning the mechanisms of lentiviral mucosal infection. One hypothesis is that the virus traverses the mucosal membrane at a lesion, and directly enters the blood stream or infects a susceptible cell at that site. [61] In non-primate neonates lentiviruses may cross the mucosal surface before normal physiologic gut closure. [62] Another pathway

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proposed is that HIV can cross an intact mucosal surface by means of M cells. [63] These cells are specialized for transepithelial transport and concentrated over intestinal mucosal lymphoid follicles and Peyer's patches. Explants of Peyer's patches from rabbit and mouse intestine have demonstrated endocytosis of HIV particles from the lumenal surface, transport of the intact virus to their basolateral membrane, and deposition of the virus into an intraepithelial space containing lymphocytic and histiocytic targets. [63]

An *in vitro* study by Bomsel [64] demonstrated that contact between various epithelial cell lines and primary peripheral blood leukocytes from HIV-infected patients resulted in rapid budding of HIV virions towards the epithelium followed by their internalization into epithelial endosome-like structures. This was mediated through the binding of HIV gp120 cell surface receptors to galactosyl ceramide at the apical membrane. As early as 30 min. after apical contact, the primary isolates crossed the epithelial cell line barrier using transcytosis and appeared in the basolateral medium. In addition, the transcytosed HIV particles productively infected mononuclear cells located at the basolateral side of the epithelial barrier. However, free HIV added apically was not internalized nor trancytosed, suggesting that cell-cell contact was required for efficient delivery of virions to the epithelial cell. However, another *in vitro* study demonstrated spontaneous binding of fluorescently labeled recombinant HIV-gp120 and CD4 cells to human enterocytes utilizing a quantitative cell adhesion assay. The binding activity was substantially enhanced by treatment of the enterocytes with cytokines representative of inflammatory conditions. [65]

Infection of mucosal dendritic cells:

Another hypothesis suggests that transmission of HIV across intact mucosa might occur via direct infection of Langerhans cells (LC). [66] In humans, skin and mucous membranes are colonized by

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these (LC) dendritic cells which play a role in the immune surveillance by acting as antigen-presenting cells. Multiple studies have demonstrated that LC can serve as targets and reservoirs for HIV *in vivo* and *in vitro*. [67-75] Studies of macaques chronically infected with SIV have also found infected cells with dendritic morphology within the stratified squamous epithelium of the vagina. [76] In addition, SIV-infected LC were found in the lamina propria and stratified squamous epithelium of foreskin from infected male macaques. [77] LC and blood dendritic cells have been shown to be efficient vectors for HIV transmission to T cells *in vitro*. [78-81] These *in vitro* experiments support the concept that mucosal LC may act as vectors for the transmission of HIV to T cells during primary infection. In an acute SIV transmission study, infected LC were found in the lamina propria of the cervicovaginal mucosa immediately subjacent to the epithelium and in draining lymph nodes within 2 days after cell-free SIV infection. [82,83] In another animal model study, resident mouse DC routed a heat inactivated lymphoctropic HIV-1 strain to the draining lymph nodes within 24 hours after vaginal or iv inoculation. [84]

An equivalent of the LC has been identified in cat stratified squamous epithelium. [85] As of yet, FIV infection of LC has not been shown, although FIV RNA and antigen have been demonstrated in association with follicular dendritic cells (FDC) in lymphoid germinal centers of infected cats. [15,86,87] However, the distinction between virus particles trapped in immune complexes on the processes of FDC via complement receptors and local viral replication within FDC remains unclear. In HIV-infected patients, viral antigens and RNA have been demonstrated in FDC. [88-90] Demonstration of FIV infection via LC would enhance the usefulness of FIV as a model to study HIV mucosal pathogenesis.

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Infection of mucosal epithelial cells:

Another potential HIV entry portal is direct infection of enterocytes. The ability of cultured fetal human enterocytes to endocytose HIV particles directly from adherent HIV-infected monocytes has been demonstrated. [91] HIV can also infect cultures of human colorectal cell lines. [92] Some researchers report that enterocytes [93-95] and enterochromaffin cells [96-98] are infected by HIV, but this is not a consistent finding in HIV-infected patients. Others have not been able to demonstrate HIV infection of intestinal epithelial cells *in vivo* [99] or in explants of well-differentiated human fetal intestine exposed to lumenal virus. [100] Due to the differences in the patients' immune status, viral load, length of infection, or cell tropism of the viral quasispecies, the subjects of the above aforementioned studies were not comparable. None of the above experiments have demonstrated transepithelial transport of HIV by normal enterocytes. Thus, there is not clear evidence to date that normal absorptive enterocytes in adults can serve as entry sites for HIV. Interestingly, HIV-1 proviral DNA has been demonstrated in oral mucosa squamous cells and in salivary mononuclear cells by IS-PCR; however, viral replication (HIV RNA) could not be detected by ISH. [101]

Mucosal transmission via exposure to lentiviral infected cells:

A number of findings suggest that mucosal transmission could occur by HIV-infected cells. The ability to detect HIV in semen correlates with the presence of leukocytes. Virus-infected cells and not cell-free virus can be found in 6-18% of semen samples. [102,103] New findings by Phillips *et al.* [104] suggest that lymphocytes may transmit HIV to CD4-negative epithelial cells (ME-80 a transformed human cervical cell line) and that in turn, these productively infected epithelial cells may transmit the virus to underlying macrophages. Studies in our laboratory indicate that exposure of cats to FIV via vaginal or rectal inoculation of infected cells efficiently induces infection, [105] although the viral pathway of entry and local cellular infection is not yet known.

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Mucosal macrophage tropism:

Several lines of circumstantial evidence suggest that an important property of transmitted virus is the ability to infect macrophages. [106-110] HIV RNA and DNA has been demonstrated in macrophages at the mucosal-stromal junction of the endocervical transformation zone, but virus positive macrophages were not detected in the vagina or endometrium. Macrophages were the predominant cells infected, whereas most lymphocytes were not infected. [111] In an other study, cervical biopsy tissue from HIV-infected women contain HIV-infected macrophages and T cells. [112] In SIV mucosal challenge studies, infected cells in the genital tract were primarily macrophages located in the submucosa of the cervix and vagina. [76,77] Thus, mucosal macrophages, especially within the reproductive tract, can become infected by HIV. Macrophages might play a role in dissemination and transmission of HIV from mucosal to regional or systemic lymphoid tissues.

Mucosal vs. systemic lentiviral replication:

The relationship of mucous membrane and systemic lymphoid tissues have to the pathogenesis of AIDS is unclear. The burden of virally infected cells in peripheral blood is too low to explain the loss of CD4 lymphocytes, especially in the early stages of the disease. [113] Thus, the search for tissue compartments with higher viral burdens than blood commenced. [88,114,115] Several researchers found tissues with higher burdens of HIV-infected cells. [115-117] In addition, lymph nodes were found to be reservoirs for HIV and sites of viral replication during the clinically latent phase of disease. [88,114,115] ISH performed on rectal biopsies from subjects in the intermediate stage of HIV infection demonstrated the highest prevalence (91%) of HIV RNA when compared to early and late stages of infection. The HIV+ cells present in both the mucosal lymphoid follicles and lamina propria were FDC. [117] The staining pattern in germinal centers of mucosal

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lymphoid follicles was identical to that seen in peripheral lymph nodes, and represented FDC trapping of HIV virions by immune complexes. [88] Thus, even though the components and function of mucosal immunity are homologous to those of systemic immunity, they involve distinct subsets of cells. [117]

Most of the studies on the initial stages of lentiviral infection have focused on peripheral blood and lymph nodes. However, the gastrointestinal tract contains the most lymphoid tissue in the body, as well as, a greater proportion of activated memory CD4 cells than in the peripheral blood or lymph nodes. [118-120] HIV has been shown to replicate most efficiently in activated memory CD4 cells. [121] Veazey *et al.* [122] found that SIV infection of rhesus monkeys resulted in profound and selective depletion of CD4 cells in the intestine within days of infection prior to such changes in the peripheral lymphoid tissues. Thus, they concluded that the intestine was the major target for SIV replication and the major site of CD4 cell loss in early SIV infection. Several studies of HIV-infected humans have also suggested that CD4 cell depletion was more pronounced or occurred sooner in the intestine than in peripheral blood. [123-125] These studies suggest that the acute phase of SIV/HIV is a disease of the mucosal immune system. Thus, therapies that target the intestinal tract and vaccines that stimulate an effective mucosal immune response may be the best early defense strategies.

Lentiviral receptors and coreceptors:

The entry of primate lentiviruses into target cells has recently shown to depend upon the interaction of the viral envelope glycoproteins with CD4 and one or more members of the G protein-coupled receptor (GPCR) family of transmembrane proteins. [126,127] *In vivo*, the transmission of HIV-1 infection generally requires viral strains that utilize the chemokine

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receptor CCR5. These strains prevail during the early course of infection. Strains isolated later in the course of progression to immunodeficency, are often CXCR4-tropic or are dual tropic for both chemokine receptors. [128,129] SIV isolates also use CCR5, but are only rarely specific for CXCR4. In addition, SIVs use two orphan members of the GPCR family: Bonzo/STRL33/TYMSTR and Bob/GPR15. [130] Additional GPCR family members have also been shown to be utilized by various strains of HIV and SIV with less efficiency and less frequency. [131-133] Several studies in HIV have found that no selection for macrophage-tropic/non-syncytium-inducing viruses occurs during sexual transmission. [134-137] Another study suggests that most macrophage-tropic HIV isolates are actually dual tropic. [138] In addition, SIV/SHIV experiments confirm that there is no restriction on mucosal transmission of variants that can replicate in T cell lines but not macrophages *in vitro*. [139,140] In both rhesus macaques and humans, T lymphocytes and macrophages in both lymphoid and nonlymphoid tissues are the major cell populations expressing HIV/SIV coreceptors. [141] While chemokine receptors are expressed primarily in hematopoietic cells, many other cell types have been shown to express one or more chemokine receptors: microglia, [142,143] endothelial cells, [144,145] placental macrophages, [146] colon, rectum, cervix, and vagina. [141] In addition, Zhang *et al.* [141] found that coreceptor-positive cells were more frequently identified in the colon than in the rectum and in the cervix than in the vagina suggesting that the expression levels of coreceptors are differentially regulated at different anatomic sites.

Unlike the primate lentiviruses, the feline homologue of CD4 does not appear to act as a primary receptor for FIV. Blocking studies utilizing antibodies that recognize diverse epitopes on feline CD4 did not inhibit infection with FIV. [147,148] In addition, ectopic expression of feline CD4 in feline fibroblasts did not render these cells susceptible to infection with lymphotropic FIV

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isolates.[149] Two studies have recently identified the shared usage of the chemokine receptor CXCR4 by FIV and HIV as well as a 94.9% amino acid sequence homology.[148,150] These results suggest a evolutionary link between FIV and HIV and a common mechanism of infection and cytopathicity for two distantly related lentiviruses that cause AIDS.

Dissertation research:

It is clear that many questions concerning HIV as well as FIV transmucosal infection remain unanswered. Thus, studying the early events in the pathogenesis of mucosal FIV infection would serve a dual purpose as an animal model for the study of mucosal HIV infection and as an experimental model for the study of a naturally occuring feline disease.

The first objective of my dissertation research was to examine the mucosal transmissibility of the virulent FIV-C-PGammer strain. Previous studies in our laboratory demonstrated mucosal transmissibility of a macrophagetropic B clade FIV.[40] However, mucosal infectivity of FIV may be strain specific as seen with HIV and SIV.[151-154] Three mucosal routes (oral-nasal, rectal, and vaginal), paralleling HIV mucosal exposure sites, were examined to identify the most susceptible infection scenario. We also hoped to reproduce in a mucosal model the accelerated disease course seen following iv FIV-C infection. In addition, we wanted to better characterize the lesions associated with the accelerated FIV-C disease course.

After identifying successful mucosal transmission routes, the final major objective was undertaken—to identify the earliest sites of FIV replication following mucosal exposure. We hypothesized that the first site of FIV replication would be local mucosal associated lymphoid

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tissue (MALT) followed by the regional draining lymph nodes. This would occur prior to systemic viral dissemination and the viral initial target cells would be mucosal dendritic cells and/or macrophages. Information from these studies would be useful in the design of therapy and intervention strategies for both HIV as well as FIV infections.

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CHAPTER ONE

FELINE IMMUNODEFICIENCY VIRUS SUBTYPE C
MUCOSAL TRANSMISSION & HOST-VIRUS RESPONSES

INTRODUCTION

FIV, a lentivirus identified in 1986, [1] causes a disease syndrome similar to that caused by HIV. [2] The clinical disease is characterized by an acute burst of viral replication, flu-like illness, lymphadenopathy, and progressive decline in CD4+ T lymphocytes, [3-6] followed by a prolonged asymptomatic phase of viral down-regulation, and terminal immunological decompensation, wasting, and clinical immunodeficiency with opportunistic infection. [2,7-11]

As with HIV, it appears FIV can be transmitted by mucosal exposure, blood transfer, and vertically, via pre-, intra-, and postpartum routes. [1,12-16] FIV has also been isolated from saliva and semen of experimentally and naturally infected cats [17-19] suggesting the potential for natural horizontal modes of transmission. In addition, FIVs of clades A and B have been transmitted by oral, rectal, or vaginal mucosal exposure [15,20-22] utilizing either cell-free or cell-associated virus inocula. While infection via the oral route has been shown in newborn kittens foster nursed by FIV-infected queens [14] or by multiple oral exposures with FIV-infected cells, [15] it is not clear whether oral transmission is a realistic route of infection, especially in post neonatal animals. Its relevance to the natural history of FIV infection and as a potential model to study transmucosal infection events and pathways needs to be determined.

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Development of the FIV model has been limited by its prolonged and variable disease course in infected cats. We have identified an FIV strain, FIV-C-PGammer (FIV-C), which induces high virus burden and rapid immunodeficiency disease onset. [23] High FIV RNA titers in plasma and tissues correlated with rapid disease progression to fatal disease [24,25] paralleling the kinetics of acute HIV infection. [24,26-28] The accelerated FIV disease could provide a rapid high virus expression model to map virus trafficking after mucosal lentiviral exposure, events at best difficult to study in humans. The objective of the present studies therefore was to determine whether FIV-C was transmissible or pathogenic after any of 3 mucosal exposure routes.

MATERIAL & METHODS

Animals & sample collection

One day old kittens from a specific-pathogen-free (SPF) breeding colony maintained at Colorado State University (Fort Collins, CO) were inoculated by atraumatic exposure of the oral and nasal mucosae with 200 μL of 200 $TCID_{50}$/mL (50% tissue culture infective dose; n = 5) cell-free infectious FIV-C plasma or 300 $TCID_{50}$/mL (n = 9) cell-free infectious FIV-C culture supernatant. Litter-matched control kittens were sham-inoculated with 200 μL of cell-free noninfectious plasma (n = 3) or cell-free noninfectious tissue culture medium (n = 4). Blood collections were performed under ketamine anesthesia every other week for the first 8 weeks post inoculation (PI) or every other week for the first 14 weeks and at week 20. Fourteen kittens (9 inoculates and 5 controls) were euthanized at 8 weeks and the remaining 7 (5 inoculates and 2 controls) at weeks 28 or 29 for necropsy and terminal sample collection.

Eight to 10 week old weanling SPF cats (n = 6) were inoculated by atraumatic exposure of the oral and nasal mucosae using a syringe to instill 1 mL of 300 $TCID_{50}$/mL cell-free infectious culture supernatant. Litter-matched control cats (n = 4) were sham-inoculated with

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an equal volume of cell-free noninfectious tissue culture medium. Blood was collected every other week for the first 8 weeks PI and then at 14 weeks or at weeks 5 and 11. Five cats (3 inoculates and 2 controls) were euthanized at 16 weeks and the remaining 5 cats (3 inoculates and 2 controls) at 21 weeks for necropsy and terminal sample collection.

Eight to 10 week old weanling SPF cats were inoculated by atraumatic exposure of the rectum using a rubber tomcat catheter to instill 1 mL of 200 $TCID_{50}$/mL cell-free infectious plasma ($n = 2$) or 300 $TCID_{50}$/mL ($n = 7$) cell-free infectious culture supernatant. Litter-matched control cats ($n = 3$) were sham-inoculated with an equal volume of cell-free noninfectious plasma ($n = 1$) or cell-free noninfectious tissue culture medium ($n = 2$). Blood was collected every other week for the first 10 or 11 weeks PI and then at 15 or 16 weeks. All cats except one were euthanized between 17 and 20 weeks for multiple tissue collection. Colic lymph node was biopsied at 37 weeks in one cat (#3587) which was observed through 52 weeks.

Eight to 10 week old weanling SPF cats were inoculated by atraumatic exposure of the vaginal mucosae using a rubber tomcat catheter to instill 500 µL of 200 $TCID_{50}$/mL cell-free infectious plasma ($n = 3$) or 300 $TCID_{50}$/mL ($n = 4$) cell-free infectious culture supernatant. Control cats were from the weanling oral-nasal and rectal inoculation studies. Blood was collected every other week for the first 12 weeks PI and then monthly thereafter. Three of the cats were euthanized early, 2 at 6 weeks and 1 at 8 weeks, due to severe immunodeficiency and secondary infections. The remaining 4 cats were transferred to other studies between 36 and 51 weeks.

Virus inocula

The cell-free plasma virus inoculum was derived from a pool of cats acutely infected with FIV-C-PGammer (subgroup C) (FIV-C). [23] The cell-free cell culture virus inoculum was obtained by coculture of naïve peripheral blood mononuclear cells (PBMC) with PBMC from

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a cat infected with the FIV-C. The plasma and supernatant were frozen in aliquots and infectivity titrated post-thawing by virus isolation coculture

Virus isolation from PBMC, lymph node cells (LNC), & thymocytes

PBMC, LNC, or thymocytes were separated by density gradient centrifugation (Histopaque 1077; Sigma, St. Louis, MO). Thymocytes and LNC were mechanically dispersed by passage through a wire strainer and resuspended in phosphate-buffered saline (PBS). Naïve donor PBMC were obtained from SPF cats and stimulated with concanavalin A (Con A, 10 μg/mL) (Sigma) for 3-7 days. One million naïve cells were cocultured in 24-well plates with either 1 x 10^6 PBMC, LNC, or thymocytes from FIV-exposed cats in RPMI medium supplemented with 20% fetal calf serum, 1% penicillin/streptomycin, 2% glutamine, 2 x 10^{-5}M 2-mercaptoethanol, and 100 units of interleukin-2/mL (Cetus/Roche, Emeryville, CA). Supernatants were collected twice weekly and assayed for FIV p26 CA by capture enzyme-linked immunosorbent assay (ELISA). [29]

Flow cytometry

Lymphocyte subset analysis was performed as described by Dean *et al.* [30] 10^4 lymphocytes were analyzed using feline CD4 and CD8 monoclonal antibodies. [31] Absolute T cell subset numbers were calculated from the total lymphocyte count.

FIV antibody assay

Whole pelleted FIV-2546 virus (subtype A/B) was applied to 96-well microtiter plates at 10 ng/well in 0.01 M borate buffer and blocked with 2% bovine serum albumin (BSA), 5% donkey serum, and 10% dried milk in Tris/EDTA/NaCl (TEN). Serum samples were serially diluted in duplicate in 100 μL of ELISA diluent (TEN, 2% BSA, 4% fetal bovine serum, 0.5% Triton X-100) and incubated at room temperature for one hour. Bound antibody was

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detected using 100 µL of 1:5000 diluted peroxidase-conjugated goat anti-cat IgG (Cappel, Organon Teknika Corp., Durham, NC) per well in ELISA diluent with 5% mouse serum for one hour at room temperature. Plates were developed with 3,3'5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories, Keene, NH) for ten minutes and stopped with 2.5 N H_2SO_4 before reading the optical density (OD) at 450 nm. Titers were expressed as the inverse of the highest serum dilution that produced an OD ≥ twice that of naïve SPF cat serum.

DNA PCR primers & probe

Primers were selected using the Oligo® Primer Analysis Software program (National Biosciences, Inc., Plymouth, MN) from the *gag* nucleotide sequence of FIV-C-PGammer obtained from J. I. Mullins (University of Washington, Seattle, WA). The first round CgagU1 and CgagL1 primers amplified a fragment 623 bp in length. The primer sequences were as follows: CgagU1 (nucleotides 65 to 80), GGGTAGGGGGAAAGAG, and CgagL1 (nucleotides 673-656), AGTGAAGTATGGCAATGG. The second round CgagU2 and CgagL2 primers amplified a fragment 301 bp in length. The primer sequences were as follows: CgagU2 (nucleotides 649-631), AAGCCGAGAGGAAAGGAA, and CgagL2 (nucleotides 367-384), GACCATCAGGAGGGTGAGT. The sensitivity of the nested PCR reaction was determined using plasmid DNA containing the FIV-C *gag* gene to be between one and ten copies of target sequence.

In order to test the specificity of the PCR products, a 22–mer probe (nucleotides 513-534), ATTATGGTTTACAGCCTTTTCG, was designed to recognize both first and second round products. The oligo was obtained with a 5' amino linker from Operon Technologies, Inc. (Alameda, CA). The probe was labeled with alkaline phosphatase (AP) using the AP-Oligonucleotide Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Following standard Southern blot transfer of PCR product from a 1.2% agarose gel to a positively charged nylon membrane and hybridization of the AP-conjugated oligoprobe using QuikHyb®

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hybridization solution (Stratagene, La Jolla, CA), the bound probe was visualized using Quantum Yield® chemiluminescent substrate (Promega, Madison, WI) and Kodak XAR autoradiography film (Eastman Kodak Company, New Haven, CT).

DNA polymerase chain reaction

DNA was extracted from PBMC or LN cells using a QIAamp blood kit (Qiagen, Inc., Chatsworth, CA). Samples were amplified by nested PCR using FIV-C specific gag primers. For both first and second round reactions, hot start polymerase chain reaction (PCR) was performed with Ampliwax® PCR Gems (Perkin Elmer Corp., Norwalk, CT) for 35 cycles of 94, 57, and 72°C for 30 seconds each. The first and second round reaction mixtures contained 3mM $MgCl_2$, 200 μM of each dNTP, 1.25X Gene Amp 10X PCR buffer II (Perkin Elmer Corp.), 2.5 units AmpliTaq® DNA polymerase (Perkin Elmer Corp.), and 0.1 μM of each first round primer or 0.05 μM of each second round primer, respectively. Product was visualized on a 1.2% agarose gel stained with GelStar® (FMC Bioproducts, Rockland, ME). Amplimer specificity was confirmed by Southern blot using an AP-labeled internal oligoprobe described above.

Statistical analysis

Data were analyzed with the Microsoft Excel® software program (Microsoft Corp., Redland, WA) using a one-tailed Student's t test with unequal variance and correlation. Significance was defined as $p \leq 0.05$.

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RESULTS

Relative efficiency of mucosal routes

FIV-C proved to be readily transmitted via oral-nasal, vaginal, and rectal routes (Table 1.1). The vaginal and oral-nasal routes were the most efficient (100% transmission); whereas, rectal inoculation was least effective (44% transmission).

Table 1.1: Summary of mucosal FIV-C transmission studies results.

Exposure Route	Age at Inoculation	#Infected / #Exposed	% Transmission
Oral-Nasal	1 day	10 / 14	71
Oral-Nasal	8-10 weeks	6 / 6	100
Vaginal	8-10 weeks	7 / 7	100
Rectal	8-10 weeks	4 / 9	44

Comparison of virus detection assays

Oral-nasal exposure:

Ten of 14 orally exposed neonatal kittens became virus-positive by virus isolation coculture (VI) and/or DNA PCR (PCR) between 2 and 4 weeks post inoculation (PI). In addition, FIV antibody titers were first detected in cats between weeks 6 and 10 which increased over time. (Table 1.2) FIV Ab titers varied between 1:100 and 1:1600 and did not correlate with viral burden. VI viral burdens in thymocytes were equal to those in PBMC in 3 of 10 cats examined, but ≥ 1 log fold lower than PBMC in 7 of the 10 cats. Four of the 14 FIV-exposed cats were virus negative by VI and PCR and did not have detectable FIV antibody. Six of 7 control animals remained virus negative by VI, PCR, and antibody assays. One control animal became PCR-positive and minimally VI positive at study termination, presumably reflecting horizontal transmission from contact-exposed FIV+ littermates. All 6 orally inoculated weanling cats became FIV positive by VI and/or PCR between 2 and 5 weeks PI. In addition, all had FIV antibody titers first detectable between 4 and 6 weeks PI which varied

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between 1:100 and 1:400 and did not correlate with viral burden. (Table 1.3) All 4 control animals remained virus negative by VI, PCR, and antibody assays.

Vaginal exposure:

By 2 weeks PI, all 7 of the vaginally inoculated cats became positive by VI and PCR. FIV antibody titers developed between 2 and 10 weeks PI; these varied between 1:100 and 1:1600 and did not correlate with viral burden. (Table 1.4) Two cats developed rapidly progressing disease courses with high viral burdens (10^4 and 10^5 TCID/10^6 PBMC), low FIV antibody titers (≤1:100), and low CD4 T cell counts (39-69 cells/μL), and were euthanized at 6 weeks PI. One of the 7 cats developed a less rapidly progressing disease course and was euthanized at 18 weeks PI.

Rectal exposure:

Two of 9 rectally inoculated cats became virus positive by VI and PCR by 2 weeks PI; FIV antibody titers developed between 6 and 8 weeks PI. (Table 1.5). Two cats were transiently VI and/or PCR-positive. One of the 2 had a transient low antibody titer (1:200) at 6 weeks PI. All 3 control animals remained virus negative by VI, PCR, and antibody assays.

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Table 1.2: VI, antibody, & PCR results after oral-nasal exposure of newborn kittens to FIV-C.

Weeks Post Inoculation (VI coculture titer / FIV Ab titer / DNA PCR)										
Cat# & Status	2	4	6	8	10	12	14	20	28/29	Thymus
3606 control	-/ nd/nd	-/-/-	-/-/-	-/-/-						-/nd/nd
3609 control	-/ nd/nd	-/-/-	-/-/-	-/-/-						-/nd/nd
3612 control	-/nd/-	-/-/-	-/-/nd	-/-/-						-/nd/nd
3617 control	-/nd/-	-/-/-	-/-/nd	-/-/-						-/nd/nd
3605a control	-/ nd/nd	-/-/-	-/-/-	-/-/-						-/nd/nd
3675 control	-/ nd/nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/nd/nd
3676 control	-/ nd/nd	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	10^6/ -/+	-/nd/nd
3677 FIV+	10^5/ nd/+	nd/-/+	10^3/ -/+	10^2/ 100/+	10^2/ 100/+	10^2/ 100/+	10^1/ 100/+	10^1/ 100/+	10^2/ 100/+	10^3/ nd/nd
3678 FIV+	10^4/ nd/+	nd/-/+	10^3/ -/+	10^2/ 100/+	10^2/ 100/+	10^1/ 400/+	10^2/ 400/+	10^2/ 1600/ +	10^3/ 1600/ +	10^3/ nd/nd
3679 FIV+	10^4/ nd/+	nd/-/+	10^3/ 100/+	10^1/ 100/+	10^1/ 100/+	10^1/ 100/+	10^1/ 100/+	10^1/ 100/+	10^1/ 100/+	10^2/ nd/nd
3680 FIV+	10^5/ nd/+	nd/-/+	10^3/ -/+	10^1/ -/+	10^2/ 100/+	10^2/ 100/+	10^3/ 400/+	10^3/ 1600/ +	10^1/ 1600/ +	10^2/ nd/nd
3681 FIV+	10^5/ nd/+	nd/-/+	10^2/ -/+	10^1/ -/+	10^3/ 100/+	10^1/ 100/+	10^2/ 100/+	10^1/ 400/+	10^1/ 400/+	10^3/ nd/nd
3605b FIV+	-/ nd/nd	10^3/ -/+	-/-/+	nd/ 100/+						10^6/ nd/nd
3607 FIV+	10^4/ nd/-	10^6/ -/+	-/-/+	10^2/ -/+						10^4/ nd/nd
3608 FIV+	10^5/ nd/+	-/-/-	-/ 100/+	10^3/ 400/+						10^4/ nd/nd
3611 FIV+	10^5/ nd/nd	10^6/ -/+	-/-/+	10^3/ 100/+						10^3/ nd/nd
3613 FIV+	-/nd/+	-/-/-	10^2/ -/+	10^1/ 100/+						10^1/ nd/nd
3610 FIV-	-/ nd/nd	-/-/-	-/-/-	-/-/-						-/nd/nd
3614 FIV-	-/nd/-	-/-/-	-/-/-	-/-/-						-/nd/nd
3615 FIV-	-/nd/-	-/-/-	-/-/-	-/-/-						-/nd/nd
3616 FIV-	-/nd/-	-/-/-	-/-/-	-/-/-						-/nd/nd

nd: not done

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Table 1.3: VI, antibody, & PCR results after oral-nasal exposure of weanling cats to FIV-C.

	Weeks Post Inoculation (VI coculture titer / FIV Ab titer / DNA PCR)					
Cat# & Status	2	4/5	6	8	11/14	16/21
3727 control		-/-/-			-/-/-	nd/-/-
3728 control		-/-/-			-/-/-	nd/-/-
3732 control	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	nd/-/-
3734 control	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	nd/-/-
3729 FIV+		-/100/+			10^3/400/+	nd/400/-
3730 FIV+		10^3/100/+			10^2/400/+	nd/400/+
3731 FIV+		10^2/100/+			10^2/400/+	nd/400/+
3733 FIV+	10^3/-/+	10^2/100/+	10^2/100/+	10^4/400/+	10^2/400/+	nd/400/+
3735 FIV+	10^4/-/+	10^4/-/+	10^2/100/+	10^3/100/+	10^1/100/+	nd/400/+
3736 FIV+	10^5/-/+	10^3/-/+	10^2/100/+	10^5/400/+	10^4/400/+	nd/400/+

nd: not done

Table 1.4: VI, antibody, & PCR results after vaginal exposure of weanling cats to FIV-C.

	Weeks Post Inoculation (VI coculture titer / FIV Ab titer / DNA PCR)											
Cat# & Status	2	4	6	8	10/11	12	16/18	20/22	25/27	29/31	36/38	51
3588 FIV+	10^4/ -/ +	10^4/ 100/ +	10^2/ 100/ +	10^5/ 100/ +	10^1/ 100/ +		10^4/ 400/ +	nd/ 400/ +				10^3/ 400/ +
3659 FIV+	10^4/ -/ +	10^3/ -/ +	10^1/ -/ +	nd/ -/ +	nd/ 100/ +	10^2/ 100/ +	10^5/ 400/ +	10^3/ 1600/ +	10^3/ 1600/ +	10^3/ 400/ +	10^4/ 1600/ +	
3664 FIV+	10^3/ nd/ +	10^3/ -/ +	nd/ -/ +	nd/ 100/ +	10^1/ 400/ +	10^2/ 400/ +	10^5/ 400/ +	10^3/ 1600/ +	10^2/ 1600/ +	10^2/ 400/ +	10^4/ 400/ +	
3666 FIV+	10^4/ -/ +	10^4/ -/ +	nd/ -/ +	nd/ 100/ +	10^1/ 400/ +	10^2/ 100/ +	10^5/ 400/ +	10^1/ 1600/ +	10^3/ 400/ +	10^2/ 400/ +	10^4/ 400/ +	
3665 FIV+	10^4/ -/ +	10^4/ 100/ +	nd/ nd/ +	nd/ -/ +	10^2/ 100/ +	10^1/ 100/ +	10^2/ 1600/ +					
3660 FIV+	10^4/ -/ +	10^3/ -/ +	10^1/ 100/ +									
3661 FIV+	10^3/ -/ +	10^3/ -/ +	10^2/ 100/ +									

nd: not done

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Table 1.5: VI, antibody, & PCR results after rectal exposure of weanling cats to FIV-C.

	Weeks Post Inoculation (VI coculture titer / FIV Ab titer / DNA PCR)										
Cat# & Status	2	4	6	8/9	10/11	15/16	17-20	37	37 Colic LN	52	Colic LN
3670 control	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	nd/-/-				nd/ nd/-
3687 control	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-				-/nd/-
3704 control	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-					-/nd/-
3706 FIV+	10^5/ -/+	10^3/ -/+	10^4/ -/+	10^3/ 100/+	10^2/ -/+	10^2/ 100/+					10^3/ nd/+
3707 FIV+	10^4/ -/+	10^3/ -/+	10^4/ 100/+	10^4/ 400/+	10^1/ 400/+	10^3/ 400/+					10^4/ nd/+
3705 FIV-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-					-/nd/-
3671 FIV-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	nd/-/-				nd/ nd/-
3673 FIV-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	nd/-/-				nd/ nd/-
3674 FIV-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	nd/-/-				nd/ nd/-
3688 FIV-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	nd/-/-				-/nd/-
3672 FIV?	-/-/+	-/-/-	-/-/-	-/-/-	-/-/+	-/-/-	nd/-/-				nd/ nd/-
3587 FIV?	-/-/-	10^6/ -/-	10^6/ 200/-	-/-/-	-/-/+	-/-/-	nd/-/-	-/-/-	-/nd/-	nd/ -/-	nd/ nd/-

nd: not done

T cell alterations in infected cats

Oral-nasal exposure:

Both newborn (Fig. 1.1) and weanling age cats (Fig. 1.2) infected oral-nasally with FIV-C developed a gradual decrease in CD4 T cell numbers over time when compared to age-matched controls. Significant statistical differences (*$p \leq 0.05$) between CD4 T cell counts of FIV-infected newborn cats versus controls were present at weeks 2, 6, 8, 14, and 20 PI. Similarly, FIV-infected weanling age cats had significant statistical differences at weeks 2, 8,

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11, and 21 PI. The uninfected newborn cats (FIV-) had CD4 T cells counts similar to controls at all time points.

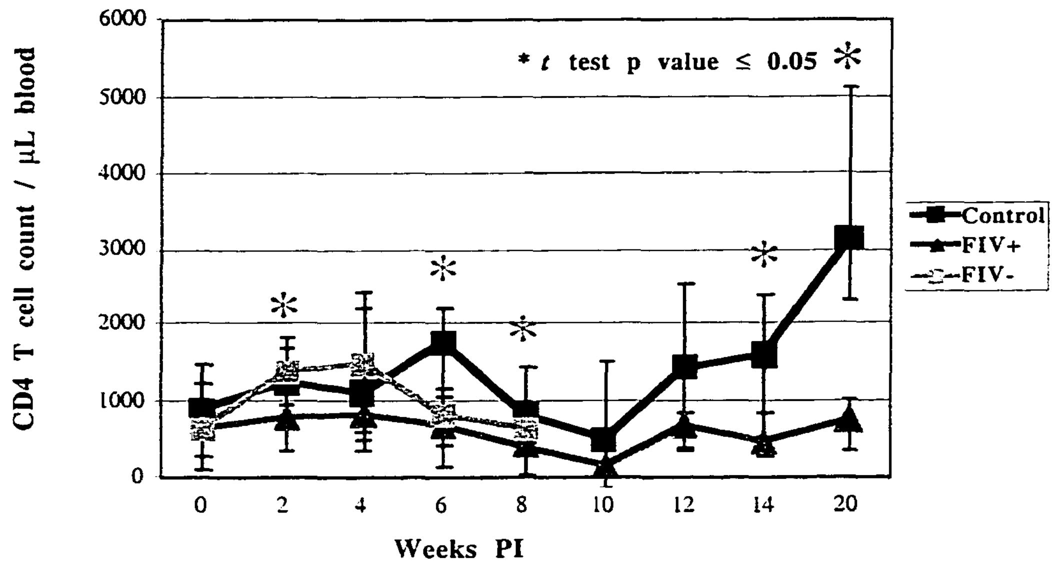

Figure 1.1: Mean CD4 T cell counts of newborn kittens exposed oral-nasally to FIV-C. FIV+ cats (▲) compared to uninfected (▩) and controls (■). Statistical significance indicated by *. CD4 depletion developed in infected cats.

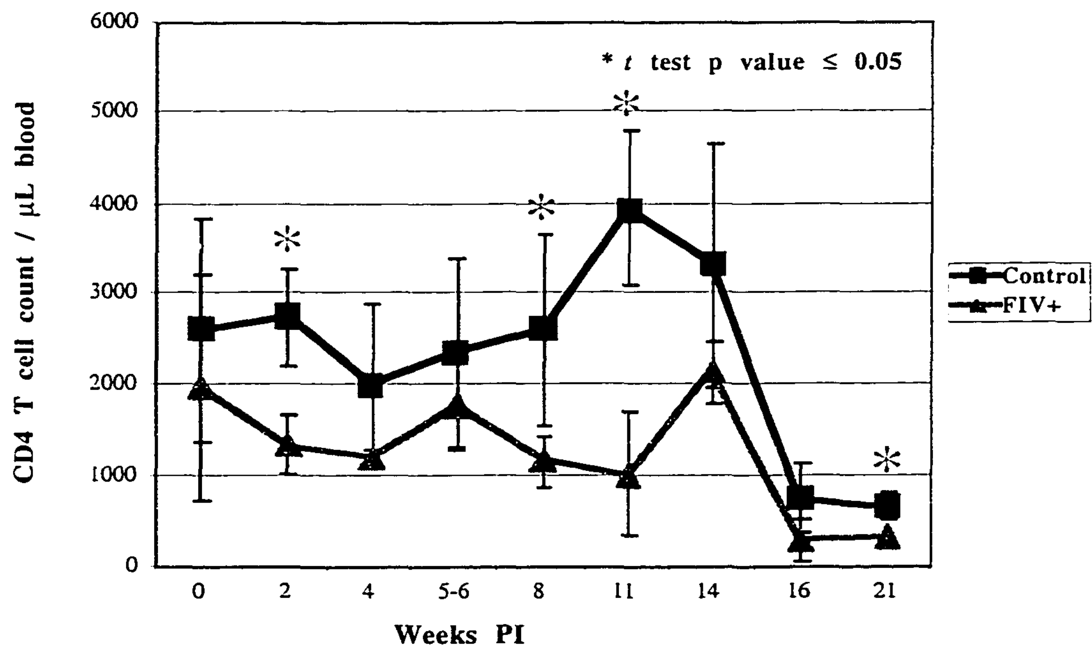

Figure 1.2: Mean CD4 T cell counts of weanlings exposed oral-nasally to FIV-C. FIV+ (▲) compared to controls (■). Statistical significance indicated by *. CD4 depletion developed in infected cats.

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Vaginal exposure:

Four of the 7 weanling age cats infected vaginally with FIV-C developed a gradual decrease in CD4 T cell numbers over time when compared to age-matched controls. The remaining 2 cats, however, developed more severe and rapid CD4 T cell declines within the first 6 weeks PI (Fig. 1.3). Statistically significant differences (*p ≤ 0.05) between CD4 T cell counts of the FIV-infected versus control cats were demonstrable at weeks 2, 4, 6, 8, 10, and 16 PI.

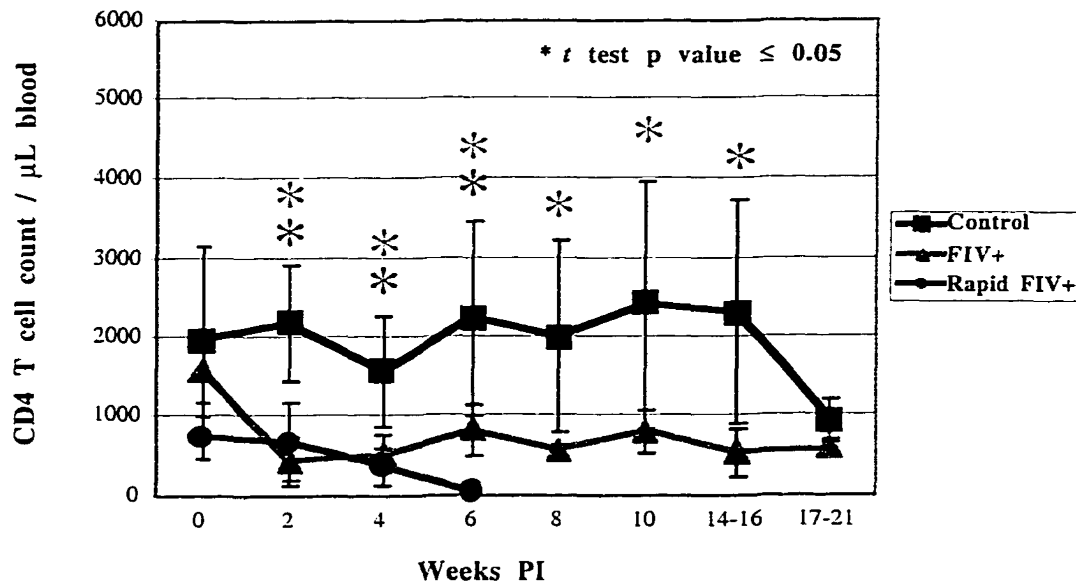

Figure 1.3: Mean CD4 T cell counts of cats exposed vaginally to FIV-C. FIV+ (▲) or rapidly progressing FIV+ (●) compared to controls (■). Statistical significance indicated by *. CD4 depletion developed in infected cats.

Rectal exposure:

Weanling age cats exposed rectally to FIV-C developed an initial decrease in CD4 T cell numbers from weeks 4 through 8 PI. However, this trend was not statistically significant when compared to age-matched controls (Fig. 1.4). Similarly, CD4 cells in cats transiently infected with FIV-C and uninfected kittens (FIV-) were not significantly different from controls.

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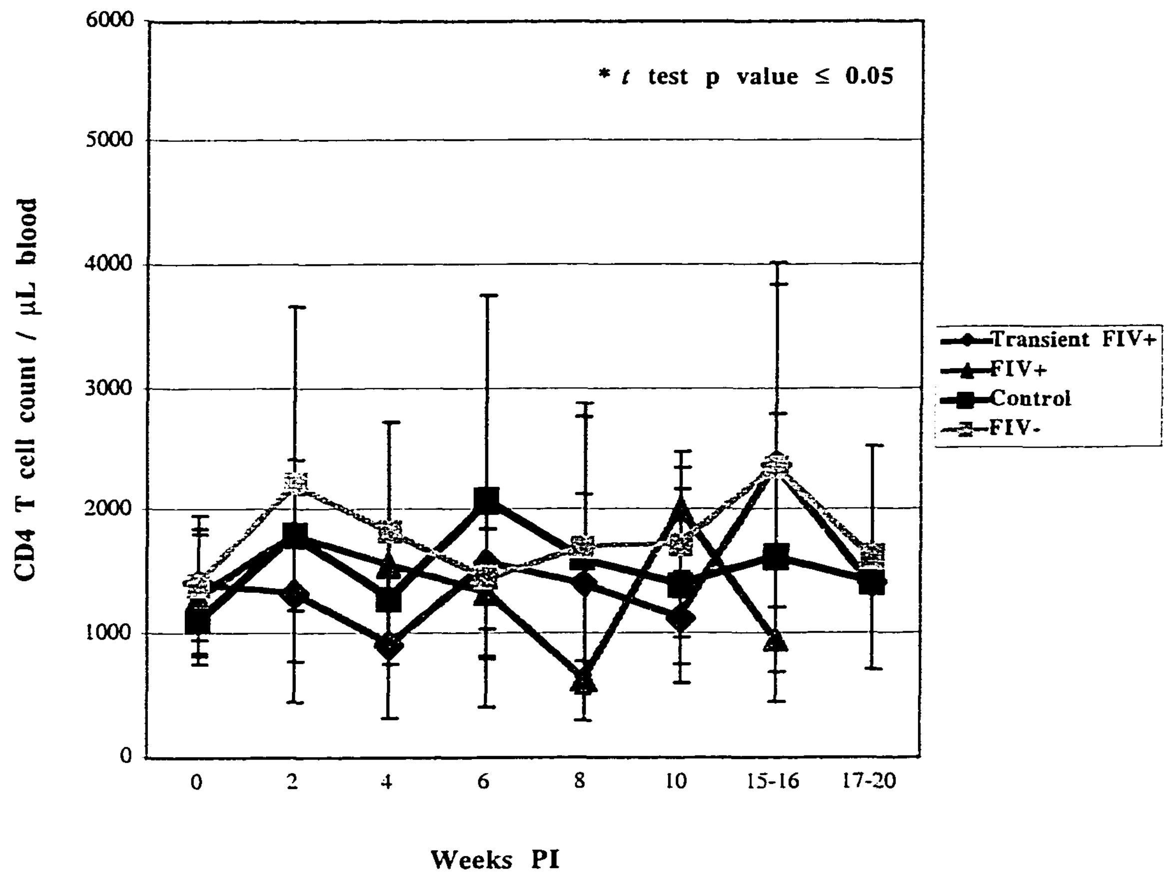

Figure 1.4: Mean CD4 T cell of cats exposed rectally to FIV-C. FIV+ (▲) or transiently FIV+ (◆) compared to uninfected cats (▩) and controls (■). Statistical significance indicated by *. No CD4 depletion evident in infected cats.

CD4:CD8 T cell ratios

Oral-nasal exposure:

Newborn kittens infected orally with FIV-C developed significantly (*p ≤ 0.05) lower CD4:CD8 T cell ratios for weeks 4 through 20 PI when compared to age-matched controls (Fig. 1.5). Weanling age cats infected oral-nasally developed significantly lower CD4:CD8 T cell ratios for weeks 4, 8, 11, and 16 PI (Fig. 1.6). The uninfected cats had CD4:CD8 ratios similar to controls at all time points.

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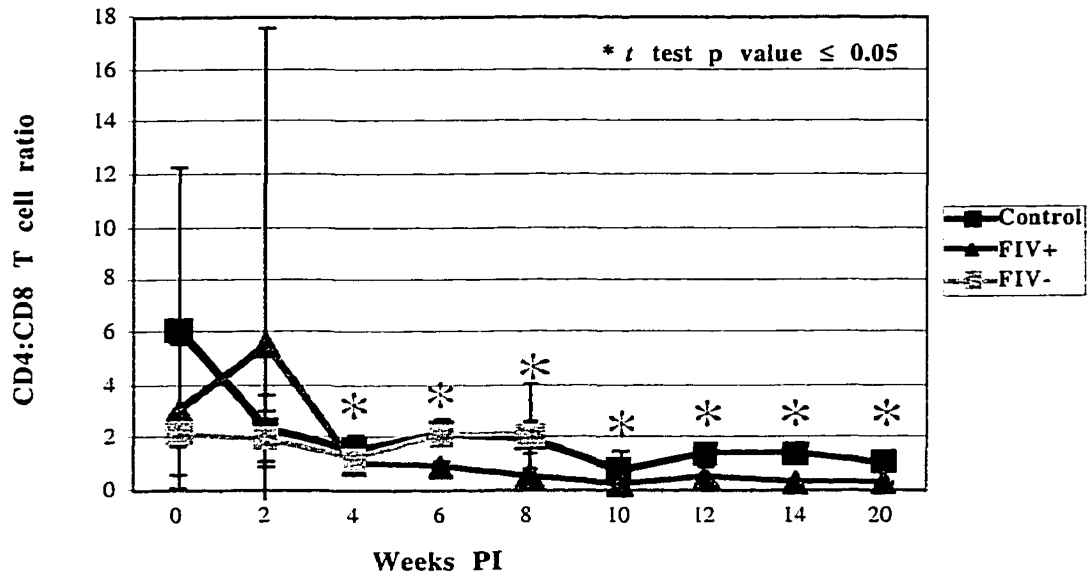

Figure 1.5: Mean CD4:CD8 T cell ratios of newborns cats exposed oral-nasally to FIV-C. FIV+ (▲) compared to uninfected cats (▒) and controls (■). Statistical significance indicated by *. CD4:CD8 T cell ratios declined in infected cats.

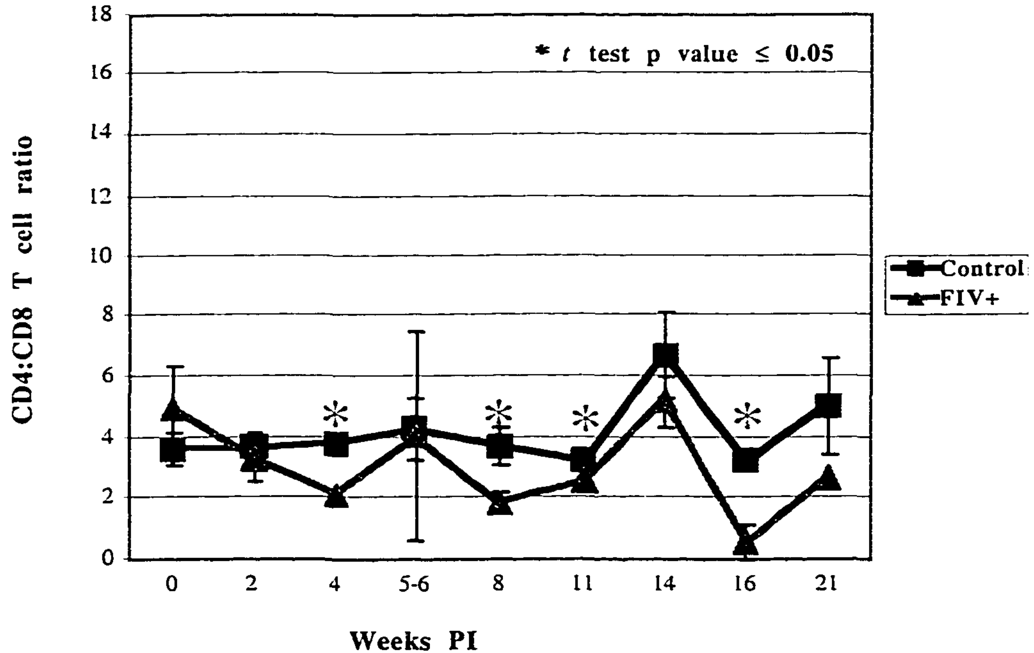

Figure 1.6: Mean CD4:CD8 T cell ratios of weanling cats exposed oral-nasally to FIV-C. FIV+ (▲) compared to controls (■). Statistical significance indicated by *. CD4:CD8 T cell ratios declined in infected cats.

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Vaginal exposure:

Four of the cats infected following vaginal exposure to FIV-C had significantly (*p ≤ 0.05) lower CD4:CD8 T cell ratios from weeks 4 through 20 PI when compared to age-matched controls (Fig.1.7). Similarly, the 2 kittens with rapidly progressing FIV had significantly lower CD4:CD8 T cell ratios for weeks 4 and 6 PI.

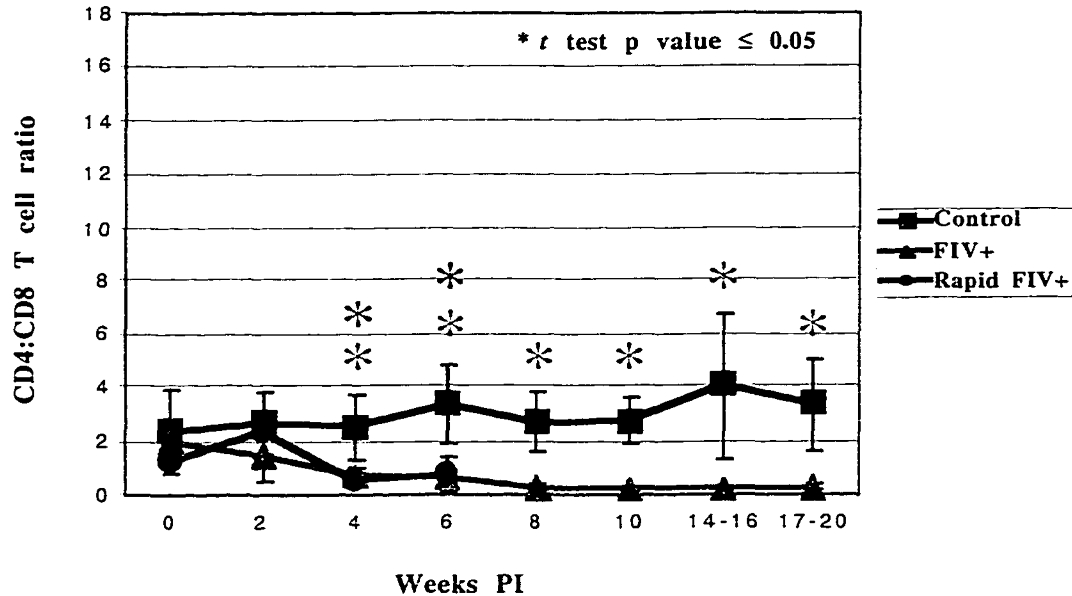

Figure 1.7: Mean CD4:CD8 T cell ratios of cats exposed vaginally to FIV-C. FIV+ (▲) or rapidly progressing FIV+ (●) compared to controls (■). Statistical significance indicated by *. CD4:CD8 T cell ratios declined in infected cats.

Rectal exposure:

The 2 cats which became persistently infected following FIV-C rectal exposure, the 2 transiently FIV-infected cats, and the cats which remained uninfected all displayed static CD4:CD8 T cell ratios over the 20 weeks PI (Fig 1.8). When compared to age-matched controls, no statistically significant differences were evident in any of the 3 FIV-exposed groups.

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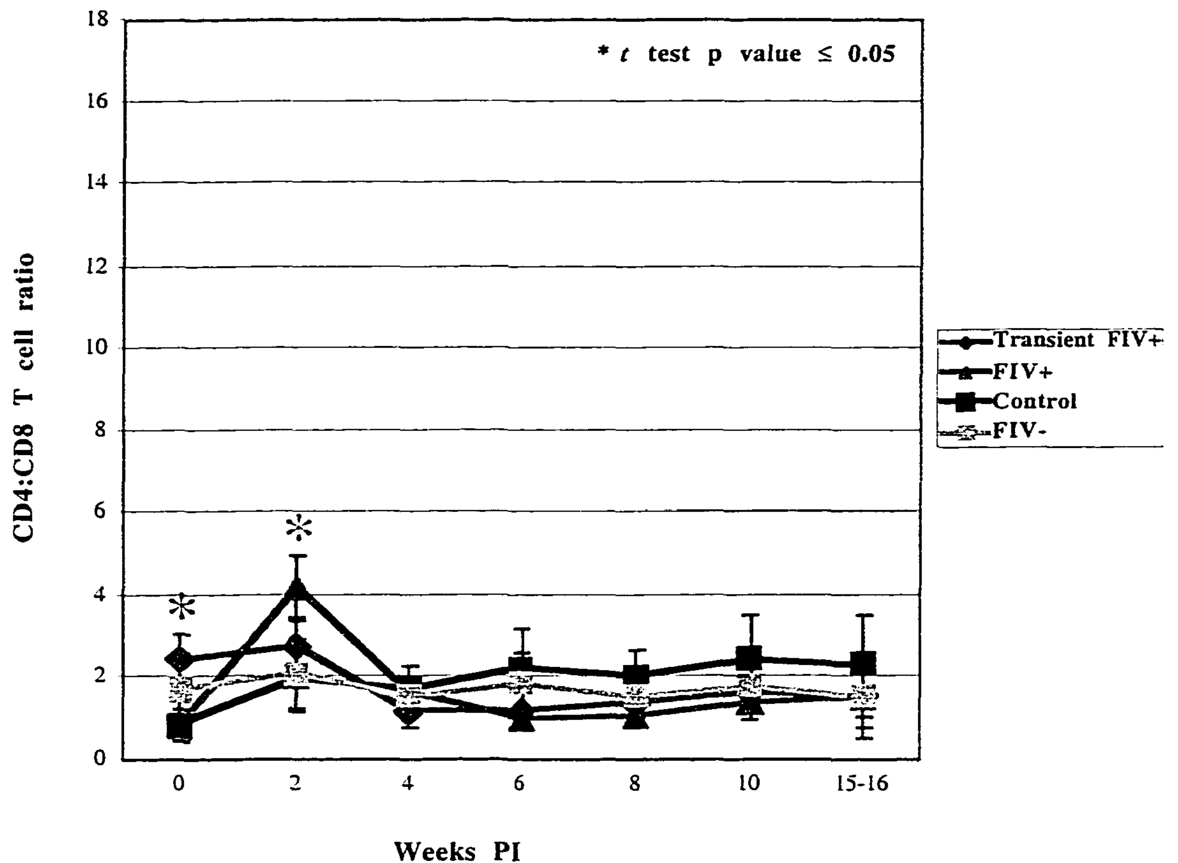

Figure 1.8: Mean CD4:CD8 T cell ratios of cats exposed rectally to FIV-C. FIV+ (▲) or transient FIV+ (◆) compared to uninfected (▩) cats and controls (■). Statistical significance indicated by *. No change in CD4:CD8 T cell ratios.

Host-virus relationships & disease courses associated with mucosal FIV-C transmission

In addition to the differences in transmission efficiency among the 3 exposure routes, three discrete disease courses were recognized as well. These were: (1) rapidly progressive infection (< 2 mos.) marked by high virus burdens, rapid CD4 cell depletion, and low antibody titers; (2) intermediate progression to infection (2 to 5 mos.) with less rapid CD4 cell decline and disease progression, and higher antibody titers; (3) conventional infection (>5 mos. to years) featuring slowly progressive CD4 T cell decline and variable FIV antibody titers; and (4) regressive infection (>5 mos. to ?) marked by transient and very low virus burdens, no CD4 cell alterations, and no or low transient antibody titers. The rapidly

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progressive disease course was only observed in cats inoculated vaginally; whereas, all three inoculation routes produced conventional FIV infections. The regressive FIV infections were observed only in cats inoculated rectally. The 4 disease categories identified after mucosal FIV-C transmission are compared in Table 1.6.

Table 1.6: Virologic and immunologic parameters defining 4 FIV-C disease courses.

Disease Course	Duration	CD4 Decline	VI Coculture	DNA PCR	Antibody Titer	# of Animals
Rapid	< 2 mos.	rapid†	10^1-10^2	+	1:100	2
Intermediate	> 2 mos. & < 5 mos.	intermediate	10^2	+	1:1600	1
Conventional	> 5 mos.	gradual†	10^1-10^6	+	1:100-1:1600	22
Regressive	?	none	10^6* / -	+* / -	1:200* / -	2

* transient positivity

† mean terminal CD4 T cell count is statistically significant compared to controls by Student's *t* test

Two of the 7 vaginally inoculated cats developed rapidly progressing disease courses, succumbing to infections by 6 weeks PI. Viral burdens were high (10^4 and 10^5 TCID/10^6 PBMC), antibody titers were low (1:100), CD4 cells declined rapidly (39-69 cells/µL). (Fig. 1.9) One vaginally inoculated animal developed an intermediately progressive disease course, succumbing to infection by 18 weeks PI. Viral burden (10^4 TCID/10^6 PBMC) and antibody titer (1:1600) were high, but CD4 cell decline (255 cells / µL) was not as rapid nor as severe as the rapid progressors.

The majority of mucosally exposed cats (n = 22) exhibited a conventional FIV disease course. Viral burdens (1 to 10^4TCID/10^6 PBMC) and antibody titers (1:100 to 1:1600) were variable and did not correlate. At study termination, the cats were not clinically ill. CD4 cell counts were >400 cells / µL; however, all weeks from 2 to 21 PI were significantly different from to controls. (Fig. 1.9).

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Two of the 9 rectally inoculated cats developed regressive FIV infections. Infection was rarely detected by VI and often only documentable by PCR. One of the 2 had a low transiently detectable antibody titer. At study termination, neither animal was clinically ill and CD4 cell counts were not statistically different from controls (Fig. 1.9). The potential duration of the regressive infection remains unknown since studies were terminated between 5 and 12 months PI.

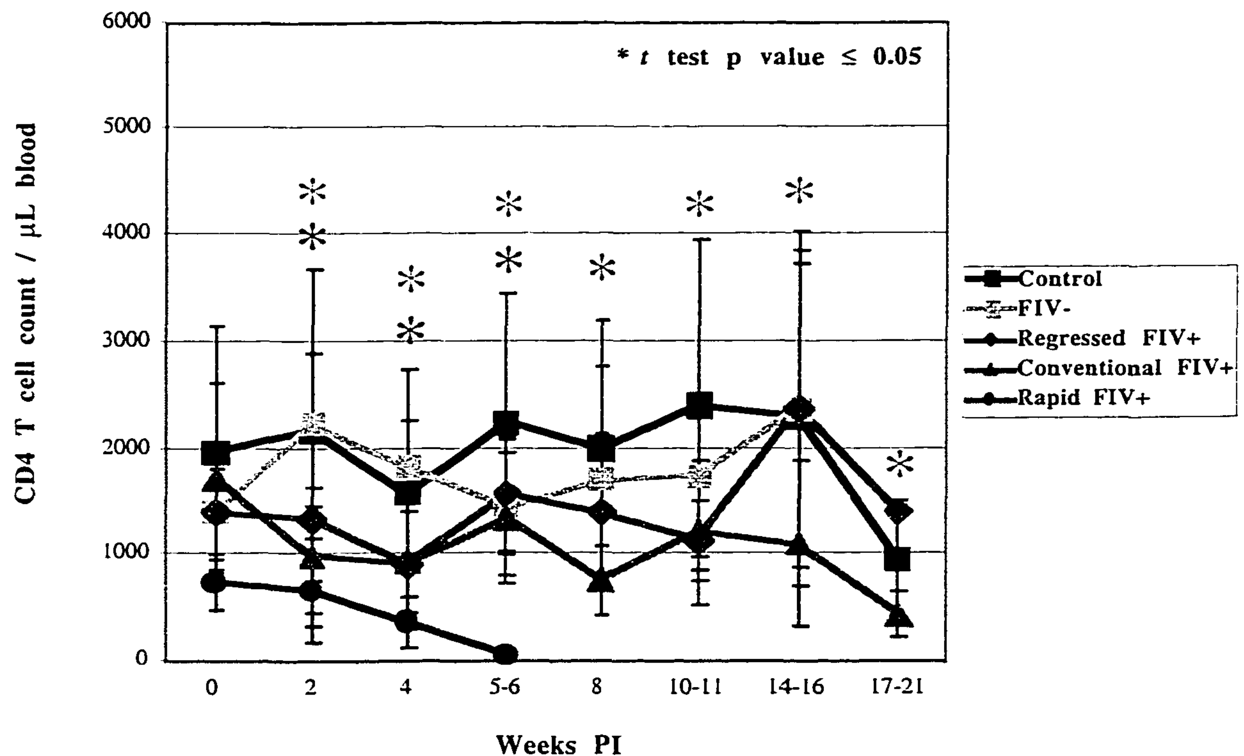

Figure 1.9: Mean CD4 T cell counts of the various FIV-C disease courses (conventional: ▲, regressed: ◆, and rapid: ●) compared to uninfected cats (▒) and controls (■). Statistical significance indicated by *. CD4 T cell decline in FIV-infected conventionally and rapidly progressing cats.

DISCUSSION

Most HIV-1 infections are acquired by mucosal exposure. [32] Here we demonstrate multi-mucosal transmissibility of a virulent clade C FIV which proved to be transmissible by all 3 exposure routes (oral-nasal, vaginal, and rectal) tested; vaginal and oral-nasal exposure were

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most efficient. These findings parallel those regarding rectal transmission in the SIV system. Adults macaques exposed to cell-free SIV orally developed systemic infection after receiving a virus dose 6,000 times lower than that required for rectal. [33,34] Pauza et al. [35,36] found that 100 to 1000 times more SIV was required to achieve infection via the rectal versus the intravenous route. Thus, transmission via the oral mucosa was more efficient than that after rectal exposure, but less efficient than intravenous inoculation. By contrast, FIV-C vaginal transmission appears more efficient than that of SIV in adult macaques. [37-40]

Only some viral strains produced systemic infection after SIV vaginal inoculation, supporting the theory that selection for viral genotypes occurs during sexual transmission of HIV. [41,42] Miller [42] presented data to suggest that viruses which are adapted to replicate to high levels rather than those highly tropic for macrophages *in vivo* are capable of being transmitted by vaginal inoculation. Our vaginal transmission results support Miller's thesis in that an FIV strain highly replicative and pathogenic after intravenous in vivo passage was very efficiently transmitted via vaginal inoculation and in some instances retained the capacity to elicit a rapidly progressive disease course similar to that seen in the parenteral studies. Overall, however, slower disease progression was noted after mucosal FIV-C transmission, again paralleling results obtained with SIVmac239 and SIVmac251. [43] Conversely, Pauza *et al.* [43] concluded that a clear dichotomy existed between virulence and transmission. Rectal inoculation of one chimeric simian-human immunodeficiency virus (SHIV) strain, $SHIV_{HXBc2}$, demonstrated very slow infection kinetics; while another $SHIV_{89.6PD}$ strain replicated more rapidly than any other isolate examined. However, both strains had similar efficiencies following rectal transmission. [43] In contrast, in our studies, while a clade B FIV was transmitted more efficiently than the clade C virus in the present studies (100% versus 44% transmission rates, respectively), [21] both strains produced similar conventional FIV disease courses. Thus, the relationship between mucosal transmissibility and pathogenicity is complex and can be dichotomous.

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The median time from HIV-1 infection until the development of AIDS in industrialized countries is ~10 years, but ranges from a few months to > 20 years. [44,45] Even though many virologic [46,47] and immunologic [48,49] factors have been implicated as contributing to this wide range, the variability in HIV-1 disease progression rate remains unexplained. Mucosal exposure of FIV-C resulted in a broader range of host-virus relationships than that observed after rapid intravenous serial passage leading us to propose 4 provisional categories of FIV infection: (1) rapidly progressive infection (< 2 mos.) marked by high virus burdens, rapid CD4 cell depletion, and low antibody titers; (2) intermediately progressing infection with less rapid CD4 decline and disease progression (2 to 5 mos.); (3) conventional (typical) infection (>5 mos. to years) featuring slowly progressive CD4 cell decline with variable FIV antibody titers; and (4) minimal, regressive infection marked by low transient virus burdens with no significant CD4 cell alterations and either no or low transiently detectable FIV antibody. All 4 patterns of persistent infection featuring rapid, intermediate, normal, or no disease progression have been described in the range of studies conducted with SIV infection in macaques. [50,51] Notably, seronegative transient viremia has been detected in macaques inoculated with low doses of pathogenic SIVmac by the vaginal, [39] rectal, [35,52] oral, [53] and intravenous [54] routes. In these studies, proviral DNA could either be detected in PBMC in the absence of virus isolation for prolonged periods following inoculation [35] or only during early stage of infection. [39,54] Various tissues in a group of transiently infected vaginally exposed macaques were PCR positive, but only 50% percent of these tissues were also RT-PCR positive. [55] A similar class of FIV-C regressive infections were identified in the current mucosal FIV-C studies, although we could not detect evidence of virus in the regional lymph nodes at study termination by VI, PCR, or in situ hybridization (ISH) (ISH data not shown), including a cat with regressive infection followed for 1 year PI. Highly regressive FIV-C infections occurred in 5.6% of all mucosally exposed cats. It appears that silent HIV infection also occurs. [56-59] Imagawa *et al.* [60,61] described a group of 27 PCR-positive seronegative men, 26 of whom became virus negative in follow-up studies. Transient HIV-seropositivity has been described in the spouse of an infected haemophiliac. [62] Seroreversion has also been described in 4 men from a multicenter AIDS cohort study. [63] In addition, there

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are several reports of viral clearance in children born to HIV-1-infected mothers. [64-68] Similarly, regression of FIV infection has been observed in 7 of 9 kittens born to 2 chronically infected mothers. [69] These regressive infections were characterized by loss of detectable antibody and virus in blood; however, FIV was detectable at low levels in lymph node and bone marrow from 5 of the 7 cats greater than 1 year of age by either VI or nested PCR, but viral RNA was not detectable by ISH. This study suggests that extremely low levels of virus may persist in tissues which may be insufficient to stimulate a detectable humoral immune response. [70] In contrast, while long-term adult HIV-infected nonprogressors have viral burdens in plasma and PBMC which are extremely low to undetectable, they also have strong and durable antiviral immune responses. [71-73] The sustained immune response observed in these individuals is thought to be driven by ongoing viral replication in tissues. These observations illustrate the varied and similar host-virus relationships of SIV and FIV infections and point to a category of regressive infections of potential importance in the evolving understanding of acquired resistance and long term survival after mucosal exposure to HIV.

Regression or resistance to infection following lentiviral exposure without mounting a protective humoral immune response suggested that cell-mediated immunity may be involved in tethering the infection. HIV-specific cytotoxic T lymphocytes (CTL) have been observed in the absence of antibody detection in a number of different exposed seronegative groups. [66,74-78] Several studies have focused on the immunologic profiles of three categories of individuals: (1) patients who reduce virus titer in the acute phase of disease, [79,80] (2) long-term nonprogressors who are HIV+ and symptomless for greater than 10 years, [81,82] and (3) repeatedly exposed seronegative individuals. [83,84] The common immunologic element shared by all 3 cohorts was strong HIV-specific Th1-like immunity and CD8-mediated HIV-specific CTL and antiviral activity in the absence of serum antibodies to HIV. Several vaccine studies conducted in the FIV and SIV systems have reported resistance to challenge correlating with virus-specific CTL activity. [85-87] CTL activity was not examined in the animals with regressive FIV-C infections. However, in study by Bucci *et al.*, [88] FIV intravaginally inoculated cats had

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a strong CD8+ immune response at 6 weeks post infection that inhibited viral replication during the acute phase of infection. The CD8+ anti-FIV activity was revealed by increased FIV replication in PBMC depleted of CD8+ lymphocytes. Two of the cats with strong CD8+ anti-FIV activity during acute infection did not seroconvert nor did they have evidence of FIV infection at later times suggesting that CD8+ immunity played a role in eliminating virus during primary transmucosal FIV infection. Similar activity has been described for CD8+ T cells in HIV infection. [89,90] Recently, Hohdatsu *et al.* [91,92] further characterized this CD8+ anti-FIV activity as being noncytolytic and mediated by a soluble factor. These findings suggest another parallel between mucosal HIV and FIV infections.

In the current FIV-C mucosal transmission studies, rapidly progressing infections occurred in 8.3% of all mucosally exposed cats. Studies of patients who progressed rapidly after seroconversion to HIV have suggested an association of this outcome with certain human leukocyte antigen alleles, [93,94] low levels of antibody to HIV-1 p24, [95,96] and plasma HIV RNA levels up to ten-fold higher than non-rapid progressors at the time of primary infection. [47,95,97-100] Although, plasma RNA level were not measured in the FIV-C rapid progressors, rapidly progressing parenterally infected cats had plasma viral burdens 1 to 2 logs higher than long-term survivors.[101] One study documented gender differences in relationship of HIV-1 viral load to progression to AIDS; women with the same viral load as men had a 1.6-fold higher risk of AIDS. [102] Interestingly, in the current FIV-C mucosal studies, both rapid progressing animals were females, whereas, both cats with regressed infections were males and the only animals to develop infection following rectal inoculation were females. Of course, these results could merely reflect the small total number of animals studied in that no sex predilection was observed in previous parenteral FIV-C studies. [23] Similarly, the variability in disease outcomes seen in the mucosal FIV-C studies could be the result of unrecognized MHC-linked resistance / susceptibility alleles expressed as individual variability in an outbred cat population. Farzadegan *et al.* [95] and others proposed several explanations for rapidly progressive disease in HIV-infected individuals: (1) rapid progressors may be infected with a rapidly replicating HIV strain, so that high levels of virus load are attained before the immune

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response controls viral replication; (2) rapid progressors may have a weak or delayed immune response to HIV-1 infection, allowing greater viremia before infection is brought under control; or (3) host factors, such as more susceptible host cells, also could permit higher viral burden to be reached in rapid progressors. [95] Certainly all of the above scenarios are plausible for outbred animal models of lentiviral infection such as SIV in macaques and FIV in cats.

In summary, the present studies demonstrate that a virulent FIV clade C isolate is readily transmissible by several portals of mucosal exposure and that any of 4 host-virus relationships may evolve with associated disease courses varying from rapid progression to regressive asymptomatic infection. These results highlight the spectrum of similarities in host-virus relationships and outcomes following mucosal exposure to the human, simian, and feline lentiviruses and reemphasize the importance of further study of this host-virus interface.

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CHAPTER TWO

PATHOLOGY OF MUCOSAL FIV-C INFECTION

INTRODUCTION

FIV, a lentivirus identified in 1986 [1], causes a disease syndrome similar to that caused by HIV. [2] The clinical phases of FIV infection parallel those staging HIV: (1) an acute phase characterized by a burst of viral replication, flu-like illness, lymphadenopathy, and progressive decline in CD4+ T lymphocytes; [3-6] (2) a prolonged asymptomatic phase featuring viral down-regulation and a continued gradual decline in CD4+ cells; and (3) a terminal phase characterized by immunological decompensation, wasting, hematologic suppression, recrudescence of plasma viremia, and clinical immunodeficiency with opportunistic infection. [2,7-11]

Development of the FIV model has been limited by its prolonged and variable disease course in infected cats. We have identified a virulent FIV strain, FIV-C-PGammer (FIV-C), which induces high virus burden and rapid immunodeficiency disease onset. [12] High FIV RNA titers in plasma and tissues were associated with rapid disease progression to fatal disease [13,14] paralleling the kinetics of acute HIV infection. [13,15-17]

In contrast to the rapid intravenous serial passages studies, mucosal exposure of FIV-C produced a broader range of host-virus relationships. [18] Four provisional categories of FIV-C infection were proposed: (1) rapidly progressive infection (< 2 mos.) marked by high virus burdens and rapid CD4 cell depletion; (2) intermediately progressing infection (2 to 5 mos.) with less rapid CD4 cell decline and disease progression; (3) conventional infection (> 5 mos.

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to years) featuring slowly progressive CD4 cell decline; and (4) regressive infection (> 5 mos. to years?) marked by very low virus burdens with no CD4 cell alterations. Similar categories of disease course have been reported in HIV-infected humans. [19]

The purpose of the following studies was to characterize the clinical and pathologic changes associated with the various disease courses detected after mucosal FIV-C exposure. In particular, the thymus was found to be a major early lymphoid system target tissue.

MATERIALS & METHODS

Animals & sample collection

The cats (*n* = 23) described in this chapter are a subset of those previously described in chapter one. [18] One day old kittens (*n* = 10) from a specific pathogen free (SPF) breeding colony maintained at Colorado State University (Fort Collins, CO) were inoculated by atraumatic exposure of the oral and nasal mucosae with 200 μL of 200 $TCID_{50}$/mL (50% tissue culture infective dose; *n* = 4) cell-free infectious FIV-C plasma or 300 $TCID_{50}$/mL (*n* = 6) cell-free infectious FIV-C culture supernatant. Eight to 10 week old SPF weanling cats were inoculated by atraumatic exposure of the oral and nasal mucosae (*n* = 6) or rectum (*n* = 4) with 1 mL of 300 $TCID_{50}$/mL cell-free infectious culture supernatant. Eight to 10 week old female SPF weanling cats (*n* = 3) were inoculated by instillation of 500 μL of 200 $TCID_{50}$/mL cell-free infectious plasma (*n* = 1) or 300 $TCID_{50}$/mL (*n* = 2) cell-free infectious culture supernatant into the vagina using a rubber tomcat catheter. Following administration of the inoculum, each animal was held in a specific position to facilitate exposure of the respective mucosal membrane for a minimum of 20 minutes: oral-nasal inoculates were positioned upright and rectal/vaginal inoculates were positioned with posterior ends elevated. Age- and litter-matched control animals receiving parallel noninfectious inocula consisted of 7 SPF newborns and 7 SPF weanling age cats.

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At study termination, the animals were euthanized for necropsy and terminal sample collection. Mucosal, regional, and systemic lymphoid tissues were harvested and fixed in 10% buffered formalin for ≤ 18 hr before histologic processing and paraffin embedding. Tissues harvested included: spleen; bone marrow; thymus; retropharyngeal, submandibular, , mesenteric, colic, and iliac lymph nodes; tonsils; gastrointestinal tract; rectal, vaginal, and oral-nasal mucosa; liver; kidneys; lungs; salivary glands; and brain. In addition, body and thymus weights were recorded, and a portion of the thymus was collected for thymocyte isolation.

Virus inocula

The cell-free plasma virus inoculum was derived from a pool of cats acutely infected with FIV-C-PGammer (subgroup C) (FIV-C) [12] The cell-free culture virus inoculum was obtained by coculture of naïve peripheral blood mononuclear cells (PBMC) with PBMC from a cat infected with the FIV-C. The plasma and supernatant were frozen in aliquots and infectivity titrated post-thawing by virus isolation coculture.

Thymocyte isolation & preparation

Thymocytes were mechanically dispersed by passage through a wire strainer, resuspended in phosphate-buffered saline (PBS), separated by density gradient centrifugation (Histopaque 1077; Sigma, St. Louis, MO), and quantified by trypan blue exclusion. The thymocytes at a concentration of 5 x 10^6 cells/mL were incubated with antibody combinations (CD4, CD8, and B cell monoclonals, see below) for one hour at 4°C, washed twice with PBS-2% fetal calf serum, and resuspended in 2% paraformaldehyde in PBS.

Flow cytometry

Thymocyte subset analysis was performed using an EPICS XL-MCL (Coulter Electronics, Hialeah, FL) flow cytometer. Twenty-thousand thymocytes were analyzed for each sample

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by a two color system using combinations of feline CD4 and CD8 monoclonal antibodies [20] and an anti-mouse CD45R, B220 (Ly 5), B cell monoclonal antibody (Cedarlane Laboratories Limited, Hornby, Ontario Canada). Thymocyte bitmap gating was based on forward and side light scatter. For each sample, the analysis gates were set to include less than 2% of thymocytes labeled with isotypic control monoclonal antibodies conjugated to flourescein isothiocyanate (FITC) or phycoerythrin (PE) (Sigma & Cedarlane Laboratories Limited) in the positive analysis regions. Results were expressed as the percentage of cells located in each quadrant.

RNA in situ hybridization

Plasmid containing either the FIV-C *gag* or *env* gene was obtained from J. I. Mullins. [21] The FIV-C genes were isolated via restriction enzyme digestion, 0.7% agarose gel electrophoresis, and Qiaex II gel purification (Qiagen, Inc., Chatsworth, CA). The purified, isolated gene fragments (*gag* = 1,353 bp, *env*A = 1,255 bp, and *env*B = 776 bp) were subcloned into the pGEM®-7Zf(+) vector (Promega, Madison, WI) containing T7 and SP6 transcription promoter sequences. Sense and antisense riboprobes were transcribed off their respected promoters incorporating digoxigenin-labeled UTP using either AmpliScribe® T7 or SP6 transcription kits (Epicentre Technologies Corp., Madison, WI).

In situ hybridization (ISH) was performed in a manner similar to that described by Hirsch et al. [22] Briefly, formalin-fixed paraffin-embedded tissues were sectioned (4-5 μM thick) and placed on positively charged glass slides (Fischer Scientific, Pittsburgh, PA). Tissue sections were deparaffinized and rehydrated sequentially with xylene, xylene-ethanol, 100% ethanol, 95% ethanol, and RNase-free water for 5 min each at room temperature. The slides were then incubated with 5 mM levamisole for 20 min, washed with SSC buffer (0.15 M NaCl, 0.015 M sodium citrate), incubated in 0.2 N HCl for 20 min, and washed again with SSC buffer. The sections were digested with 25 μg/mL proteinase K in buffer containing 10 mM Tris (pH 7.4) and 2 mM $CaCl_2$ for 10 min at 37°C. Digestion was stopped with 0.1 M glycine in PBS.

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Slides were washed with PBS, incubated in 0.1 M triethanolamine-0.25% acetic anhydride solution for 10 min, washed in 2X SSC, incubated in 0.1 M Tris (pH 7.4)-0.1 M glycine solution for 15 min and washed in 2X SSC. Prehybridization was done at 50°C for 10 min with hybridization solution containing 50% deionized formamide, 1X SSC, 1X Denhardt's solution, 5 mM $NaPO_4$ (pH 6.8), 0.1% sodium dodecyl sulfate, 250 μg of salmon sperm DNA/mL, 5% dextran sulfate, and 250 μg/mL tRNA. Riboprobes were added at 1 ng/μL, the sections coverslipped heated to 65°C for 5 min, chilled for 10 min on ice, and hybridized overnight at 55°C. Following hybridization, the coverslips were removed and the slides washed with 4X SSC-50% formamide for 1 hr at 50°C and then in 2X SSC for 5 min. An RNase mixture (1 unit RNase T_1 and 20 μg/mL RNase A/mL for 30 min at 37°C) was used to digest excess probe and wash steps were repeated. Slides were blocked for 1 hr in a buffer containing 2% horse serum, 0.3% Tween 20, 100 mM Tris (pH 7.4), and 150 mM NaCl. Hybridized probe was detected with a sheep anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim, Indianapolis, IN) diluted 1:500 in blocking buffer for 1 hr at room temperature. After washing, the sections were incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)-nitroblue tetrazolium (NBT) substrate overnight in the dark. Finally, the sections were washed in 10 mM Tris-HCl- 10 mM EDTA solution (pH 8.1), counterstained with nuclear fast red, dehydrated in graded ethanols, cleared in xylene, and mounted with Clear*Mount® (American Master "Tech" Reagent Company Inc., Lodi, CA). Negative controls included tissue sections from uninfected cats and application of sense riboprobes on tissue sections from FIV-infected and uninfected cats.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was employed to identify individual thymocytes undergoing apoptotic cell death within thymic tissue sections. A protocol based on the method of Gavrieli *et al.* was utilized in this study [23] Formalin-fixed paraffin-embedded tissues were sectioned (4-5 μM thick) and placed on positively charged glass slides (Fischer Scientific). Tissue sections were deparaffinized in

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xylene and rehydrated in graded ethanols to dH_2O. The slides were then incubated with 5 mM levamisole for 20 min and washed in PBS. The slides were digested with 25 µg/mL proteinase K in buffer containing 10 mM Tris (pH 7.4) and 2 mM $CaCl_2$ for 10 min at 37°C. Digestion was stopped with 0.1 M glycine in PBS and slides were rinsed with PBS. The TUNEL cocktail was applied using a coverslip and the slides incubated at 37°C for 1 hr. The TUNEL cocktail consisted of 20 mM potassium cacadylate, 25 mM Tris-HCl, bovine serum albumin (BSA) 250 µg/mL, 1 mM $CoCl_2$, 5 µM Digoxigenin-dUTP (Boehringer Mannheim), and 25 units terminal transferase. Following incubation, the slides were washed several times with PBS and rinsed in 100 mM Tris (pH7.4)- 150 mM NaCl solution. Slides were blocked for 1 hr in a buffer containing 2% horse serum, 0.3% Tween 20, 100 mM Tris (pH 7.4), and 150 mM NaCl. The TUNEL reaction was detected with a sheep anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim) diluted 1:500 in blocking buffer for 1 hr at room temperature. After washing, the sections were incubated with BCIP/NBT substrate overnight in the dark. Finally, the sections were washed in 10 mM Tris-HCl- 10 mM EDTA solution (pH 8.1), counterstained with nuclear fast red, dehydrated in graded ethanols, cleared in xylenes, and mounted with Clear*Mount® (American Master "Tech" Reagent Company Inc). Controls included thymic tissue sections from age- and litter-matched uninfected cats, as well as, tissue sections processed as described above, but omitting the TdT enzyme.

Image analysis

Tissue sections containing ISH positive cells or TUNEL positive cells were visualized using an Olympus VANOX-S microscope with a mercury light source. Video images were captured with a low-light cooled charged-coupled device (CCD) camera (Sony Photocamera DKC-5000), the Adobe Photoshop® software program (Adobe Systems Inc., Mountain View, CA), and a Power Macintosh G3® (Apple Computer, Cupertino, CA). The scanned images were imported into the MetaMorph® digital imaging software program (Universal Imaging, Westchester, PA). The positive staining cells were differentiated from non-staining cells with

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the "threshold" and "measure objects" tools of the MetaMorph software. The pixel size of staining and non-staining cells were determined by "binorizing" the thresholded cells and using the "measure objects" tool to determine the average number of pixels per cell (object). Both the positive threshold (staining cells) and the negative threshold (non-staining cells) values were measured for each image and recorded on a Microsoft Excel® spreadsheet (Microsoft Corp., Redland, WA). From the average pixel size information determined for each cell population, the number of positive and negative staining cells were calculated and compared as a ratio (1 positive cell to # negative cells). For each tissue section, five different microscopic fields were evaluated as described above and the results averaged.

Immunohistochemistry for cell phenotype

A protocol based on the methods of Beebe *et al.* was utilized in these studies. [24] Formalin-fixed paraffin-embedded tissues were sectioned (4-5 μM thick) and placed on positively charged glass slides (Fischer Scientific). Tissue sections were deparaffinized in xylene, rehydrated in graded ethanols to dH_2O and rinsed in 0.05 M Tris (pH7.4). The slides were digested with 250 μg/mL proteinase XXIV (Sigma) in 0.50 mM Tris (pH 7.4) for 15 min at room temperature. Slides were washed in TBT buffer (50 mM Tris, 150 mM NaCl, 0.5% BSA, 0.3% Tween 20, pH7.6). Nonspecific proteins were blocked with 5% goat serum, 5% horse serum, 1% BSA, and 1 mg/mL cat IgG (Sigma) in 50 mM Tris-HCl- 150 mM NaCl (TBS). In addition, endogenous avidin and biotin binding activity were blocked using a commercial kit (Vector Laboratories, Burlingame, CA). The slides were then incubated for 1 hr at room temperature with one of the following primary antibodies. T cells were identified using a polyclonal rabbit anti-human CD3 (Dako Corp., Carpentaria, CA) at 1:100 dilution in TBS. Macrophages were detected with a monoclonal antibody MAC387 (Dako Corp.) at a 1:100 dilution in TBS. Dendritic cells were labeled with a rabbit anti-cow S-100 antibody (Dako Corp.) at a 1:250 dilution in TBS. The primary antibody was then detected using either the mouse IgG (MAC387) or rabbit IgG (CD3 and S-100) Vectastain® ABC-AP Kits (Vector Laboratories) according to manufacturer's instructions. The resulting immobilized

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antibody/streptavidin conjugated to alkaline phosphatase complexes were detected with Vector® Red (Vector Laboratories) as a chromagen. Tissues were counterstained with Vector® Methyl Green (Vector Laboratories), dehydrated in graded ethanols, cleared in xylenes, and mounted with Clear*Mount® (American Master "Tech" Reagent Company Inc). Negative controls included serial sections of tissue to which either irrelevant mouse IgG_1 or rabbit IgGs had been applied matching the concentration of the primary antibody and processed in parallel with experimental sections.

Statistical analysis

Data was analyzed with the Microsoft Excel® software program (Microsoft Corp., Redland, WA) using a one-tailed Student's *t* test with unequal variance and correlation. Significance was defined as $p \leq 0.05$.

RESULTS

Pathologic changes associated with FIV-C disease courses

Following mucosal exposure to FIV-C by either oral-nasal, rectal, or vaginal routes, 4 different disease courses were recognized. Thus, 4 provisional categories of FIV infection were proposed: (1) rapidly progressive infection (< 2 mos.) marked by high virus burdens and rapid CD4 cell depletion; (2) intermediately progressing infection (2 to 5 mos.) with less rapid CD4 cell decline and disease progression; (3) conventional infection (> 5 mos. to years) featuring slowly progressive CD4 cell decline; and (4) regressive infection (> 5 mos. to ?) marked by very low virus burdens with no CD4 cell alterations.

Despite the range of disease courses observed following mucosal FIV-C infection, the pathologic changes were similar varying chiefly in severity. The most commonly occurring lesions (seen in 91% of the mucosally FIV-C infected cats) were generalized lymphoid

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hyperplasia, bone marrow hyperplasia, and thymic atrophy. The second most common lesions observed in these cats were splenic lymphoid hyperplasia and thymic follicle formation, occurring in 83% and 78%, respectively. A third cadre of miscellaneous lymphoid and mucosal lesions were confined to ≤ 30% of the cats.

Of the 18 cats with the conventional FIV disease course, all exhibited thymic atrophy and thymic follicle formation and varying degrees of lymphoid hyperplasia in lymph nodes, bone marrow, and spleen. In addition, 3 had lymph node sinus histiocytosis, 3 had mild lymphoid depletion in lymph nodes, and 4 had multifocal areas of necrosis in mesenteric lymph nodes. (Table 2.1) Lymphoid follicle formation was detected in 7 of the 18 in bone marrow. One cat had focal ulcerative dermatitis. Histologic and/or gross examples of thymic depletion and variable degrees of lymph node hyperplasia are demonstrated in Figs. 2.1, 2.2, and 2.3. In addition, sinus histiocytosis is demonstrated in Fig. 2.4.

The intermediate and rapidly progressive FIV disease courses were marked by a mixture of lymphoid hyperplasia and depletion in multiple lymphoid tissues (spleen, lymph node, tonsil, and mucosal associated lymphoid tissue (MALT) of the intestinal tract). (Fig. 2.5) By contrast, lymphoid depletion unaccompanied by hyperplasia was seen only in the lymph nodes from cats with conventional/slow FIV disease progression. In all instances, rapid progression was correlated with severe thymic atrophy, lymphoid necrosis in tonsils and either cecum or jejunum, and bone marrow hyperplasia. In addition, suppurative necrotizing lesions were detected in several organs including oral mucosa, jejunum, cecum, submandibular lymph node, uterus, and lung. Also, sinus histiocytosis was detected in lymph nodes from 2 of 2 rapid progressors.

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Table 2.1: Summary of pathologic changes associated with 4 disease courses detected in cats (n = 23) infected mucosally FIV-C.

Cat#	Disease Course	Lymph Node Hyperplasia	Lymph Node Histiocytosis	Lymphoid Depletion	Lymphoid Necrosis	Bone Marrow Hyperplasia	Bone Marrow Lymph Follicles	Spleen Hyperplasia	Thymus Depletion	Thymus Lymph Follicles	Ulceration	Abscessation
3660	Rapid	1		S,LN T,M	T cec	2			4		cec uter	lung
3661	Rapid	1	retro	S,LN T,M	T jejun	1			4		jejun	lung subm
3665	Intermediate	1	retro	S,LN T,M		2			4		oral	
3706	Conventional	3	mes colic			2	1	3	1	2		
3707	Conventional	2		LN		1		3	2	2		
3729	Conventional	2				2	2	2	2	2		
3730	Conventional	2				3	2	3	1	1		
3731	Conventional	2				3	2	3	2	2		
3733	Conventional	2			mes	2		3	2	2		
3735	Conventional	2			mes	1		3	3	3		
3736	Conventional	2				1		2	2	2		
3613	Conventional	2			mes	1		2	2	2		
3611	Conventional	2				2		2	2	1		
3608	Conventional	2				3		2	1	1		
3607	Conventional	2		LN		3		1	3	2		
3605b	Conventional	2	mes	LN		3		2	3	3		
3677	Conventional	2				3		2	3	3		
3678	Conventional	2				3	2	2	2	2		
3679	Conventional	3				3	3	3	3	3	skin	
3680	Conventional	2			mes	3		3	3	3		
3681	Conventional	3	retro			3	2	3	3	3		
3672	Regressive							1				
3587	Regressive											
	# of Cats	21	5	6	6	21	7	19	21	18	4	2
	% of Cats	**91**	22	26	26	**91**	30	83	**91**	78	17	9

LN: lymph node; mes: mesenteric LN; retro: retropharyngeal LN; S: spleen; T: tonsil; M: mucosal associated lymphoid tissue; cec: cecum; jejun: jejunum; uter: uterus; subm: submandibular LN; 1: mild; 2: moderate; 3: marked; 4: severe.

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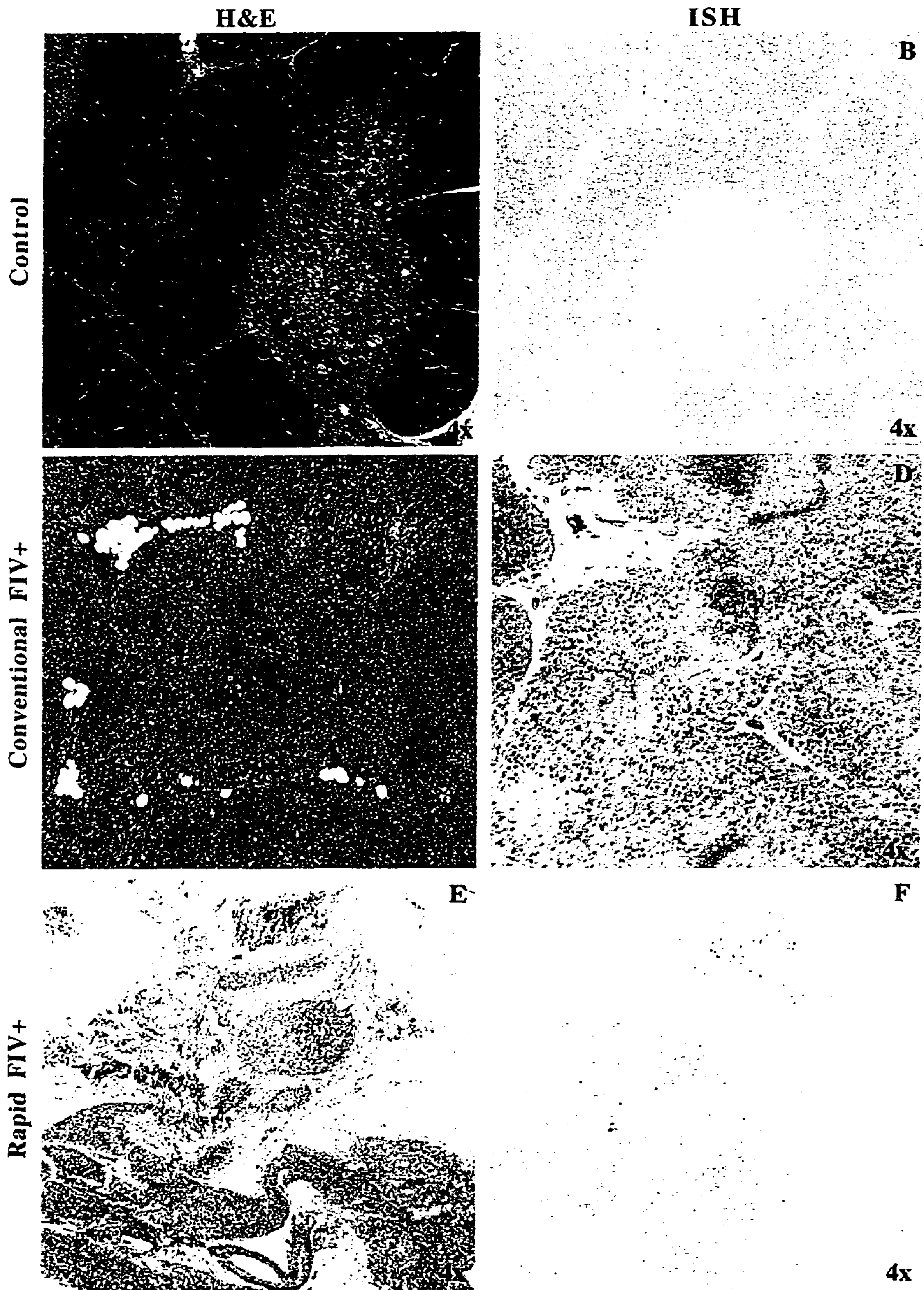

Figure 2.1: Histologic evidence for FIV-C induced thymocyte depletion (panels C&E) and viral RNA-bearing remaining thymocytes detected by ISH (panels D&F) in thymuses from

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cats with conventional (panels C&D) and rapid (panels E&F) FIV disease courses, respectively, compared with thymus controls from an uninfected animal (panels A&B).

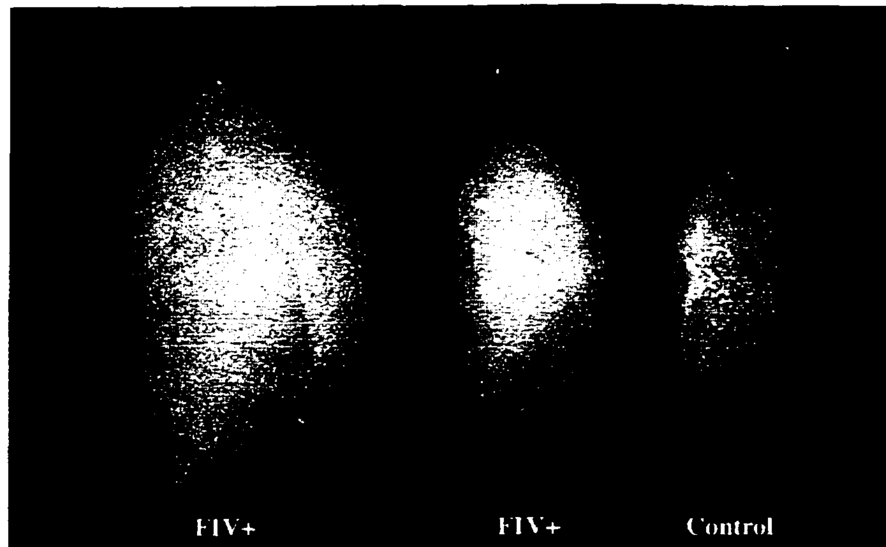

Figure 2.2: Marked (left) and moderate (middle) degrees of gross retropharyngeal lymph node hyperplasia in FIV-C-infected cats compared to the equivalent lymph node from an age- and litter-matched uninfected control animal (right).

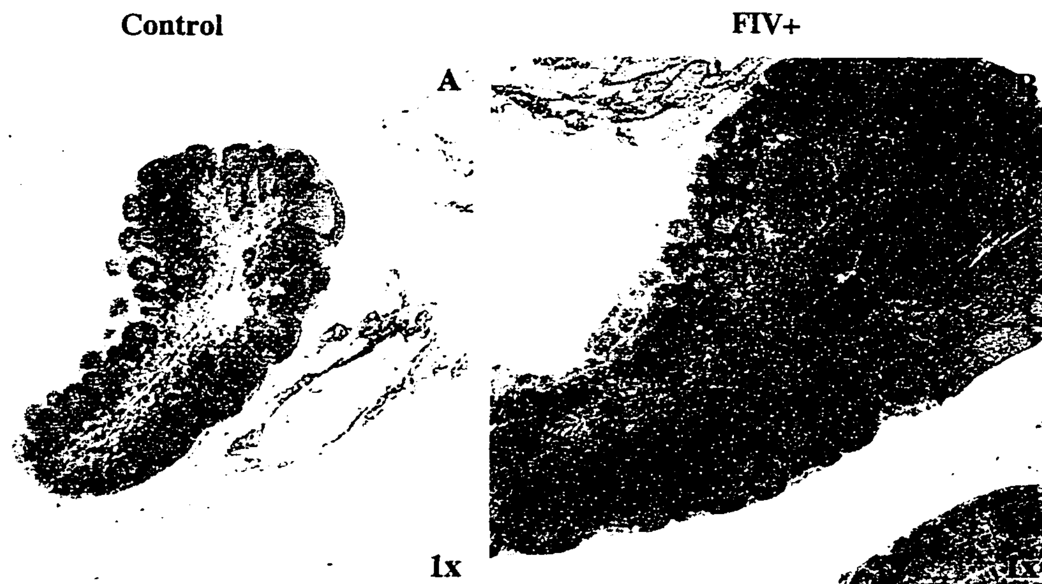

Figure 2.3: Histology of an internal iliac lymph node from an FIV-C-infected cat (panel B) demonstrates hyperplastic changes consisting of 2° follicles with expanded parafollicular regions and distended hypercellular medullary cords compared to the corresponding lymph node from an age-matched control animal (panel A).

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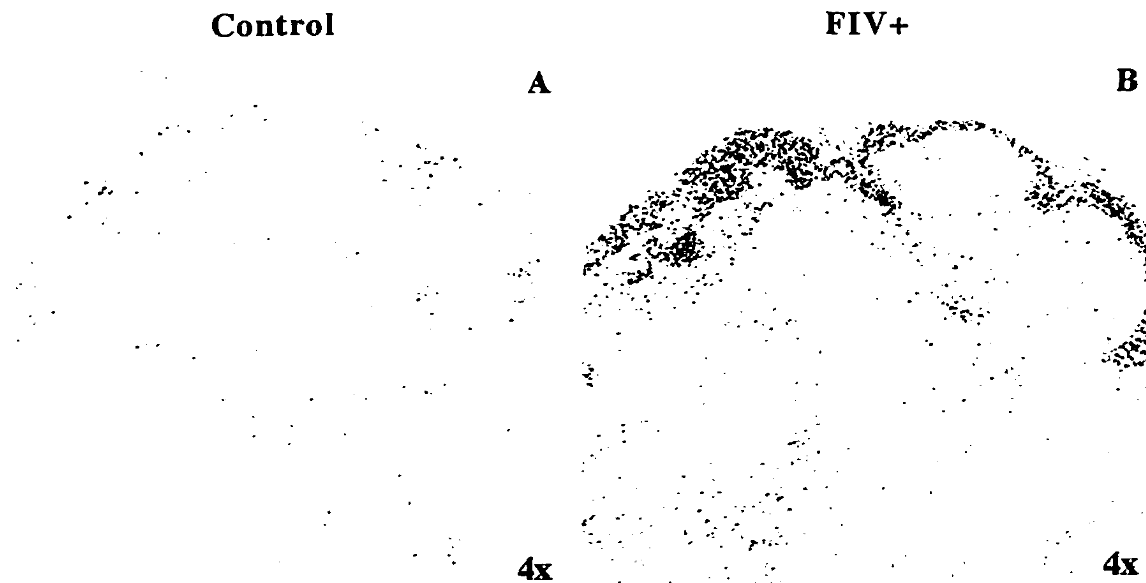

Figure 2.4: Numerous MAC387+ macrophages filled the subcapsular sinus region of a mesenteric lymph node from an FIV-infected cat (panel B) compared to the corresponding lymph node from an age- and litter-matched control animal (panel A).

Thymic depletion induced by FIV-C

Qualitative aspects of thymic depletion: histologic changes

Depicted in the left panels of Fig. 2.1 are thymic changes associated with FIV-C infection in comparison to a control animal. Thymuses from control animals had normal architecture with clear distinction between cortical and meduallary regions and without follicle formation. However, thymuses from FIV-infected cats with conventional disease course had variable degrees of cortical and medullary dissolution with follicle formation occurring at cortical-medullary junctions and in subcapsular regions. Cats with rapidly progressive infection had a complete loss of thymus architecture with minimal thymic tissue remaining amongst the connective and adipose tissue, and follicle formation was absent. In addition, thymuses from all FIV-infected cats had decreased numbers of Hassel's corpusels compared to age-matched controls.

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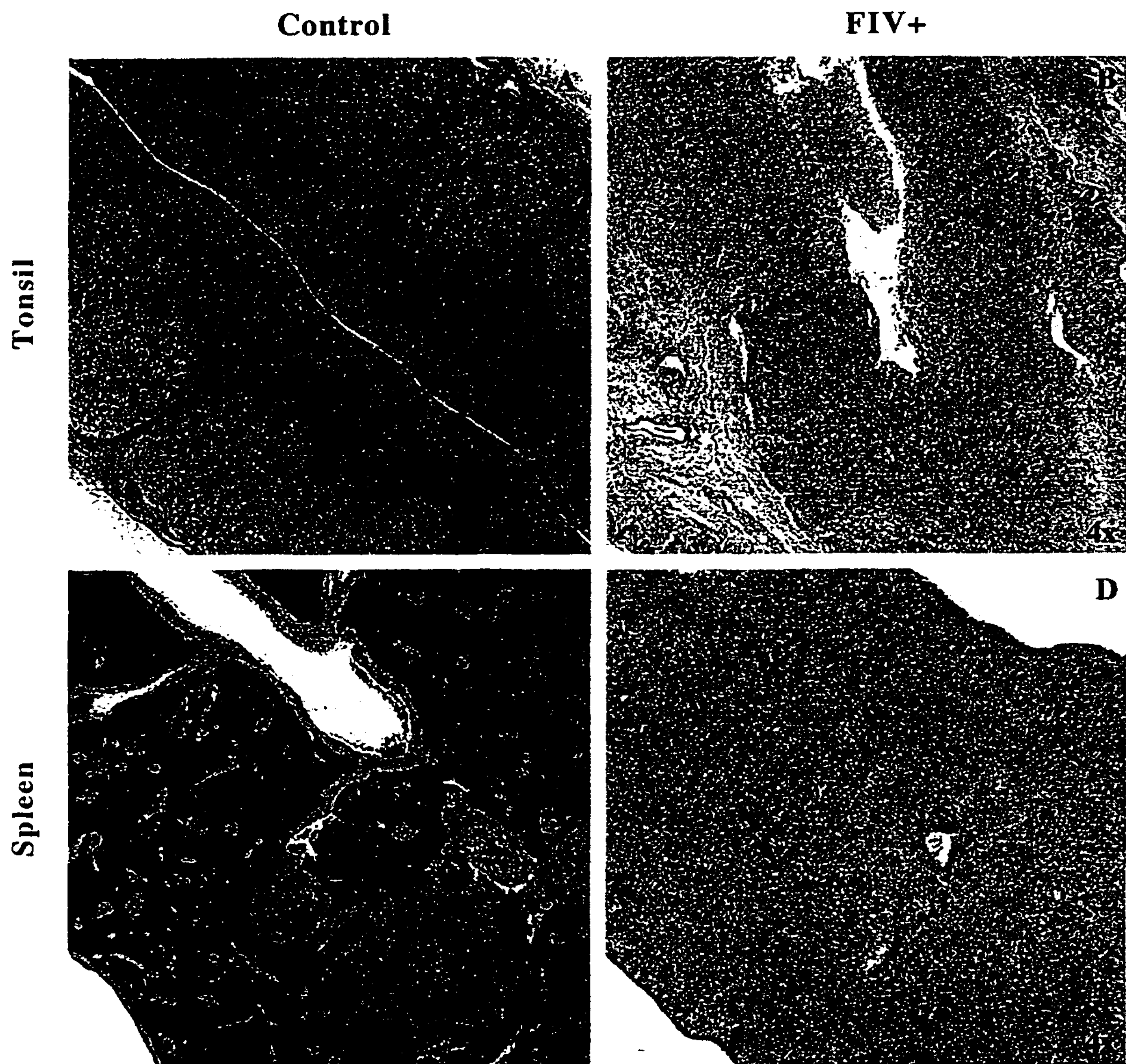

Figure 2.5: Lymphoid tonsilar (panel B) and splenic (panel D) depletion in a FIV-C rapid progressor was characterized by the loss of normal architecture, the absence of follicular structures, and overall hypocellularity compared to the corresponding tissues from a control animal (panels A&C).

Quantitative aspects: thymic weight comparisons

Thymic depletion was a prominent feature of FIV-C infection. By comparing thymic weight to whole body weight, cats with accelerated FIV infections could be distinguished from those with conventional infections, because the rapid progressors had more severe thymic atrophy and lower body weights. In addition, FIV-infected cats were distinct from age-matched

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uninfected animals, since controls had intact thymuses and better overall body condition. (Fig. 2.6)

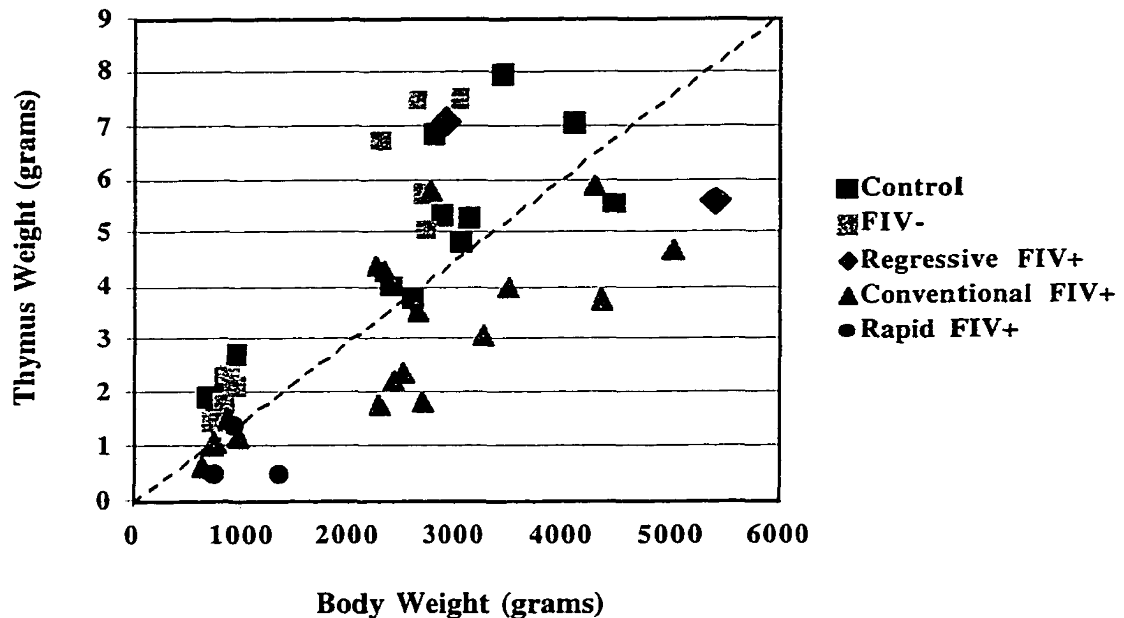

Figure 2.6: Thymic weight versus body weight of mucosally FIV-C-infected cats with regressive (◆), conventional (▲), or rapid (●) disease courses compared to controls (■) and uninfected cats (▩). Persistently FIV-infected cats (▲,●) and uninfected animals (■,▩) segregated on opposite sides of the dashed diagonal line which bisects the x-y intercept indicating differences in thymic and body weights between the 2 groups.

The average thymic weight / body weight ratios of cats having either conventional or rapidly progressive FIV infections were statistically significant when compared to controls by a Student's *t* test; whereas, uninfected cats and cats with regressed infections showed no statistical difference from controls. By comparing thymic weight / body weight ratios to terminal CD4 cell counts, cats with accelerated FIV infections and those with conventional infections could be distinguished from age-matched controls and uninfected cats. (Fig. 2.7) The cats with rapidly progressive FIV infection could be distinguished from those with conventional FIV infection, because the rapid progressors had more severe thymic atrophy and lower CD4 cell counts.

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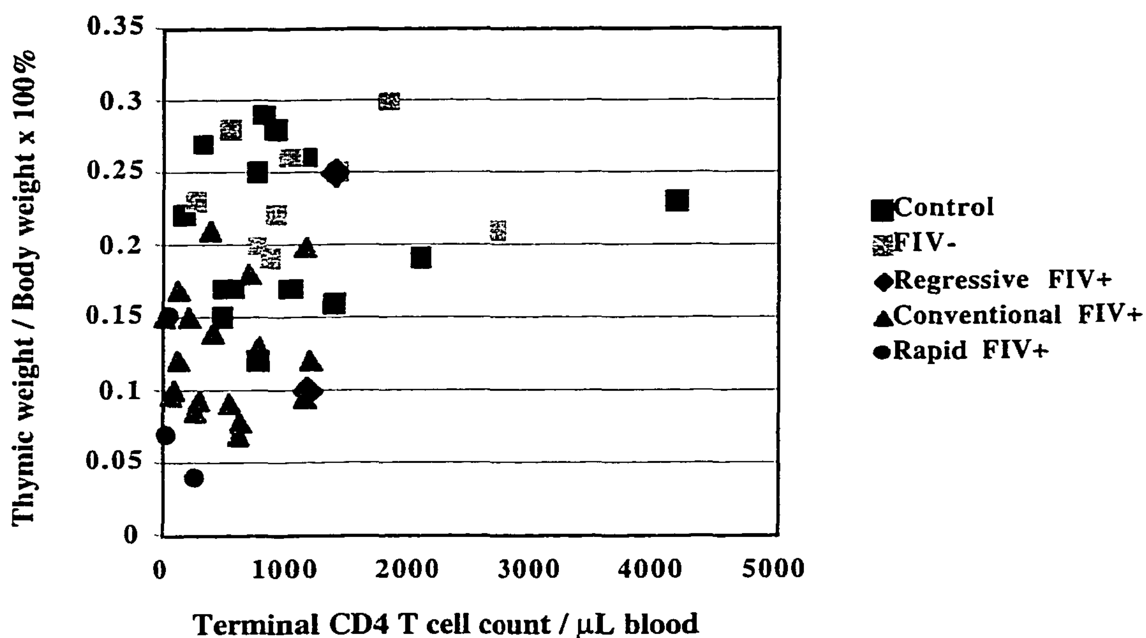

Figure 2.7: Thymic weight / body weight x 100% ratio versus terminal CD4 cell counts of mucosally FIV-C-infected cats with regressive (◆), conventional (▲), or rapid (●) disease courses compared to controls (■) and uninfected cats (▩). Persistently FIV-infected cats (▲,●) and uninfected animals (■,▩) clustered in different areas on the graph. The FIV-infected cats clustered in the lower left quadrant reflecting lower terminal CD4 cell counts and lower ratios due to smaller thymuses.

Quantitative aspects: thymic phenotype alterations

Four thymocyte populations, CD4+/CD8+, CD4+, CD8+, and B cells , from 13 mucosally FIV-infected cats and 9 uninfected cats were assessed via flow cytometry. (Fig. 2.8) In FIV-infected cats, CD4+/CD8+ and CD4+ thymocytes were significantly decreased and B cells were significantly increased. The CD8+ population was slightly increased compared to controls, but this difference was not statistically significant.

Qualitative aspects: thymic phenotype alterations

Immunohistochemical staining for CD3+ thymocytes demonstrated loss of cortical-medullary architecture and highlighted the presence of non-staining B cell follicles. (Fig. 2.9) In cats with both conventional and rapid disease courses, the number of non-staining immature

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(CD3-) thymocytes was decreased, leaving primarily mature (CD3+) thymocytes framing B cell follicles. Whereas in control thymuses, immature (CD3-) thymocytes were primarily in the cortices of lobules and mature (CD3+) thymocytes were in medullary regions.

The S100+ dendritic cells were decreased in the atrophic thymuses from cats with conventional and rapid FIV disease courses. (Fig. 2.9) By contrast, thymuses from control cats contained numerous dendritic cells in the medullary regions with lesser numbers in the lobule cortices.

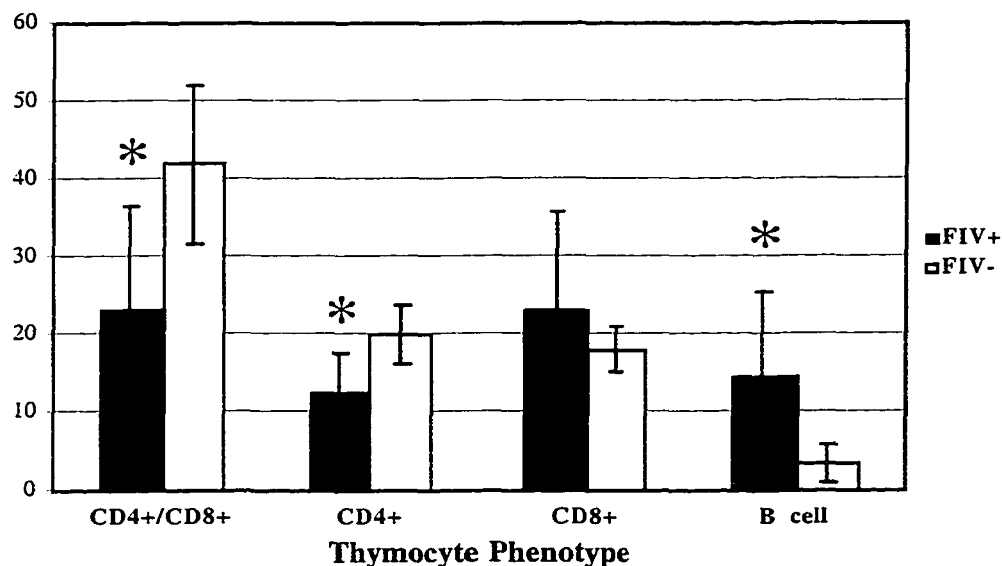

Figure 2.8: Comparison of CD4/CD8, CD4, CD8, and B cell thymocyte populations for FIV-C-infected cats (■) and uninfected cats ( ). CD4+/CD8+ and CD4+ populations were significantly decreased (indicated by *) and B cell population significantly increased.

Quantitative aspects: thymic apoptosis

The in situ terminal dUTP nucleotide end labeling (TUNEL) assay identified numerous apoptotic cells in atrophic thymic tissue of FIV-infected cats. (Fig. 2.10) Thymic tissue from age- and litter-matched control cats displayed the expected pattern (cortex and cortical-medullary junction staining [25]) and degree of apoptosis expected in weanling animals. Image analysis (utilizing Metamorph® software) revealed for every 1 TUNEL+ apoptotic thymocyte

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in uninfected control cats there were an estimated 1,260 non-apoptotic thymocytes. By contrast thymuses of FIV-infected cats contained for every 1 TUNEL+ apoptotic thymocyte approximately 48 non-apoptotic thymocytes. (Table 2.2) The apoptotic thymocytes were primary located in the cortex and follicular germinal centers and to a lesser extent in the medulla. A significant difference was confirmed ($p \leq 0.05$, Student's *t* test) between the degree of apoptosis occurring in FIV-infected cats as compared to controls.

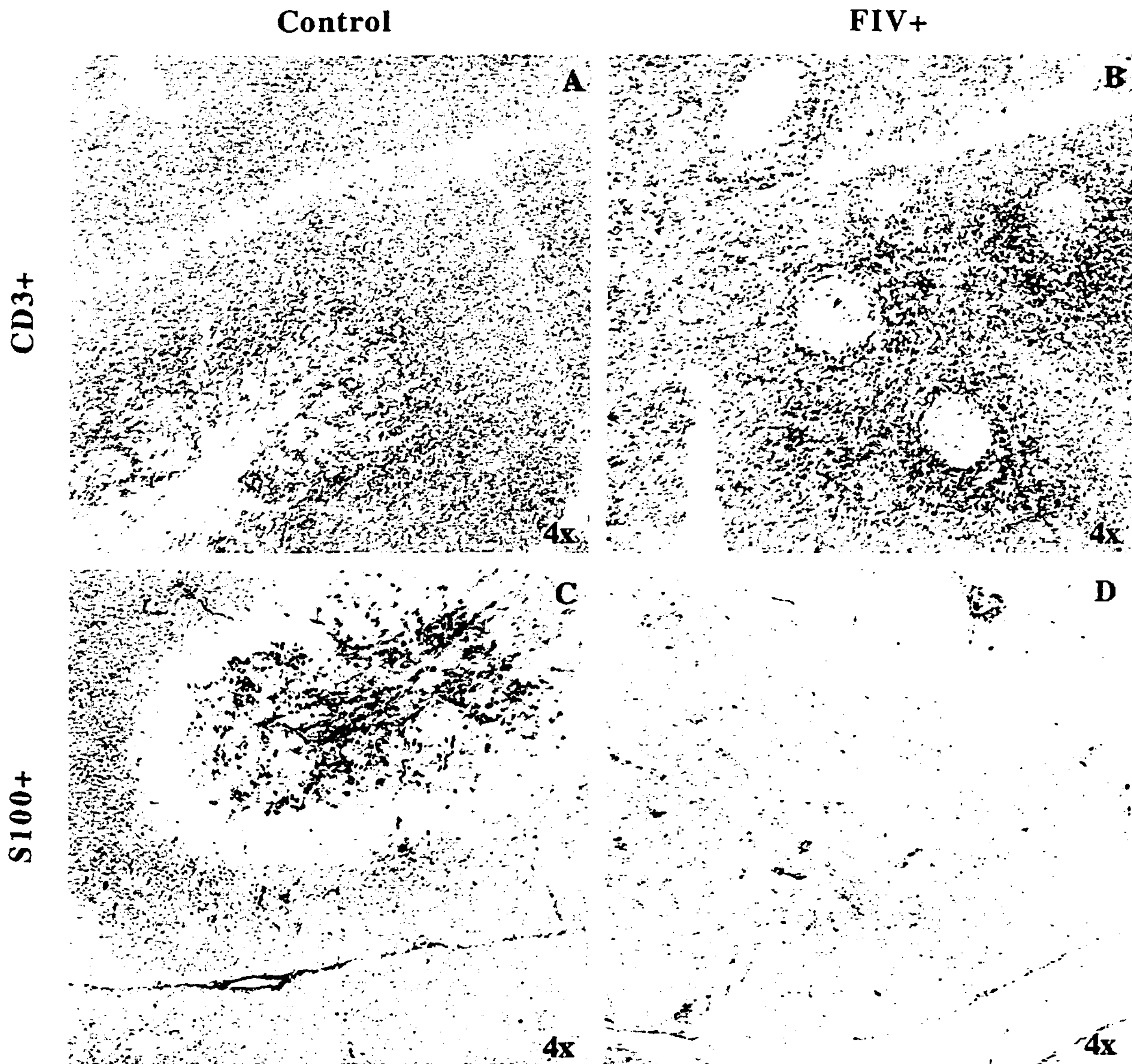

Figure 2.9: Decreased CD3-negative cortical thymocytes (panel B) and S100+ medullary dendritic cells (panel D) were demonstrated in FIV-infected cats versus uninfected controls (panels A&C). In atrophic thymic tissue from an FIV-infected cats, mostly red-staining CD3-positive thymocytes remained, highlighting the non-staining follicles. Numerous red-staining dendritic cells with their web of processes were evident in the medulla of thymic tissue from a control animal.

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Table 2.2: Image analysis of TUNEL+ apoptotic thymocytes reported as a ratio of apoptotic+ to non-apoptotic thymocytes and as percentage of the thymocytes examined.

Cat#	FIV Disease Status	1 TUNEL+ Cell : # Negative Cells	% TUNEL+ Cells
3605b	+	1 : 48	2.08
3607	+	1 : 264	0.38
3608	+	1 : 88	1.14
3611	+	1 : 71	1.41
3605a	-	1 : 1,260	0.08
3609	-	1 : 417	0.24

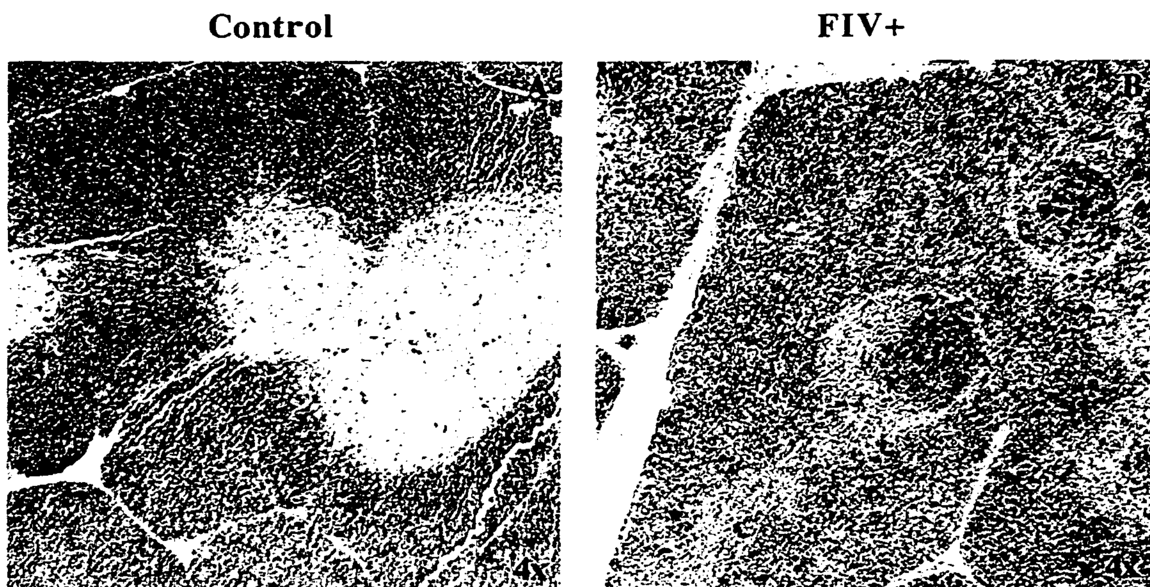

Figure 2.10: TUNEL analysis of apoptotic thymocytes in an FIV-C-infected cat (panel B) compared to an uninfected control (panel A). Numerous dark purple-staining apoptotic cells were identified in the remnant cortex and follicles of thymic tissue from an FIV-infected cat. Apoptotic cells were detected to a much lesser degree in thymic tissue from a control animal.

Quantitative aspects: thymic viral load via (RNA) in situ hybridization (ISH)

The right panels of Fig. 2.1 demonstrate thymic viral burden by RNA ISH. Thymic tissue from cats with conventional FIV infection had numerous FIV *gag/env* RNA-bearing cells throughout the atrophic parenchyma. Image analysis was used to enumerate the frequency FIV virus-bearing thymocytes compared to uninfected cells. For every 1 FIV+ thymocyte, there were 21 non-staining cells. (Table 2.3) By contrast, thymic tissue from a cat with

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rapidly progressive FIV disease course contained approximately 1 FIV+ thymocyte for every 218 non-staining cells. The severely atrophic thymuses tissue from rapid and intermediate progressors had fewer virus-bearing cells compared with thymuses from cats with conventional FIV disease course, probably reflecting the terminal stage of disease at tissue collection. The frequency of FIV *gag/env* RNA in thymocytes of animals with conventional infections was variable ranging from 1 FIV+ thymocyte for every 18 to 401 uninfected cells. Thus, viral replication was prominent in thymic tissue of FIV-infected cats which was not already severely depleted of thymocytes.

Table 2.3: Image analysis of ISH+ thymus viral burden in FIV-infected cats reported as a ratio of FIV+ to uninfected thymocytes and as a percentage of the thymocytes examined.

Cat #	FIV Disease Course	1 FIV+ Cell : # Uninfected Cells	% FIV+ Cells
3605b	conventional	1 : 112	0.89
3608	conventional	1 : 401	0.25
3677	conventional	1 : 21	4.76
3678	conventional	1 : 116	0.86
3679	conventional	1 : 23	4.35
3681	conventional	1 : 18	5.56
3665	intermediate	1 : 334	0.30
3661	rapid	1 : 218	0.46

DISCUSSION

Previous work in our laboratory demonstrated that rapid *in vivo* passage of a subgroup C FIV isolate (FIV-C-PGammer) resulted in rapidly progressive, fatal disease in greater than 50% of infected cats. [19] The accelerated disease was characterized by rapid and progressive CD4 cell loss, lymphadenopathy, weight loss, lymphoid depletion, susceptibility to opportunistic infections, and severe thymic atrophy. These changes mirrored those seen in accelerated and/or terminal HIV infection. Subsequent mucosal inoculation with FIV-C also resulted in rapidly progressive disease, but only in 8.3% of infected animals. [25] Instead, a wider spectrum of disease courses occurred, spanning from rapid to regressive and asymptomatic.

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The goal of the current study was to characterize the lesions associated with this range of mucosal FIV-C disease courses.

The thymus was a major viral target organ in the mucosal FIV-C infection. In particular, the CD4+/CD8- and CD4+/CD8+ thymocyte populations were selectively decreased. Similar findings have been reported in other FIV and SIV studies. [26-32] Severe thymic atrophy characterized by selective depletion of CD4-bearing thymocyte populations in pediatric AIDS patients. [33-35] As with FIV infection, the severely depleted tissue from children who have died of AIDS contains minimal evidence of virus replication. [36,37] In both instances, it appears that loss of CD4 cells left only the least susceptible thymocyte populations (CD4-/CD8+ and CD3+) or infiltrating CD4-/CD8+/CD3+/CD1- inflammatory cells [26,27,38-40] remained. Immunohistochemistry demonstrated in both conventional and rapid progressors a population of mostly CD3+ thymocytes (single positive mature thymocytes/lymphocytes [41]) remaining in the cortex; whereas controls had a large population of non-staining cortical cells (CD3- immature thymocytes [41]). These data suggest that the remnant thymocyte population consists of primarily single positive mature thymocytes having a CD4-/CD8+ phenotype. This is consistent with other studies in FIV-infected cats reporting thymic cortical involution associated with virus replication. [24,26,27] Likewise, thymuses with discernible morphology in FICV-C infected cats exhibited only rare FIV-bearing cells in the medullary region. In contrast, several reports in SIV and HIV infection have identified most virus-replicating thymocytes in the medullary region and more rarely in the cortex. [31,40,42] Taken together, the immunohistochemistry, flow cytometry, and ISH findings, suggest that FIV-C has preferential tropism for immature thymocytes.

The thymus may contribute to the pathogenesis of AIDS by serving as a reservoir for virus replication and a site for centralized T lymphocyte depletion. [43] Rapid progression and abnormal peripheral T lymphocyte subset development in pediatric HIV infections suggests thymic dysfunction. [34,44,45] In addition, HIV-infected adults receiving antiretroviral therapy do not have restored thymic and peripheral CD4 function. [35] Dua *et al.* [46] suggested that

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thymocyte depletion in FIV-infected cats may precede peripheral blood CD4 lymphocyte depletion. Similarly, Woo et al. [27] concluded that FIV infection in juvenile cats reduced the ability of the thymus to replenish the peripheral T cell pool contributing to the peripheral lymphocytopenia. Thus, the preferential depletion of immature thymocytes in FIV-C-infected cats would contribute to elimination of peripheral CD4 cells and subsequent immunodeficiency

Similar to the observations made for FIV-C-infected cats, several studies have shown that thymocytes are highly susceptible to productive HIV infection and that a selective depletion of CD4-bearing thymocytes occurs. [34,41,47-50] Immature human CD4+/CD8+ thymocytes express the highest levels of both CD4 and CXCR4 and are preferentially infected and depleted by syncytium-inducing (SI) HIV-1 strains *in vitro*. [51] SI isolates appear to replicate faster, to higher titers, and more cytopathically in immature and mature CD4+ thymocytes than do non-SI isolates. [52] Similarly, a highly replicative, cytopathic, tissue-specific HIV variant was isolated from the thymus of an HIV-infected patient. [41] Hence, the FIV-C-PGammer isolate may be analogous to such T tropic HIV SI strains.

Thymic lymphoid follicles containing RNA-bearing cells was a feature of FIV-C infections with the exception of the depleted thymic tissue from rapid progressors. Thymic lymphoid follicle formation has been described previously for FIV, HIV, and SIV infections. [24,26,27,46,53-56] In contrast to our findings, Orandle *et al.* [26] reported an absence of FIV-bearing cells in the thymic lymphoid follicles. In addition, decreased S100+-staining medullary dendritic cells as well as a decreased number of Hassel's corpusels were observed for FIV-C infected cats compared to controls. The cells of the thymic microenvironment, which include epithelial cells, macrophages, B cells, and interdigitating dendritic cells, are necessary for intrathymic proliferation and maturation. [57-59] Thymic epithelial cells are structurally and functionally altered in simian and feline lentiviral infections. [26,27,29,60-62] In the early course of SIV infection, ultrastructural analysis revealed vacuolization, shrinkage, and cytolysis of thymic cortical epithelial cells and interdigitating dendritic cells. [28] Thymic dendritic cells

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can be infected with HIV-1 in vitro, support viral replication, and transmit viral infection only following cell-to-cell contact. [63] Most likely the changes observed in the thymic microenvironment associated with FIV-C infection contributed to the resulting disease process. Also, the above findings suggest that dendritic cells are susceptible to FIV-C infection and cytopathicity.

FIV-C mRNA burden revealed by ISH did not correlate with the degree of thymic atrophy, suggesting that viral cytopathicity was not the only mechanism contributing to thymic depletion. In fact, loss of CD4+/CD8+ lymphocytes independent of cell infection has been reported in HIV disease. [49] In addition, thymic tissue from FIV-C-infected cats had significantly higher level of apoptosis when compared to age- and litter-matched control cats. Apoptotic cells were diffusely located in the atrophic thymic tissue, but were also prominent in the follicular germinal centers, as described for the lymph nodes of HIV-infected individuals. In SIV-infected macaques, increased apoptosis was seen in the thymic cortex by 7 to 14 days PI followed by depletion of thymocyte progenitors (CD34+ and CD4+/CD8+) by day 21. However, absolute numbers of CD4+ and CD8+ cells in the peripheral blood changed little over the 50 day experiment. [40] Sarli *et al.* [64] found a higher apoptotic index in the thymic cortex (especially in the follicle germinal centers) of FIV-infected cats versus controls. Thymic dendritic cells release, upon infection with primary HIV isolates and laboratory strains, soluble factors which induce killing of primary CD4+ and CD8+ thymocytes and activated but not resting PBMC. This cytotoxic factor-mediated cell death involves the direct/indirect contribution of FasL and TNF-α. [65] In addition, FasL and TNF-α have been shown to play a role in the HIV-1-infection-macrophage-dependent apoptosis of CD4 cells from HIV-1-infected individuals. [66] Cross-linking of CD4 molecules by HIV gp120 triggers apoptosis in noninfected CD4 cells as well as lamina propria T cells. [67-69] Even though FIV apparently does not utilize the CD4 receptor, [70-73] reduction in the number of CD4-bearing cells has been documented following FIV infection. [74] Apoptosis has been demonstrated in PBMC including B cells and CD8 cells in both FIV and HIV infections. [74-80] In addition, disease progression in FIV-infected cats is associated with increased production

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of TNF-α which has been shown to induce apoptosis in a cell line chronically infected with FIV. [79,81,82] Multiple HIV studies have demonstrated an association between apoptosis and cell loss. [83-88] Holznagel *et al.* [89] reported that the degree of *in vitro*-induced apoptosis was closely related to FIV-mediated T cell depletion and lymphocyte activation *in vivo*, and could serve as an indicator of FIV disease progression. Similarly, the degree of HIV-induced apoptosis correlates with CD4 cell decline and disease progression. [90,91] Thus, apoptosis is likely to be another mechanism likely contributing to FIV-C and to other lentivirus-associated thymic CD4 depletion.

Spectrum of lymphoid cell alterations ranging from hyperplasia to depletion were observed in FIV-C-infected cats. At one extreme were cats with regressive FIV-C infection in which little or no alterations were detected. At the other extreme were rapid progressors in which lymphoid hyperplasia was variably mixed with lymphoid depletion and necrosis. The latter was primarily observed in the tonsil and in mucosal associated lymphoid tissue (MALT) of the intestine. Follicular hyperplasia followed by progressive follicular involution and depletion has been described in HIV infection. [92-94] Similar lymph node alterations have also been described in FIV infection. [3,11,95-99] These changes correlate with clinical stages of HIV infection. [92,100,101] Our findings with FIV-C are in accord with those in HIV infection and with the conclusions of Rideout *et al.* [99] that FIV lymph node histology reflects clinical disease stage.

Many of FIV-C-infected cats with conventional disease courses had lymphoid follicle formation in bone marrow and sinus histiocytosis. Increased secretion of IL-6 by macrophages in lymph nodes and bone marrow of HIV-infected patients has been linked to polyclonal B cell activation and plasmacytosis. [102-104] B cell sinus reaction (formerly called immature sinus histiocytosis) has been frequently identified in HIV lymphadenitis. [105] Even though these cells have histiocytic morphology, they have been identified as B cells rather than of macrophage/histiocytic lineage. [106,107] In 6 of the FIV-C infected cats with either conventional or rapid disease courses, sinus histiocytosis (macrophage) was observed in the

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subcapsular sinus and parafollicular regions. Similarly, expansion of the paracortical macrophage population and B cell hyperplasia accompanied by histiocytic hyperplasia have been described in lymph nodes of HIV-infected patients. [108-111] Thus, the lymph node reactive changes observed in FIV-C-infected cats parallel those reported in HIV-infected people.

In addition to profound lymphoid depletion, rapidly progressing FIV-C-infected animals had intestinal and mucosal ulcerations with necrosis and disseminated bacterial infections. [12] Rapidly fatal infection characterized by severe intestinal mucosal damage has been reported in macaques infected with SIVsmmPBj14. [112-114]. In contrast to the cats with accelerated FIV-C disease, SIVsmmPBj14-infected macaques die within 21 days of a systemic shock-like syndrome with severe diarrhea. [12,112-114] Rapid disease progression with lymphoid depletion has also been described in SIV-infected macaques; more analogous to FIV-C disease, these animals succumb to AIDS after a few months rather than weeks of infection. [115-117] While there have been occasional reports of individual cats developing rapid disease following FIV infection; prior to FIV-C studies this response was rare and not reproducible. [1,96,118] Even though the rapid FIV-C disease occurred less frequently after mucosal infection as in SIV infection, [119] the resulting disease was equivalent to that associated with intravenous passage, suggesting that in at least some instances the virulent genotype can cross the mucosal barrier. While rapid progression in SIVmac239 and SIVsmmPBj14 involves amino acid substitution in the *nef* gene leading to enhanced disease in resting T cells, [120-122] the viral genetic basis of the acute FIV-C-PGammer disease has not been resolved, because efforts to generate infectious molecular clones have thus far failed. Further understanding of the molecular basis of FIV-C acute pathogenicity must await successful cloning of the virulent genotype.

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CHAPTER THREE

EARLY PATHOGENESIS OF MUCOSAL FIV INFECTION

INTRODUCTION

The early events in transmucosal HIV infection remain unknown and are at best difficult to study in humans. Thus, the earliest target cells and trafficking of viral infection after of mucosal lentiviral exposure can best be explored in an animal model. Model studies could also aid in identifying stages in lentiviral infection progression at which antiviral therapy remains highly effective. The objective of these studies, therefore, was to employ feline immunodeficiency virus (FIV) infection to study of early events in mucosal lentivirus transmission.

FIV causes a disease syndrome similar to that caused by HIV. [1,2-5] As with HIV, FIV can be transmitted by mucosal exposure, blood transfer, and vertically. [6-11] FIV has been transmitted by 3 mucosal routes (oral, rectal, vaginal) with either cell-free or cell-associated virus inocula. [10,12-15] FIV replication has been demonstrated in multiple target cells: T lymphocytes, [9,16-20] macrophages, [2,21-24] B cells, [25] megakaryocytes, [26] salivary gland ductular epithelium, [27] kupffer cells, [28] endothelial cells, [29,30] astrocytes, and microgia. [31,32]

Several theories have been proposed concerning the mechanisms of lentiviral mucosal infection. One is that the virus traverses the mucosal membrane at a lesion, and enters the blood or susceptible cells at that site. [33] Perhaps in non-primate neonates lentiviruses cross the mucus membrane before physiologic gut closure. [34] Other proposed pathways by which

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HIV could cross an intact mucosal surface include translocation by M cells, [35] transcytosis, [36] endocytosis, [37] infection of epithelial cells, [38-44] and infection of Langerhans cells (LC). [45]

Studies of macaques chronically infected with SIV have also identified infected cells with dendritic morphology in the stratified squamous epithelium of the vagina. [46] LC and blood dendritic cells (DC) have been shown to be efficient vectors for HIV transmission to T cells *in vitro*. [47-50] Frankel *et al*. [51] demonstrated CD3+ T cells in contact with multinucleated syncytia co-expressing S100 and p55, dendritic cell markers, and HIV-1 Gag protein at the mucosal surface of adenoids from HIV-infected patients. These observations support the concept that mucosal LC may act as vectors for the transmission of HIV to T cells during primary infection. In an acute SIV transmission study, infected LC-like were found in the lamina propria of the cervicovaginal mucosa immediately subjacent to the epithelium and in draining lymph nodes within two days after cell free SIV infection. [52,53] An equivalent of the LC has been identified in cat stratified squamous epithelium. [54] FIV infection of LC has not yet been shown, although FIV RNA and antigen have been demonstrated in association with follicular dendritic cells (FDC) in lymphoid germinal centers. [2,55,56] Similarly, in HIV-infected patients, viral antigens and RNA have been demonstrated in FDC. [57-59]

Several lines of circumstantial evidence also suggest that an important property of transmitted virus is the ability to infect macrophages. [60-64] Macrophages also bear the chemokine receptor CCR5, used by macrophage-tropic HIV clinical isolates. [65-67] HIV RNA and DNA has been demonstrated in macrophages at the mucosal-stromal junction of the endocervical transformation zone, but virus positive macrophages were not detected in the vagina or endometrium. Macrophages were the predominant cells infected, whereas most lymphocytes were not infected. [68] In SIV mucosal challenge studies, infected cells in the genital tract were primarily macrophages located in the submucosa of the cervix and vagina. [46,69]. Thus, macrophages might play a role in dissemination and transmission of lentiviruses from mucosal to regional or systemic lymphoid tissues.

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The entry of primate lentiviruses into target cells depends upon the interaction of the viral envelope glycoprotein with CD4 and one or more members of the G protein-coupled receptor (GPCR) family of transmembrane proteins. [70,71] *In vivo*, the transmission of HIV-1 infection is thought to require viral strains that utilize the chemokine receptor CCR5. These macrophage-tropic strains prevail during the early course of infection, whereas viruses isolated later in the progression to immunodeficiency are often tropic for the chemokine receptor CXCR4 or dual tropic. [72,73] However, several studies in HIV have found that no selection for macrophage-tropic, non-syncytium-inducing viruses occurs during sexual transmission. [74-77] While chemokine receptors are expressed primarily in hematopoietic cells, many other cell types have been shown to express one or more chemokine receptors, including microglia, [78,79] endothelial cells, [80,81] placental macrophages, [82] and colonic, rectal, cervical, and vaginal epithelia. [83] Unlike the primate lentiviruses, the feline homologue of CD4 does not appear to act as a primary receptor for FIV, [84-86] however, 2 studies have recently identified shared usage by FIV and HIV of the chemokine receptor CXCR4 which displays a 94.9% amino acid sequence homology between the species. [85,87]

The objective of the current study was to examine the earliest sites of FIV replication following mucosal exposure. We employed infection models using a clade C FIV isolate (FIV-C-PGammer), reasoning that this virulent, highly replicative, and mucosally transmissible isolate would provide the best opportunity to detect very minimal early sites of viral replication. Our intent was that information gained from these studies would be useful in development of intervention strategies for both HIV and FIV infections.

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MATERIALS & METHODS

Animals & sample collection

Eight to 10 week old weanling cats from a specific pathogen free (SPF) breeding colony maintained at Colorado State University (Fort Collins, CO) were exposed to FIV-C by instillation of either: (a) 2 mL of 300 $TCID_{50}$/mL (50% tissue culture infective dose) cell-free infectious culture supernatant (*n* = 18); or (b) 4 x 10^5 infectious peripheral blood mononuclear cells (PBMC) (*n* = 8) onto the oral and nasal mucosae using a syringe. Following inoculation with virus, each animal was positioned upright to facilitate oral-nasal exposure and to prevent aspiration.

Female SPF weanling age kittens were exposed to FIV by depositing either: (a) 500 µL of 300 $TCID_{50}$/mL cell-free infectious FIV-C culture supernatant (*n* = 7); (b) 500 µL of 400 $TCID_{50}$/mL cell-free infectious FIV-B culture supernatant (*n* = 5); or (c) 2 x 10^5 FIV-C infectious PBMC (*n* = 3) into the vagina using a rubber tomcat catheter. Following instillation of virus inoculum, each animal was positioned with its posterior end elevated for 20 minutes to facilitate exposure of the vaginal mucosa.

At 1 to 2 day intervals within the first 12 days post inoculation (PI), cats were necropsied and multiple tissues collected including mucosae and local, regional, and systemic lymphoid tissues and fixed in 10% buffered formalin for ≤ 18 hr before histologic processing and paraffin embedding. Tissues harvested included: spleen; bone marrow; thymus; retropharyngeal, submandibular, gastric, tracheobronchial, mesenteric colic, sacral, iliac, and popliteal lymph nodes; tonsils; liver; gastrointestinal tract; lungs; salivary glands; oral-nasal and vaginal mucosa; and brain. Parallel tissue samples were also frozen in liquid nitrogen for nucleic acid extraction. Equivalent samples of contralateral organs were dissected for formalin fixation and freezing. Small portions of the retropharyngeal and submandibular

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lymph nodes from 11 of the 26 oral-nasally inoculated cats and 2 control cats were collected in media for virus isolation coculture.

Virus inocula

Three viral inocula were employed: (1) cell-free virus prepared as supernatant from coculture of naïve PBMC with PBMC from a cat infected with the FIV-C-PGammer; [88] (2) cell-free virus prepared as supernatant from coculture of naïve PBMC with PBMC from a cat infected with the FIV-B-2542 (subtype B); [89] and (3) cell inoculum was derived from PBMC isolated from an acutely FIV-C-infected cat (cat #3823). The total number of PBMC inoculated was recorded and the number of infectious PBMC inoculated was calculated following titration by dilutional DNA PCR. Oral inoculates received 4 x 10^5 infectious PBMC and vaginal inoculates received 2 x 10^5 infectious PBMC.

Virus isolation from PBMC & lymph node cells (LNC)

PBMC and LNC cells were separated by density gradient centrifugation (Histopaque 1077; Sigma) from heparinized samples or phosphate-buffered saline (PBS) and washed twice with PBS. LNC were mechanically dispersed by passage through a wire strainer and resuspended in PBS. Naïve donor PBMC were obtained from SPF cats and stimulated with concanavalin A (Con A, 10 µg/mL) (Sigma) for 3-7 days. One million naïve cells were cocultured with either 1 x 10^6 PBMC or LNC from FIV-exposed cats in RPMI medium supplemented with 20% fetal calf serum, 1% penicillin/streptomycin, 2% glutamine, 2 x 10^{-5} M 2-mercaptoethanol, and 100 units of interleukin-2/mL (Cetus/Roche, Emeryville, CA). Cell were culture in 24-well plates. Supernatants were collected twice weekly and assayed for FIV p26 capsid antigen by capture enzyme-linked immunosorbent assay (ELISA). [90]

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DNA PCR primers & probes

Primers were selected using the Oligo® Primer Analysis Software program (National Biosciences, Inc., Plymouth, MN) from the *gag* nucleotide sequence of FIV-C-PGammer obtained from J. I. Mullins (University of Washington, Seattle, WA). The first round CgagU1 and CgagL1 primers amplified a fragment 623 bp in length. The primer sequences were as follows: CgagU1 (nucleotides 65 to 80), GGGTAGGGGGAAAGAG, and CgagL1 (nucleotides 673-656), AGTGAAGTATGGCAATGG. The second round CgagU2 and CgagL2 primers amplified a fragment 301 bp in length. The primer sequences were as follows: CgagU2 (nucleotides 649-631), AAGCCGAGAGGAAAGGAA, and CgagL2 (nucleotides 367-384), GACCATCAGGAGGGTGAGT. The sensitivity of the nested PCR reaction was determined using plasmid DNA containing the FIV-C *gag* gene to be between one and ten copies of target sequence.

In order to test the specificity of the PCR products, a 22–mer probe (nucleotides 513-534), ATTATGGTTTACAGCCTTTTCG, was designed to recognize both first and second round products. The oligo was obtained with a 5' amino linker from Operon Technologies, Inc. (Alameda, CA). The probe was labeled with alkaline phosphatase (AP) using the AP-Oligonucleotide Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Following standard Southern blot transfer of PCR product from a 1.2% agarose gel to a positively charged nylon membrane and hybridization of the AP-conjugated oligoprobe using QuikHyb® hybridization solution (Stratagene, La Jolla, CA), the bound probe was visualized using Quantum Yield® chemiluminescent substrate (Promega, Madison, WI) and Kodak XAR autoradiography film (Eastman Kodak Company, New Haven, CT).

A second set of nested FIV-C *gag* gene specific PCR primers and complementary probe were designed as described above for analysis of DNA extracted from formalin-fixed paraffin-embedded tissues. The first round PCgagU1 and PCgagL1 primers amplified a fragment 393 bp in length. The primer sequences were as follows: PCgagU1 (nucleotides 475 to 495),

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GAAAAGGCAAGAGAAGGGTT, and PCgagL1 (nucleotides 867-847), GGCATAATCTGAAGGTCC. The second round PCgagU2 and PCgagL2 primers amplified a fragment 144 bp in length. The primer sequences were as follows: PCgagU2 (nucleotides 671-692), GACCATTGCCATACTTCACTGC, and PCgagL2 (nucleotides 815-793), CTTTTATAGCCGCCAACTTTCC. The sensitivity of the nested PCR reaction was determined using plasmid DNA containing the FIV-C *gag* gene to be between one and ten copies of target sequence. A 25–mer probe (nucleotides 723-698), TGAGTCAGCCCTATCCCCATTATCT, was designed to recognize both first and second round products.

DNA polymerase chain reaction

DNA was extracted from PBMC, frozen tissue samples, or from formalin-fixed paraffin-embedded tissues using a QIAamp tissue kit (Qiagen, Inc., Chatsworth, CA). Samples were amplified by nested PCR using FIV-C specific *gag* primers. For DNA extracted from PBMC or frozen tissue samples the first set of nested FIV-C *gag* primers amplifying a final product size of 301 bp was used. For both first and second round reactions, hot start polymerase chain reaction (PCR) was performed with Ampliwax® PCR Gems (Perkin Elmer Corp., Norwalk, CT) for 35 cycles of 94, 57, and 72°C for 30 seconds each. The first and second round reaction mixtures contained 3mM $MgCl_2$, 200 μM of each dNTP, 1.25X Gene Amp 10X PCR buffer II (Perkin Elmer Corp.), 2.5 units AmpliTaq® DNA polymerase (Perkin Elmer Corp.), and 0.1 μM of each first round primer or 0.05 μM of each second round primer, respectively. For DNA extracted from formalin-fixed paraffin-embedded tissues the second set of nested FIV-C *gag* primers amplifying a final product size of 144 bp was used. For both first and second round reactions, hot start polymerase chain reaction (PCR) was performed with Ampliwax® PCR Gems (Perkin Elmer Corp., Norwalk, CT) for 35 cycles of 94, 54, and 72°C for 30 seconds each. The first and second round reaction mixtures contained 3mM $MgCl_2$, 200 μM of each dNTP, 1.25X Gene Amp 10X PCR buffer II (Perkin Elmer Corp.), 2.5 units AmpliTaq® DNA polymerase (Perkin Elmer Corp.), and 0.5 μM of

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each first round primer or each second round primer. Products were visualized on a 1.2% agarose gel stained with GelStar® (FMC Bioproducts, Rockland, ME). Amplimer specificity was confirmed by Southern blot using an AP-labeled internal oligoprobes described above.

RNA in situ hybridization & tyramide signal amplification

Plasmid containing either the FIV-C *gag* or *env* gene was obtained from J. I. Mullins. [91] The FIV-C genes were isolated via restriction enzyme digestion, 0.7% agarose gel electrophoresis, and Qiaex II gel purification (Qiagen, Inc., Chatsworth, CA). The purified, isolated gene fragments (*gag* = 1,353 bp, *env*A = 1,255 bp, *env*B = 776 bp) were subcloned into the pGEM®-7Zf(+) vector (Promega, Madison, WI) containing T7 and SP6 transcription promoter sequences. Sense and antisense riboprobes were transcribed off their respected promoters incorporating digoxigenin-labeled UTP using either AmpliScribe® T7 or SP6 transcription kits (Epicentre Technologies Corp., Madison, WI).

In situ hybridization (ISH) was performed in a manner similar to that described by Hirsch *et al.* [92] Briefly, formalin-fixed paraffin-embedded tissues were sectioned (4-5 μM thick) and placed on positively charged glass slides (Fischer Scientific, Pittsburgh, PA). Tissue sections were deparaffinized and rehydrated sequentially with xylene, xylene-ethanol, 100% ethanol, 95% ethanol, and RNase-free water for 5 min each at room temperature. The slides were then incubated with 5 mM levamisole for 20 min, washed with SSC buffer (0.15 M NaCl, 0.015 M sodium citrate), incubated in 0.2 N HCl for 20 min, and washed again with SSC buffer. The sections were digested with 20 μg/mL proteinase K in buffer containing 10 mM Tris (pH 7.4) and 2 mM $CaCl_2$ for 10 min at 37°C. Digestion was stopped with 0.1 M glycine in PBS. Slides were washed with PBS and endogenous peroxidase was blocked with 5% H_2O_2 in methanol for 30 min. The slides were then incubated in 0.1 M triethanolamine-0.25% acetic anhydride solution for 10 min, washed in 2X SSC, incubated in 0.1 M Tris (pH 7.4)-0.1 M glycine solution for 15 min, and washed in 2X SSC. Prehybridization was done at 50°C for 10 min with hybridization solution containing 50% deionized formamide, 1X SSC, 1X

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Denhardt's solution, 5 mM $NaPO_4$ (pH 6.8), 0.1% sodium dodecyl sulfate, 250 μg of salmon sperm DNA/mL, 5% dextran sulfate, and 250 μg/mL tRNA. Riboprobes were added at 1 ng/μL, the sections coverslipped heated to 65°C for 5 min, chilled for 10 min on ice, and hybridized overnight at 55°C. Following hybridization, the coverslips were removed and the slides washed with 4X SSC-50% formamide for 1 hr at 50°C and then in 2X SSC for 5 min. An RNase mixture (1 unit RNase T_1 and 20 μg/mL RNase A/mL for 30 min at 37°C) was used to digest excess probe and wash steps were repeated. Slides were blocked for 1 hr in a buffer containing 2% horse serum, 0.3% Tween 20, 0.5% Boehringer blocking agent (Boehringer Mannheim, Indianapolis, IN), 100 mM Tris (pH 7.4), and 150 mM NaCl. Hybridized probe was detected with a sheep anti-digoxigenin peroxidase-conjugated antibody (Boehringer Mannheim) diluted 1:100 in blocking buffer for 1 hr at room temperature. The sections were washed in TNT buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) three times for 5 min each. At this point, Renaissance® Tyramide Signal Amplification (TSA) Indirect ISH kit (NEN Life Science Products, Boston MA) was employed according to manufacturer's directions. Briefly, the tyramide reagent, diluted 1:50 in the diluent provided in the NEN kit, was applied to the slides for 10 min. After three washes with TNT buffer, the streptavidin-alkaline phosphatase, diluted 1: 2,500 in the provided diluent, was applied for 30 min. After three more washes with TNT buffer, the sections were incubated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)-nitroblue tetrazolium (NBT) substrate for 2 hr in the dark. Finally, the sections were washed in 10 mM Tris-HCl- 10 mM EDTA solution (pH 8.1), counterstained with nuclear fast red or methyl green, dehydrated in graded ethanols, cleared in xylene, and mounted with Clear*Mount® (American Master "Tech" Reagent Company Inc., Lodi, CA). Negative controls included tissue sections from uninfected cats and application of sense riboprobes on tissue sections from FIV-infected and uninfected cats.

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RESULTS

FIV-C cell-free oral-nasal transmission: study outline & comparative assay sensitivity

Eighteen 8 to 12 week old kittens were inoculated oral-nasally with cell-free FIV-C-PGammer (FIV-C) and sacrificed at intervals from 1 to 10 days PI for harvest of and mucosal, regional, and systemic lymphoid tissues were harvested. FIV DNA was sought by nested PCR using DNA extracted from frozen and/or formalin-fixed paraffin-embedded tissues. Assay sensitivity was ≤ 10 copies FIV (based on an FIV template plasmid DNA spiked into genomic DNA from naïve feline PBMC). Comparison of results on PBMC and LNC indicated that sensitivity of PCR appeared to be ≥ that of VI. The retropharyngeal and submandibular lymph nodes and PBMC from 13 oral-nasally infected cats were also assayed by virus isolation coculture (VI). On 4 occasions, VI was negative and PCR was positive for a given tissue sample (Table 3.1, *). Also, in 2 of the 4 cases (Table 3.1, (+)), the tissues were only positive once out of several separate PCR reactions suggesting very low proviral burden. Selected tissues were also examined by tyramide enhanced in situ hybridization (ISH) for viral mRNA using FIV-C specific *gag* and *env* riboprobes. On numerous occasions, rare positive virus-bearing cells were identified in tissues that were negative via PCR. Thus, utilization of a signal amplification with ISH increased detection sensitivity to ≥ that of PCR.

Detection of provirus after oral-nasal exposure to cell-free FIV-C

FIV-C was first detected by PCR in blood (4 of 10), bone marrow (2 of 10), retropharyngeal lymph node (3 of 10), tonsil (1 of 10), spleen (1 of 10), tracheobronchial lymph node (1 of 10), and mesenteric lymph node (1 of 10) of individual animals between 1 and 6 days PI. (Table 3.2) Despite intensive PCR assays on multiple regional and systemic lymphoid tissues, FIV was detectable sporadically until 8 days PI. Detection of viral DNA prior to day 8 was surprisingly sporadic and included occasional presence in: PBMC (only), retropharyngeal lymph node, and bone marrow. In 10 tissue samples, FIV DNA was only detected once out

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of multiple PCR reactions "(+)" indicating low proviral burden. Seven of the these 10 barely detectable FIV DNA-containing tissues were harvested prior to 6 days PI.

Table 3.1: VI versus PCR sensitivity for the detection of early FIV-C oral-nasal infection.

Cat#	Day PI	VI Retro LN	PCR Retro LN	VI Subm LN	PCR Subm LN	VI PBMC	PCR PBMC
3797	1	-	-	-	-	-	-
3791	2	-	(+)*	-	-	-	(+)*
3798	3	-	-	-	-	-	-
3792	4	-	-	-	-	-	-
3799	5	-	-	-	-	-	-
3793	6	-	+*	-	-	-	-
3802		-	-	-	-	-	-
3794	8	+	+	-	-	+	+
3803		+	+	+	+	+	+
3795	10	+	+	+	+	+	(+)
3804		+	+	-	-	-	+*
3790	Control	-	-	-	-	-	-
3796		-	-	-	-	-	-

VI: VI coculture; Retro LN: retropharyngeal lymph node; Subm LN: submandibular lymph node; PBMC: peripheral blood mononuclear cells; PI: post inoculation; [illegible]and +: PCR positive; –: PCR negative; +*: PCR+ not VI+; (+) PCR+ only once.

Detection of FIV-C by ISH after oral-nasal exposure to cell-free FIV-C

FIV-C RNA was detected in retropharyngeal lymph node as early as 2 day PI and in 7 of the 9 cats examined between days 1 and 6 PI. (Table 3.2) Overall, viral RNA was most frequently detected in retropharyngeal lymph node (i.e. 12 of 15 animals examined between days 1 and 10 PI). RNA-bearing cells were next most commonly detected in tonsil (8 of 9 animals) and then tracheobronchial lymph node (7 of 11 animals) between days 1 and 10 PI.

For all time points examined, ISH either correlated with or exceeded nested PCR (or VI) results in identifying rare virus-bearing cells. In 16 cases, ISH was positive for a given tissue when PCR was negative. (Table 3.2)

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FIV-C mRNA+ staining cells were primarily located in the cortical regions of lymph nodes and the parafollicular regions of tonsils. In lymph nodes, FIV+ cells most frequently appeared as individual positive cells or in clusters of 2 to 5+ positive cells within the germinal centers of follicles or less commonly in the interfollicular areas. (Fig. 3.1) In the tonsil, FIV+ cells were detected in the interfollicular areas as individual positive cells. However, on occasion, FIV+ cells were also present in the subepithelial tissue immediately adjacent to the crypt lymphoepithelial surface. (Fig. 3.1) In at least half of the cases, the contralateral lymphoid tissue was negative for FIV RNA-containing cells.

Table 3.2: Detection of FIV-C by nested DNA PCR and ISH between days 1 and 10 after oral-nasal exposure to cell-free virus.

Cat#	Day PI	Tonsil	Retropharyngeal LN	Submandibular LN	Gastric LN	Mesenteric LN	Tracheobronchial LN	Lung	Spleen	Thymus	Bone marrow	Popliteal LN	PBMC
LLk1	1	-	-	-	-	-	-	-	-	-	+	nd	+
3797	1	-	-	-	-	-	-	-	-	-	-	-	-
LLk2	2	-	-	-	-	-	■	-	-	-	-	nd	(+)
3791	2	-	■ (+)	-	-	-	-	-	-	-	-	-	(+)
LLk3	3	-	■	-	-	-	■	-	-	-	-	nd	-
3798	3	-	-	-	-	-	-	-	-	-	-	-	-
LLk4	4	(+)	■ (+)	-	-	-	-	-	-	-	+	nd	-
3792	4	■	■	-	-	-	-	-	(+)	-	-	-	-
LLk5	5	-	■	-	-	-	■	-	-	-	-	nd	(+)
3799	5	-	-	-	-	-	+	-	-	-	-	-	-
3793	6	-	■ +	■	■	-	-	-	-	-	-	-	-
3802	6	■	■	■	-	+	-	-	-	-	-	-	-
3855	7	■	■	■	■	-	■	-	-	-	-	-	-
3857	7	■	-	-	■	-	-	-	-	-	-	-	-
3794	8	■ +	■ +	-	-	+	■ +	-	+	-	+	+	+
3803	8	■ +	■ +	■ +	■ +	(+)	+	+	+	-	+	+	+
3795	10	■ +	■ +	■ +	■ +	+	■ +	-	+	+	+	+	(+)
3804	10	■ +	■ +	-	■	+	■	-	+	+	+	-	+
3790	Ctl	-	-	-	-	-	-	-	-	-	-	-	-
3796	Ctl	-	-	-	-	-	-	-	-	-	-	-	-

ISH: in situ hybridization; PI: post inoculation; LN: lymph node; Ctl: control; nd: PCR not done; +: PCR positive; –: PCR negative; (+): PCR+ only once; ■: ISH+; : ISH–; ❐: ISH not done.

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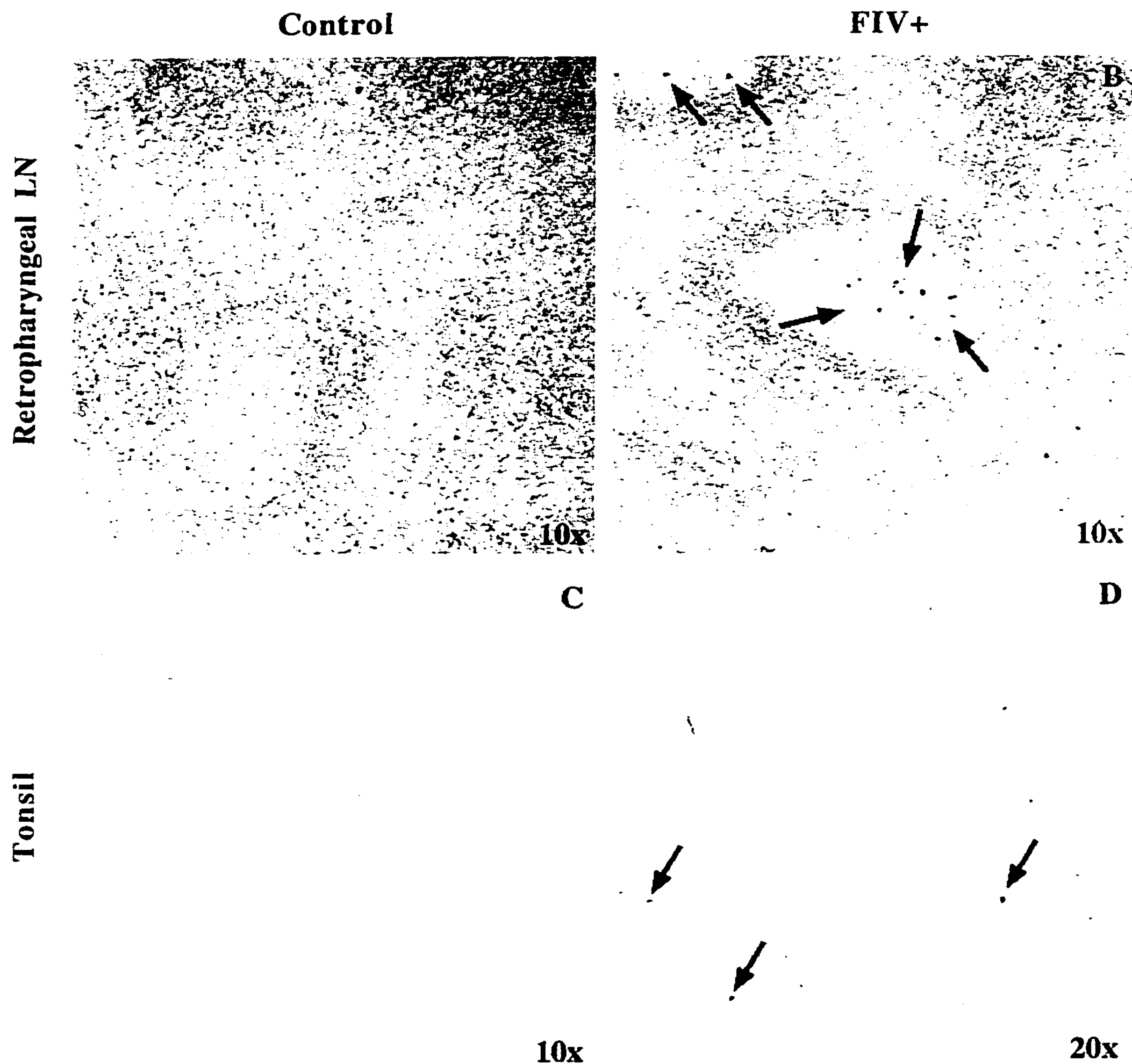

Figure 3.1: Early FIV-C ISH+ target cells (arrows) in the retropharyngeal lymph node from a day 10 PI cat (panel B) and tonsil from a day 6 PI cat (panel D) compared to controls (panels A & C).

FIV-C cell oral-nasal transmission: study outline

Eight 8 to 12 week old kittens were inoculated oral-nasally with PBMC harvested from a cat with acute FIV-C infection. The cats were sacrificed at intervals from 3 to 9 days PI, and mucosal, regional, and systemic lymphoid tissues were harvested. FIV DNA and viral mRNA were assessed in specific tissues by PCR and ISH, respectively. Table 3.3 summarizes PCR and ISH findings.

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Detection of provirus after oral-nasal exposure to FIV-C-infected cells

FIV-C was first detected by PCR in tracheobronchial lymph node, spleen, and bone marrow in 3 of 6 cats between days 3 and 7 PI. (Table 3.3) PBMC and tracheobronchial lymph node were the most frequently positive tissues in 3 of 4 cats between days 7 and 9 PI. By 9 days PI, FIV DNA was detected in local and/or regional lymphoid tissues and PBMC in each of 2 cats examined. In 6 of 10 PCR+ tissue samples, FIV DNA was detected once "(+)" out of multiple PCR reactions, indicating DNA levels at the margin of assay sensitivity

Detection of FIV by ISH after oral-nasal exposure to FIV-C-infected cells

FIV-C RNA was detected in the retropharyngeal lymph node in 5 of 6 cats between days 5 and 9 PI. (Table 3.3) Tonsil was virus positive by 9 days PI in each of 2 animals examined.

In one case PCR and ISH data correlated; tonsil from cat #3824 was positive for both assays. However, ISH identified rare FIV-C+ cells in the 6 other tissues which were PCR negative. (Table 3.3)

As in the cell-free infection study, FIV-C mRNA+ cells were localized to follicles and/or interfollicular areas adjacent to the subcapsular sinus of the retropharyngeal lymph node. (Fig. 3.2) Similarly, FIV+ cells were frequently observed as individual cells and/or clusters of 2 to 5+ cells in the mantle zones and germinal centers of tonsilar follicles. (Fig. 3.2) In the 2 cats examined, FIV+ cells were also identified in the subepithelium of the tonsil adjacent to the crypt lymphoepithelial surface.

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Table 3.3: Detection FIV-C by nested DNA PCR and ISH between days 3 and 9 after oral-nasal exposure to cell-associated virus.

Cat#	Day PI	Tonsil	Retropharyngeal LN	Submandibular LN	Gastric LN	Mesenteric LN	Tracheobronchial LN	Lung	Spleen	Thymus	Bone marrow	PBMC
3814	3	-	-	-	-	-	-	-	-	-	-	-
3815		-	-	-	-	-	-	-	-	-	+	-
3816	5	-	[ISH+] [illegible]	-	-	-	-	-	-	-	-	-
3817		-	-	-	-	-	-	-	-	-	-	-
3818	7	-	[ISH+] -	-	-	-	-	-	(+)	-	-	-
3819		-	[ISH+] [illegible]	-	-	-	+	-	-	-	-	-
3820	9	[ISH+] -	[ISH+] [illegible]	+	-	-	(+)	-	-	-	-	(+)
3824		[ISH+] (+)	[ISH+] -	-	+	(+)	-	-	-	-	-	(+)
FIV+ 3823		+	[ISH+] +	+	+	+	+	+	+	+	+	+

PI: post inoculation; LN: lymph node; nd: PCR not done; +: PCR positive; –: PCR negative; (+): PCR+ only once; ▨: ISH+; : ISH–; ❒: ISH not done.

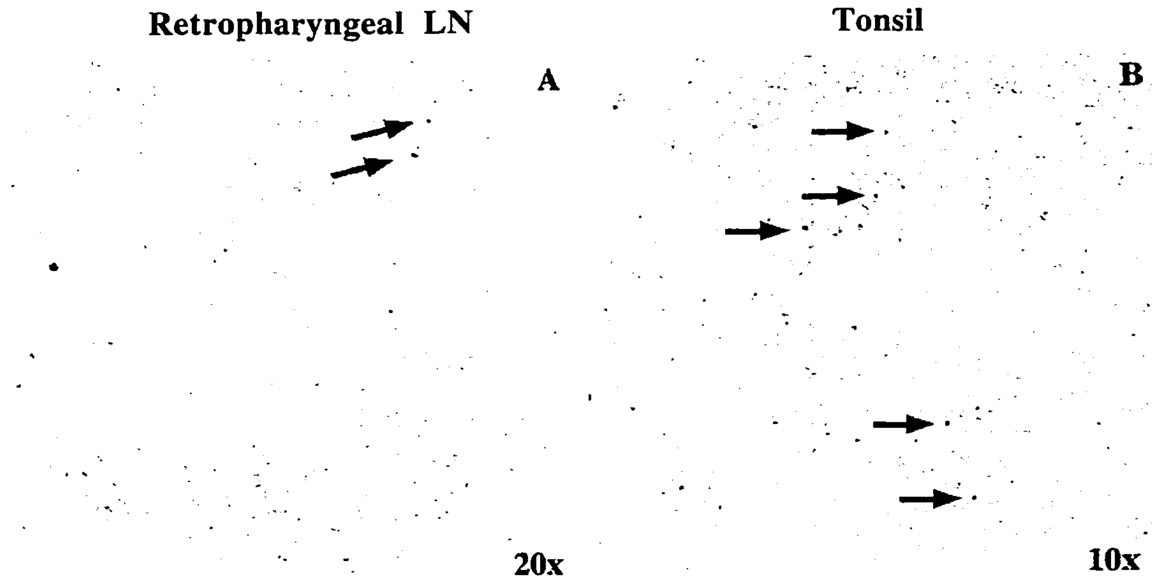

Figure 3.2: Early FIV-C ISH+ target cells (arrows) in the retropharyngeal lymph node (panel A) and tonsil (panel B) from a cat 9 days following oral-nasal exposure to PBMC from an FIV-C-infected donor cat.

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Summary of target tissue infection frequency following oral-nasal FIV exposure

The frequency of target tissue infection following oral-nasal FIV-C exposure is summarized for both cell-free and cell-associated infection studies. (Table 3.4) In the majority of the animals (31%) virus was detectable in only lymph nodes draining the inoculation site. In another group of animals (n = 7; 27%), virus was identified in regional lymph nodes and primary lymphoid organs (spleen or bone marrow) or PBMC . Thus, in 58% of oral-nasally exposed cats, infection was detected in regional lymph nodes prior to widespread virus dissemination to distant lymphoid tissues. In 15% of cats exposed oral-nasally to cell-free FIV-C, systemic infection was evident at between 8 or 10 days PI.

Table 3.4: Summary of FIV-C target tissue scenarios following oral-nasal exposure.

Target Tissue(s)	# of Cats	% of Cats
Regional LN(s) only	8	31
Regional LN(s) + PBMC	4	15
Regional LN(s) + 1° Lymphoid organ(s)	3	11.5
1° Lymphoid organ(s) only	1	4
1° Lymphoid organ(s) + PBMC	1	4
1° Lymphoid organ(s) + PBMC + Regional LN(s)	1	4
Systemic infection	4	15

LN: lymph node; regional LN: LN(s) draining oral/nasal mucosal tissues; PBMC: peripheral blood mononuclear cells; 1° lymphoid organ: spleen or bone marrow.

FIV vaginal transmission studies: study outline

Fifteen 8 to 12 week old female kittens were inoculated vaginally with either cell-free FIV-B (n = 5), cell-free FIV-C (n = 7), or PBMC harvested from a cat with acute FIV-C infection (n = 3), and sacrificed at intervals between 1 to 12 days PI. FIV DNA and viral mRNA were assessed by PCR and ISH, respectively. Table 3.5 summarizes PCR and ISH findings.

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Detection of provirus after vaginal exposure to FIV

FIV-B was first detected in vaginal mucosa and spleen from 2 of 4 cats between days 1 and 2 PI. (Table 3.5) By day 7 PI, both vaginal mucosa, the draining internal iliac lymph node, and PBMC had detectable viral DNA; however, FIV-B DNA was not present in other systemic lymphoid tissues.

As early as 3 days PI, FIV-C DNA was detected in the draining internal iliac lymph node from 1 cat, and in the internal iliac lymph nodes from 2 of 6 cats between days 4 and 12 PI; however, viral DNA was not detectable in vaginal tissues. (Table 3.5) In 2 of 4 PCR+ tissue samples identified between days 3 and 8 PI, FIV DNA was detected once "(+)" out of multiple PCR reactions. Widespread virus dissemination occurred in a cat 12 days PI.

FIV-C DNA was present in the internal iliac lymph node 4 days following vaginal exposure to PBMC from an acutely FIV-infected cat. (Table 3.5) Similarly, virus DNA was detected in the internal iliac lymph node and less frequently in PBMC from a cat 8 days PI.

Detection of FIV by ISH after vaginal exposure to FIV

FIV-B RNA was first detected in the vaginal mucosa as early as 1 and 2 days PI. Virus-bearing cells were seen in the internal iliac lymph node at day 7 PI. In contrast, FIV-C RNA was detected in an internal iliac lymph node as early as 3 days PI, and in internal iliac lymph nodes from 9 of 10 cats between 4 and 12 days PI; whereas, virus-bearing cells were identified in vaginal tissue from 3 animals on only days 6, 8, and 12 PI.

In all cases, ISH was positive when PCR was positive. However, in 8 cases, ISH revealed rare virus positive cells when PCR of those tissue was negative.

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The distribution of FIV-B-2542 paralleled that of FIV-C in that mRNA+ cells were primarily located in the cortex of internal iliac lymph nodes. Most frequently, the virus-bearing cells were detected in follicles and/or the interfollicular regions adjacent to the subcapsular sinus. (Fig. 3.3) In vaginal mucosal tissue, FIV+ cells were rare and most often found in the submucosa. On occasion, virus positive cells could be seen within the mucosal epithelium or in the mucosal associated lymphoid tissue (MALT). (Fig. 3.3) Most of these FIV+ cells had stellate morphology resembling that of dendritic cells.

Table 3.5: Detection of FIV-B/-C by nested DNA PCR and ISH between days 1 and 12 after vaginal exposure to cell-free or cell-associated virus.

Cat#	Inoculum: FIV Strain & Cell-free or Cell	Day PI	Internal Iliac LN	Sacral LN	Mesenteric LN	Spleen	Thymus	Bone marrow	Vaginal mucosa	PBMC
3593	**B** cell-free	1	-	-	nd	-	-	-	+ (ISH+)	-
3594	**B** cell-free		-	-	nd	-	-	-	-	-
3598	**B** cell-free	2	-	-	nd	+	-	-	- (ISH+)	-
3599	**B** cell-free		-	-	nd	-	-	-	-	-
3604	**B** cell-free	7	+ (ISH+)	-	nd	-	-	-	+ (ISH+)	+
3851	**C** cell-free	3	- (ISH+)	-	-	-	-	-	-	-
3853	**C** cell-free	4	- (ISH+)	-	-	-	-	-	-	-
3623	**C** cell-free		-	-	nd	-	-	-	-	-
3858	**C** cell-free	5	+ (ISH+)	(+)	-	-	-	-	-	-
3859	**C** cell-free	6	- (ISH+)	-	-	-	-	-	-	-
3860	**C** cell-free	8	- (ISH+)	-	(+)	-	-	-	-	+
3651	**C** cell-free	12	+ (ISH+)	+	nd	+	+	-	- (ISH+)	+
3825	**C** cell	4	+ (ISH+)	-	-	-	-	-	-	-
3826	**C** cell	6	- (ISH+)	-	-	-	-	-	- (ISH+)	-
3827	**C** cell	8	+ (ISH+)	-	-	-	-	-	- (ISH+)	(+)

ISH: in situ hybridization; PI: post inoculation; LN: lymph node; nd: not done; +: PCR positive; –: PCR negative; (+): PCR+ only once; ▩: ISH+; : ISH–; ☐: ISH not done.

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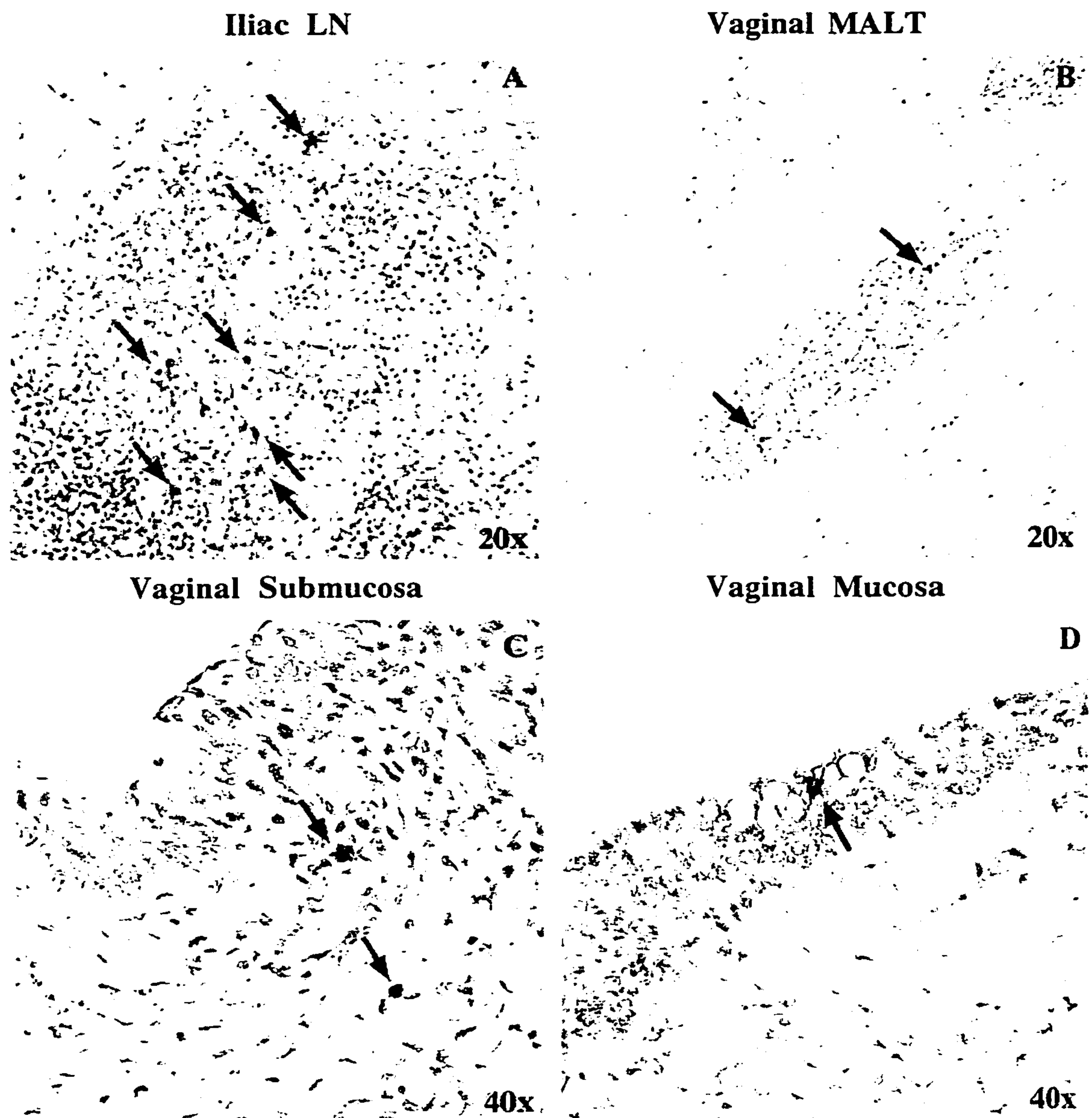

Figure 3.3: Early FIV-C ISH+ target cells (arrows) in the iliac lymph node (panel A) and vaginal mucosal tissue (panels, B: MALT, C: submucosa, D: muocsa) from a cat 12 days following vaginal exposure to cell-free FIV-C.

Summary of target tissue infection frequency following vaginal FIV exposure

The frequency of target tissue infection following vaginal FIV exposure is summarized for cell-free and cell-associated studies. (Table 3.6) The majority of the animals (33%) had detectable virus in "only" regional lymph nodes. In 1 animal (7%), virus was detected "only" in vaginal tissue. Only one of vaginal FIV inoculates developed a systemic infection

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between 1 and 12 days PI. Whereas, 4 cats (27%) had FIV present in the vaginal mucosa and in either lymphoid tissue or PBMC. Thus, in 40% of cats exposed to FIV via the vaginal mucosa, there was evidence of virus detectable in mucosa or regional lymph nodes prior to virus dissemination and amplification in systemic lymphoid tissues.

Table 3.6: Summary of FIV target tissue scenarios following vaginal exposure.

Target Tissue(s)	# of Cats	% of Cats
Regional LN(s) only	5	33
Vaginal mucosa only	1	7
Vaginal mucosa + Regional LN + PBMC	2	13
Vaginal mucosa + LN + PBMC	1	7
Vaginal mucosa + Spleen	1	7
Systemic infection	1	7

LN: lymph node; regional LN: LN(s) draining vaginal mucosal tissues; PBMC: peripheral blood mononuclear cells.

DISCUSSION

It has been estimated that more than 30 million people are infected with HIV-1 worldwide and there are approximately 16,000 new cases daily. [93,94] Sexual transmission at the genital epithelium is the most common mode of HIV-1 infection. [95] Although infection is transmitted most frequently across the genital mucosa, numerous reports suggest that infection can also be transmitted across the oral mucosa as a result of genital-oral sex. [96-99] Little is known concerning how HIV crosses the mucosal barrier, which cells are its initial targets, and how virus dissemination ensues. For obvious reasons, such issues are difficult to study in humans. Thus, an animal model in which a naturally occurring species-homologous lentivirus analogous to HIV could be used to address these questions would be valuable.

In the current study, mucosal, regional, and systemic lymphoid tissues of cats exposed either oral-nasally or vaginally to cell-free or cell-associated FIV and were examined by PCR, VI, and RNA ISH at 11 specific time points during the first 12 post inoculation (PI) in attempt to track early viral localization and progression. Several target tissue scenarios resulted

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following oral-nasal or vaginal exposure. (Tables 3.4. and 3.6) In 29 out of 43 (67%) cats studied, viral-infected cells were demonstrable in mucosal tissue and/or regional lymph node(s) within 12 days following oral FIV exposure. In 13 of these cats (30%) virus was detectable in tissues within the first 5 days PI. These data indicate that virus replication occurs early and frequently in mucosal and regional lymphoid tissues following mucosal lentiviral exposure. After vaginal mucosal exposure, FIV-bearing cells were identified within mucosal epithelium (Fig. 3.3 D), lamina propria (Fig. 3.3 C), and MALT (Fig. 3.3 B) of the vagina by 1 to 2 days PI. The morphology of the mucosal-associated cells was either stellate (in mucosal epithelium, lamina propria, MALT), small round (in MALT), or oval (in lamina propria) consistent with the phenotype of dendritic cells, lymphocytes, or macrophages, respectively. However, dual ISH and immunohistochemistry will be needed to definitively identify the phenotype of these early FIV-bearing cells. All aforementioned cell phenotypes have been identified as potential HIV targets in the human female reproductive tract. [100]

Acute intravaginal SIV infection in rhesus macaques provides some additional insights and parallels to the present studies with FIV regarding the sequence of cellular events that occur in the earliest stages of infection. [52,53] The first recognized cellular targets of SIV were described as having dendritic-like morphology and were located in the lamina propria subjacent to the cervicovaginal epithelium. It was then speculated that these cells may fuse with CD4 lymphocytes and spread the infection to deeper tissues. Within 2 days after infection, SIV was identified in the draining internal iliac lymph nodes, and by 5 days systemic dissemination was detected by PBMC coculture. [52] In HIV-1 infection, the time from mucosal exposure to initial viremia is variable ranging from 4 to 11 days paralleling observations in the FIV and SIV systems. [101] However, within 24 hours following vaginal inoculation of mice with murine dendritic cells pulsed with heat-inactivated HIV-1 *in vitro*, the virus was detected in the iliac and sacral draining lymph nodes. These data suggested that dendritic cells could route HIV to the lymph nodes following mucosal exposure without being productively infected and without utilizing the human CD4 molecule. [102] Another study demonstrated that dendritic cells could capture HIV via CD4- and coreceptor-

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independent mechanisms. [103] Thus, dendritic cells could function as Trojan horses transporting virus from mucosa to local/regional lymphoid tissues where numerous susceptible cells reside.

Both the SIV and mouse studies have suggested a role for dendritic cells in the initiation of a primary lentiviral infection [52,53,102]. However, these studies did not demonstrate virus in the epithelium nor how it crossed the epithelium. In the SIV study, the virus-bearing cells were not formally identified as dendritic cells. Similarly, in the mouse study, viral sequences were detected via RT-PCR in the draining lymph nodes, but these sequences were not shown to be associated with a specific cell type. SIV was detected not only in the draining lymph node of all the animals studied, but was also found in the spleens and axillary lymph nodes of all the animals. This pattern of virus distribution is more suggestive of systemic viral dissemination following vaginal SIV exposure rather than initial viral spread to the regional draining lymph nodes. Obviously more studies need to be done to further clarify and validate the former findings.

In cats inoculated oral-nasally with FIV, virus-infected cells were first detected in the tonsils at 4 to 6 days PI. The FIV+ cells were located subjacent to the crypt lymphoepithelium (Fig. 3.1 D), in the parafollicular regions (Fig. 3.2 B) and within germinal centers. Similarly, in histocultures of human tonsil infected with HIV-1, viral particle production started between 5 to 6 days after infection, increased through day 8, and plateaued at day 13. [104] Several studies have demonstrated active replication of HIV-1 at the lymphoepithelial surface of the tonsil and adenoids. Most of the cells were small, multinucleated, and double labeled for HIV-1 RNA and S100, a dendritic cell marker. [105,106] In buccal biopsies from HIV-infected individuals, provirus was identified in lymphocytes, Langerhans cells, and epithelial cells. [107] Evidence has also been reported that human tonsilar stromal cells are infectable by HIV-1, but do not shed virus. [108] Thus, the detection, albeit rare, of FIV-positive cells beneath tonsilar lymphoepithelium after oral exposure to virus is consistent with findings in HIV infection and could suggest tonsilar mucosa as a portal of entry

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In the current study, virus-bearing cells were detected in regional draining lymph nodes as early as 2 days following oral-nasal exposure to cell-free FIV-C. The FIV+ cells were most often located in the parafollicular regions and within germinal centers (Fig. 3.1 B). In contrast, we did not detect virus-bearing cells in regional draining lymph nodes of cats inoculated with PBMC from an FIV-C-infected donor until 7 days after exposure. These findings suggest that cell-free virus is more efficient at crossing oral/nasal/gastrointestinal mucosa than is cell-associated virus. Studies of SIV infection in neonatal and adult rhesus macaques demonstrate that oral exposure to relatively small amounts of cell-free virus results in infection.[109,110] It seems more likely that the cell-associated virus inoculum constituted a lower effective virus challenge than did the cell-free inoculum. It seems plausible that inoculation of more FIV-infected cells (> 4 $\times 10^5$ infectious PBMC) would have resulted in greater chance for transmission. To achieve oral transmission of FIV Moench *et al.*[12] applied a second oral inoculation using 10-fold more virus infected cells (2×10^7 *in vitro* infected cells) than the first inoculation. We inoculated the cats in our oral-nasal study with a total of 2×10^7 PBMC; however, our inoculum was isolated from a cat acutely infected with FIV-C and was determined by PCR to contain 4×10^5 infectious PBMC. In contrast, cell-mediated transmission with HIV to 3 chimpanzees was reported following application of only a few hundred cells to an intact cervix.[111] Similarly, in the present study cats inoculated vaginally with ~1/2 the number of FIV+ PBMC inoculated via the oral-nasal route developed infection in the regional lymph nodes by 4 days PI - - 3 days earlier than that achieved oral-nasally. It is possible that cell-free and cell-associated virus may utilize different pathways to cross different mucosal barriers. Also, cell-free virus may be more readily transmitted via the oral-nasal route than cell-associated virus.

We did not detect evidence of epithelial cell infection after FIV exposure via the oral-nasal or vaginal mucosae. However, FIV-bearing cells possessing non-epithelial morphology were visualized by ISH within the vaginal epithelium. (Fig. 3.3 D) An *in vitro* study utilizing cervix-derived epithelial cells demonstrated that transepithelial passage of cell-free HIV-1 occurred within 1 hour following apical introduction. The virus that traversed the cervical

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epithelium remained infectious; however, there was no evidence that the cervical epithelium became HIV-infected. The ability to cross the cervical epithelial barrier could correlate with the absence of tight junctions. [112] In another in vitro study, cervix-derived epithelial monolayers were productively infected following culture with monocytes infected with primary macrophages–tropic HIV isolates. The monocytes migrated between the epithelial cells secreting virus along their path. [113] In a murine *in vivo* study, mouse peritoneal lymphoid cells from one strain were inoculated into the vaginal vault of another mouse strain; labeled donor cells were visualized in the vaginal epithelium, submucosa, and iliac lymph node by 24 hours PI. [114] In a similar study, double-stained activated lymphocytes and macrophages inoculated into the vaginal vaults of mice traversed the vaginal epithelium within 4 hours and could be identified in the submucosal connective tissue and in the iliac lymph nodes. It was speculated that such migration may be involved in sexual transmission of HIV. [115] Our data supports both pathways of transepithelial virus, since vaginal FIV transmission was achieved via both cell-free and cell-associated inocula.

In a few orally exposed cats virus was detected in gastric and/or mesenteric lymph nodes prior to systemic infection, potentially suggesting that FIV gained entrance via traversing the gastrointestinal mucosae. One possible mechanism by which FIV could cross the intestinal mucosa following oral inoculation is via epithelial transcytosis. Bomsel [36] demonstrated that as early as 30 minutes after apical contact, primary virus isolates generated from primary peripheral blood leukocytes from HIV-infected patients can cross an epithelial cell line barrier using transcytosis involving both gp120 and the galactosyl-ceramide receptor, but not CD4. However, cell-free HIV produced in liquid culture without cell-cell contact was neither internalized [37] nor transcytosed. [36] In contrast, a recent *in vitro* study utilizing P6 HeLa cell line, HIV-1 infection and trancytosis was demonstrated; CD4 and galactosylceramide were not involved in theses processes. Instead, a molecule(s) identified by the monoclonal antibody C57a9-9 was associated with both processes. [116] Several other groups have demonstrated that HIV can infect epithelial cell lines in the absence of the CD4 molecule. [37,38,117,118] Yahi *et al.* [118] reported that HIV used galactosyl ceramide for CD4-independent entry into enterocytes.

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This group later found that infection was more effective if HIV-infected T cells were cocultured with the epithelial target cell line. [119] In contrast to the epithelial cell line studies, tissue cultures of fetal intestine revealed productive infection of only mononuclear lamina propria cells and not epithelial cells. [120] Similarly, most studies examining intestinal biopsies from HIV-infected patients revealed HIV-infected cells predominantly in the lamina propria and only rarely if at all in the epithelial layer. [40,121-124] These findings are consistent with our findings suggesting that the phenotype(s) of initial FIV-bearing cells is/are likely to be non-epithelial than epithelial.

RNA ISH was more sensitive in identifying virus-infected cells and tissues than was DNA PCR. In contrast, Kotler et al. [122] found a lack of correlation between ISH and DNA PCR such that PCR detected HIV more frequently. This was explained by PCR detects latently infected cells while RNA ISH detects productively infected cells. The difference between our results and those of Kotler may reflect the stage of disease examined (acute versus asymptomatic) or sensitivity of the assays employed in each case. In addition, Kotler demonstrated p24 staining in occasional epithelial cells via immunohistochemistry (IHC), but HIV RNA was not detected in the epithelium using ISH. The different results could certainly be explained by epithelial cell absorption of HIV antigens from the intestinal lumen or the interstitial compartment without direct virus production. The specific binding of HIV gp120 to gut epithelial cells has been shown *in vitro* [125] and epithelial cells can absorb intact molecules and present antigens in association with MHC II. [126] Electron microscopic studies have shown internalization of viral particles during *in vitro* infection of a small intestinal epithelial cell line. [37] Fackler et al. described an increase in HIV-1 p24 but not proviral load in the intestinal mucosa compared to peripheral blood in HIV-infected patients. [127] Enhanced local virus production was the most likely explanation for the high mucosal p24 concentration. In fact, increased mucosal HIV RNA expression has been demonstrated in the esophagus versus blood. [128] Taken together, these data suggest that lentiviral expression can vary between tissue compartments and that relatively few proviral-containing cells can produce abundant viral RNA/protein.

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In summary, studies of a mucosal FIV-C infection model have demonstrated virus-bearing cells in mucosal tissues and regional draining lymph nodes as early as 1 to 2 days following oral or vaginal mucosal exposure. The early FIV mucosal target cells had morphology consistent with dendritic cells; whereas, virus-bearing cells in the lymphoid tissues had morphology more heterogeneous and potentially consistent with dendritic cells, lymphocytes, or macrophages. Even though virus positive cells were visualized in the vaginal epithelium and subjacent to the tonsil lymphoepithelium, epithelial cell infection per se was not observed. Additional concurrent viral RNA and cell phenotype labeling experiments will be required to more definitively identify these early FIV target cells. The present work establishes a stage for future studies which can focus in greater detail on the initial virus-target cell events following exposure to cell-free or cell-associated FIV. Such events include: mucosal epithelial cell receptor involvement, virus and cell transepithelial migration, virus transcytosis, and the role of dendritic cells as virus targets or virus transporters.

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