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DISSERTATION

FELINE IMMUNODEFICIENCY VIRUS MODEL OF MATERNAL-FETAL HIV-1 TRANSMISSION

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Colorado State University

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ARLIN B. ROGERS ENTITLED FELINE IMMUNODEFICIENCY VIRUS MODEL OF MATERNAL-FETAL HIV-1 TRANSMISSION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

FELINE IMMUNODEFICIENCY VIRUS MODEL OF MATERNAL-FETAL HIV-1 TRANSMISSION

Maternal-fetal human immunodeficiency virus type-1 (HIV-1) transmission contributes to the AIDS pandemic. Chemotherapy reduces maternal-fetal HIV-1 transmission, but its impact has been limited to industrialized countries. New intervention approaches require improved understanding of the determinants affecting fetal infection outcomes. We used the feline immunodeficiency virus (FIV) model of maternal-fetal HIV-1 transmission to elucidate basic mechanisms of intrauterine lentivirus infection which are not amenable to study in humans .

Fetuses, placentas, and blood from cats infected with FIV-B-2542 were assayed at defined gestational intervals. Most fetal infections occurred in the third trimester. Fetal tissues frequently targeted by FIV were blood mononuclear cells, brain, and thymus; bone marrow, spleen, and liver were less frequently targeted. Surrogate maternal hematologic and viral load markers did not vary with gestational stage. Therefore, fetal and/or placental factors may determine transmission timing. Infection prevalence in term fetuses was equivalent to that seen in vaginally delivered offspring. Thus, most vertical FIV-B-2542 transmission occurs in utero.

Tissues from juvenile cats acutely infected with FIV-B-2542 and FIV-C-Pgmr were used to develop improved immunohistochemistry and DNA in situ hybridization assays. Virus detection was coupled with phenotype labeling to identify specific cells targeted by FIV. Most identified cells containing FIV were T lymphocytes. Macrophages contained virus less commonly. Dendritic cells comprised the smallest percentage of identified FIV^+ cells. Bone marrow contained many unidentified FIV^+ cells, probably leukocyte progenitors.

Vertical FIV transmission studies were extended to include clades A and C. FIV-A-Petaluma and FIV-C-Pgmr were both transmitted to >60% of term fetuses. FIV-C-Pgmr was detected in all placentas and sampled fetal tissues. FIV-A-Petaluma, by contrast, exhibited little tropism for placenta and was never detected in fetal brain or liver. FIV provirus, but not protein, was detected in placental and fetal tissues in situ, suggesting little active virus replication occurs in these tissues. Fetal tissue FIV sequestration was common. By extension, the blood-based assays used to define the timing of perinatal HIV-1 infection likely underestimate transplacental transmission incidence. Further study of maternal-fetal FIV transmission will shed additional light on the mechanisms of intrauterine HIV-1 transmission.

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DEDICATION

To Kasey.

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INTRODUCTION

Mother-to-child HIV-1 transmission

In the year 2000, 5.3 million new people were infected with human immunodeficiency virus (HIV), raising the total to 36.1 million people living with HIV/AIDS worldwide [1] . About half of adults newly infected with HIV in 2000 were women. Children under the age of 15 years comprised more than 10% of new infections. Over 1.4 million children currently are living with HIV/AIDS. Tragically, 500,000 children died of AIDS in 2000, meaning that one in four children with HIV dies each year [1] . The number of deaths per year of adults with HIV is less than one in ten. Since the beginning of the epidemic 4.3 million children, equal in number to the total population of Colorado, have died of HIV/AIDS.

Nearly all pediatric AIDS cases result from mother-to-child HIV type-1 (HIV-1) transmission. Vertical transmission is a major contributor to the number of new HIV-1 infections and AIDS-related deaths globally. While HIV-1 can be transmitted during parturition and via breast feeding [2, 3] , *in utero* transmission may account for up to half of all infections of newborns [4] . HIV-1 has been isolated from aborted fetal tissues and placentas, and from cord blood of full-term babies [5-8] .

More than 80% of children infected with HIV-1 live in sub-Saharan Africa. Because 55% of adults with HIV-1 in sub-Saharan Africa are women, the number of newly infected children will continue to mount [1] . The high rate of infection of sub-

Saharan African women is compounded by the fact that many endemic HIV-1 strains have a known propensity for vertical transmission [9] . An alarming rise in HIV-1 vertical transmission parallels overall population infection increases in South Africa [10] . While sub-Saharan Africa justifiably receives the world's attention, vertical HIV-1 transmission silently afflicts other regions including Latin America and the Caribbean [11] , and southeast Asia [12] . Even in the United States, the Centers for Disease Control and Prevention estimates that nearly 20,000 children were living with maternally-acquired HIV-1 at the end of 1997 [13] .

Multiple factors influence the risk of vertical HIV transmission. Maternal viral load appears to be the best predictor of transmission risk [14, 15] . Women with advancing HIV disease are also more likely to transmit virus to their children [16, 17] . One marker of advancing HIV-1 infection is high viral genotypic heterogeneity resulting from replicative mutations. Women with high viral heterogeneity more often deliver infected infants than do women with low viral diversity [18] . Certain HIV-1 clades are also associated with higher rates of vertical transmission than are others [19] . Fetal/neonatal immunity may also influence the outcome of HIV exposure. One study demonstrated that 25% of uninfected children born to HIV-1-infected mothers harbored cytolytic T-cell activity against HIV [20] . Another study identified strong β-chemokine responses in response to HIV antigens in cells from HIV-negative infants, but not in HIV-positive infants, of HIV+ women [21] . Thus no single factor determines whether or not a given infant will be infected with HIV-1.

Intervention of mother-to-child HIV-1 transmission

In utero HIV exposure cannot be prevented through behavioral modification, physical barriers, or surgical intervention. Antiviral chemotherapy is the only tool presently available to reduce the risk of fetal infection. In spite of intensive research, vaccines and immunotherapies for reducing fetal infections appear years away from clinical application [4] . The gold standard of mother-to-child HIV intervention is the AIDS Clinical Trial Group 076 protocol (ACTG 076). ACTG 076 prescribes institution of zidovudine orally in pregnant women at mid-gestation, intravenously during parturition, with continuation in the infant orally for a total of 26 weeks of therapy [22, 23] . This regimen reduces rates of mother-to-child HIV-1 transmission in women who do not breast-feed from about 3-in-12 births to 1-in-12, a reduction of two-thirds [22] . Unfortunately, this regimen is prohibitively expensive for most developing countries. Even in industrialized countries, efforts to increase public awareness about the benefits of chemoprophylaxis do not always lead women to seek access to anti-HIV drugs [24] . Moreover, chemotherapeutic intervention of mother-to-child HIV-1 transmission is not without its own risks. For example, reverse transcriptase inhibitors such as zidovudine may be toxic to fetuses, especially to cellular mitochondria [25-30] , and also exhibit tumorigenic potential [31] . Protease inhibitors likewise demonstrate potential teratogenicity in animal models [32] .

In order to investigate chemoprophylaxis regimens more practical for application in developing countries, a number of trials of short-course therapies of zidovudine and other drugs have been performed. These protocols have ranged in duration from a few weeks to a few days of treatment for mother and/or infant. Although none of the short-

course regimens has resulted in the two-thirds reduction in infant infections seen with ACTG 076, many have led to reductions in infant infections by about one-quarter to one-half [23, 33-35] . Institution of maternal therapy even a few hours before birth can result in therapeutic drug levels in the fetus. AZT levels in the fetal compartment are equivalent to those of the mother within 60—90 minutes following maternal administration [36] . A single dose of nevirapine given to the mother just prior to birth, and another given to the neonate 2 days later, can reduce perinatal infections by about a half, at a cost of around US$4 per intervention [37] .

Regrettably, continuing virus transmission during the breast-feeding period leads to infection of infants who may have been protected from the virus perinatally [38] . Because formula supplies in developing countries are frequently unsafe, and because of stigmatization and other cultural norms, cessation of breast-feeding by HIV+ mothers is not a widely viable option in sub-Saharan Africa. This is especially unfortunate in light of the findings of a recent study which showed that up to 44% of infant infections might be prevented through formula feeding [39] .

Ineffective intervention strategies

Just as important as learning what might be effective in preventing vertical HIV-1 transmission, is knowing what is not effective. For example, studies agree that maternal antibody titers and neutralizing antibody status play little or no role in determining fetal infection outcome [40] . Passive immunoglobulin therapy has been uniformly disappointing [41, 42] . Microbicidal vaginal cleansing has been advocated as an inexpensive strategy for reducing perinatal infections in resource-poor countries, but has shown little or no effectiveness [43] . Nutrient deficiencies, particularly vitamin A

deficiency, have been anecdotally associated with increased rates of vertical HIV-1 transmission [44] . However, carefully controlled randomized studies have failed to demonstrate significant benefit of vitamin supplementation for prevention of infant infections in regions of endemic malnutrition [45, 46] . Thus, simple remedies such as vaginal cleansing and vitamin supplementation are unlikely to have a great impact on reducing vertical HIV-1 transmission in sub-Saharan Africa and other developing regions.

Challenges in further reducing vertical HIV-1 transmission and the need for animal models

One of the great challenges in preventing perinatal HIV-1 transmission is encouraging women to themselves be tested for the virus. Many women are unable or unwilling to be tested, or are unaware that they might be at risk. Sadly, many women only learn of their own HIV-positive status by consenting to receive results of tests performed on their infants [47] . Not only do HIV^+ women risk transmitting the virus to their infants, but HIV infection itself over prolonged periods appears to reduce fertility [48] . For these reasons, the HIV pandemic has reversed most of the increases over the previous 50 years in average lifespan in sub-Saharan Africa [1] . Perinatal HIV-1 transmission rates are influenced by numerous variables. These include increasing rates of infection of women of child-bearing age, identification of pregnant HIV+ women, emergence of viral strains with a propensity for vertical transmission, supplies of drugs and patient compliance, access to appropriate medical care, breast-feeding, and socio-cultural customs. Therefore, there are no simple solutions for further reductions in the global incidence of mother-to-child HIV-1 transmission.

Little is known about the early events surrounding maternal-fetal HIV-1 transmission. Hypotheses about transplacental HIV transmission are derived largely from anecdotal and in vitro observations. Primary cultures of placental and fetal macrophages can be infected with HIV-1 in vitro [49] . Likewise, primary cultures of fetal-derived syncytiotrophoblast support HIV-1 replication in vitro [50, 51] . Reports on fetal HIV-1 timing and cellular tropisms are sparse and contradictory [52, 53] . New avenues must be explored to identify methods for preventing infant infections in environments where current chemoprophylaxis regimens are impractical. In order to identify new targets for intervention, we need a better understanding of the basic interactions between virus, mother, placenta, and fetus. Because mechanistic studies cannot be performed in humans, elucidation of the in vivo pathogenesis of intrauterine lentiviral transmission requires animal models.

Animal models of maternal-fetal HIV-1 transmission

There are two mammalian lentiviruses which induce AIDS-like disease in their hosts: simian immunodeficiency virus (SIV) infection of Asian-origin non-human primates, and feline immunodeficiency virus (FIV) infection of domestic and wild felids. SIV is most like HIV in terms of genomic organization and cellular receptor usage. For these reasons SIV (or chimeric simian/human immunodeficiency virus [SHIV]) is an excellent animal model of AIDS. However, because of the high cost of SIV/primate research, relatively small numbers of animals may be devoted to any given study. Moreover, non-human primates pose a zoonotic risk to their handlers, and certain primate species are endangered. But the biggest drawback of the SIV model in regards to vertical transmission modeling is the extremely low prevalence of fetal infection [54] . Maternal-

fetal SIV transmission is rare, even though the virus readily infects placental cells in primary in vitro culture systems [55, 56] . Most well documented cases of vertical SIV transmission are attributable to the breast-feeding route [57] . For these reasons, little or no research is currently being performed to evaluate naturally occurring transmission of SIV from mother to fetus.

The FIV model

Feline immunodeficiency virus (FIV) induces a disease syndrome in cats virtually identical to that caused by HIV-1 in humans [58-60] . The acute phase is characterized by a burst of viral replication, flu-like illness, lymphadenomegaly, and an early progressive decline in CD4+ T lymphocytes [61, 62] . The acute phase is followed by a chronic asymptomatic or latent phase featuring viral down-regulation despite a continued decline in CD4+ T cells. The terminal phase of FIV disease, equivalent to human AIDS, is characterized by immunologic decompensation, wasting, hematologic abnormalities, upsurge in plasma viremia, increased incidence of cancers, and clinical immunodeficiency with opportunistic infections [59, 60, 63] . Other clinical features of FIV infection may include encephalopathy (similar to AIDS-dementia complex), and lymphadenopathies which are initially hyperplastic and later depletive in nature [60, 63-66] . Thus, feline FIV disease mirrors all the major hallmarks of human HIV-1 infection.

Vertical FIV transmission

Early published reports suggested that vertical transmission of FIV was rare, occurring only in those female cats (queens) infected acutely during pregnancy [60, 67-70] . During collection of North American field isolates of FIV, our laboratory identified

highly virulent isolates and captured them into specific pathogen-free (SPF) cats [71]. In contrast to the majority of known laboratory virus isolates at the time, which belonged to FIV *env* clade A, some of these newly captured isolates represented clade B and C subtypes [72]. Employing a semi-quantitative competitive RNA polymerase chain reaction (PCR) assay analagous to an early diagnostic test for HIV [73], the clade B and C FIV isolates were shown to reach 10^7 or more viral RNA copies/mL plasma during acute infection. These isolates were associated with rapid disease progression and fatal immunodeficiency [71, 74]. Two isolates, FIV-AB-2771 and FIV-B-2542, were selected for subsequent vertical transmission studies.

The FIV isolates described above were efficiently transmitted from mother-to-offspring *in utero*, through breast-feeding, and possibly during parturition [75, 76]. The rate of virus transmission to offspring was equivalent in both acutely and chronically infected maternal cohorts (47% and 51%, respectively) [75, 76]. In the studies cited above, one group of cesarean-derived kittens from chronically infected queens exhibited a transplacental transmission prevalence of 95% [76]. In this cohort of queens, CD4+ T cell counts were significantly lower (200 cells/μl) than in two otherwise similar cohorts which delivered kittens vaginally (597 and 748 cells/μl, respectively; $p < 0.001$) [76]. One difference between the groups was that the duration of infection in the queens delivering by cesarean section was longer than that in the other cohorts. The high rate of *in utero* FIV transmission in the cesarean-derived kittens was attributed to advancing FIV disease in the queens rather than mode of delivery. Although not determined in the cited studies, heavy maternal viral burdens may have played a role in the high rate of vertical FIV transmission in queens with advancing disease.

In summary, certain FIV isolates are transmitted from mother to fetus with high frequency. Cats are less expensive to purchase and maintain than are non-human primates, and cats have more offspring per reproductive cycle, allowing for greater statistical power at a lower cost than similar studies in non-human primates. Thus, FIV may be the best model currently available for exploring the basic pathogenesis of maternal-fetal HIV-1 transmission.

Vaginal lentivirus infection

Because most HIV-1 isolates transmitted to fetuses were originally acquired by the mother through heterosexual activity, a brief review of vaginal lentivirus infection is warranted. Macaques vaginally inoculated with $SIV_{mac}251$ exhibit virus in intraepithelial dendritic cells (Langerhans cells) within 1 hour; virus translocates to regional lymph nodes within 18 hours [77] . As early as day 2 following vaginal inoculation, $SHIV^+$ cells are detected in cortical parafollicular cells of lymph nodes of pig-tailed macaques [78] . Even tonsillar dendritic cells can be found harboring SIV following intravaginal inoculation [79] . Although dendritic cells may be the first mucosal cells targeted by HIV-1, $CD4^+$ T cells quickly expand into the majority of productively infected cells both at the site of inoculation and in regional lymph nodes [80] . Like HIV-1 and SIV, some FIV isolates are infective vaginally [81-83] .

Although horizontal FIV transmission is believed to occur through bites [84] , mucosal infection, including sexual transmission, is possible. FIV has been recovered from semen of acutely infected cats [85, 86] . Because FIV isolates which are transmitted vertically have also been shown to be transmitted mucosally [76, 81, 82, 87, 88] , the feline system can be used to model all phases of mother-to-child HIV-1 transmission,

from intravaginal infection of the mother to transplacental, intrapartum, and milk-borne infection of the offspring.

Viral receptors

A brief review of current thinking concerning lentivirus/cell interactions in humans and animals will help set the stage for the subsequent discussion of maternal-fetal lentivirus transmission. The range of HIV-1 isolates infecting women, and subsequently their infants, is determined at least in part by the cellular receptors the viruses bind. The discovery of fusin (now CXCR4) in 1996 as a co-receptor for HIV [89] has led to an explosion of research into the role of chemokines and their receptors in HIV pathogenesis.

CXCR4 is the major co-receptor for syncytium-inducing varieties of HIV-1, the viral phenotype which prevails in end-stage disease and AIDS [90-92] . Like HIV-1, laboratory-adapted FIV isolates bind the chemokine receptor CXCR4 and induce fusion of cells bearing those receptors [93-98] . Indeed, FIV induces fusion of cells transfected with human CXCR4 [97] and will infect human cells in vitro [99, 100] . The bicyclam drug AMD3100, which competitively binds CXCR4, potently inhibits infection of Crandall feline kidney cells by a cell culture-adapted strain of FIV, and less potently inhibits thymocyte infection by a thymocyte-selected FIV isolate [101] . Other investigators have found that inhibitors of CXCR4 block both laboratory-adapted and certain primary isolates of FIV [102] . Whether or not CXCR4 is the major receptor for FIV in vivo, utilization of this highly conserved chemokine receptor by both HIV-1 and FIV clearly strengthens the validity of the feline model.

The other major co-receptor used by primary HIV-1 isolates is CCR5 [103] . A widely held paradigm, which emerged from early studies, states that CCR5-utilizing (or "R5") viruses are the first to cross mucosal surfaces and be taken up by new hosts [104, 105] . After months or years of replication and mutation, CXCR4-utilizing (or "X4") viruses emerge. Owing to their higher rates of replication and special tropism for T cells, X4 isolates accelerate immune system destruction, leading to opportunistic infections and other AIDS-defining signs. However, the notion that only R5 HIV isolates are transmitted mucosally is being revised. For example, a SHIV construct containing a clade E HIV env phenotype is capable of infecting macaques when inoculated vaginally, even though the virus preferentially utilizes the CXCR4 receptor [106] . In the SIV/macaque system, dual chemokine receptor and macrophage tropism did not predict the ability of a given isolate to be infectious when administered vaginally, while the degree to which an isolate replicated when inoculated intravenously did predict vaginal transmission [107-109] . Thus chemokine receptor usage is only one factor affecting the ability of a lentivirus to induce primary infection.

As with HIV-1 and SIV, CXCR4-usage is not an absolute requirement for FIV. A mutant strain of FIV-PPR selected by passage through an IL-2 dependent cell line demonstrates a change in cell tropism, absence of inhibition by the CXCR4 ligand stromal derived-factor 1 (SDF-1), and indications of usage by a CC chemokine receptor [110] . In vitro and mutagenic selection of macrophage-tropic FIV isolates leads to a loss of CXCR4 usage, yet these isolates continue to infect lymphocytes in addition to monocyte/macrophages [111] . This is not surprising since feline lymphocytes, like those of other species, express both CXC and CC chemokine receptors [112] . FIV-B-2542

readily infects monocyte/macrophages in vitro and in vivo [113] and is also transmitted vertically [76, 88, 114] . However, care must be taken when extrapolating in vitro tropisms to in vivo infectivity. For example, in one study of multiple FIV clones, in vitro macrophage-tropism correlated poorly with in vivo infectivity [115] .

Placenta

The importance of the placenta in preventing or facilitating fetal infection is the subject of intense controversy. Some researchers suggest that the placenta is the gatekeeper of fetal exposure to HIV-1 [116] . The intact placenta appears impermeable to passage of HIV [117] . Certain HIV-1 and SIV strains will infect placental macrophages (Hofbauer cells) and synctiotrophoblast in primary placental cultures in vitro [49-51, 55] . Chorioamnionitis is a demonstrated risk factor for vertical HIV-1 transmission in some settings [118] , possibly due in part to upregulation of ICAM-1 by syncytiotrophoblast in the presence of the inflammatory cytokine milieu, with subsequent maternal mononuclear cell adhesion [119] . Placental trophoblasts are selectively infected by non-syncytium-inducing (NSI), R5 strains of HIV-1 [120] . Preferential infection of infants similarly by NSI viral variants has been attributed to placental selection [116] . Whether limited by the placenta or at some other level, it is clear that only a small subset of HIV-1 phenotypes are capable of inducing productive infant infections [121] .

There is also abundant evidence pointing to a diminished role of the placenta in determining fetal infection risk. Small breaks in placental epithelium are ubiquitous at the time of birth and do not correlate with vertical transmission [13] . Indeed, virtually all placentas of HIV+ women contain virus, regardless of fetal infection outcome [122] . R5 HIV-1 isolates are most commonly first identified in newly infected people following

horizontal as well as vertical transmission [103]. Moreover, co-receptor usage may inadequately explain selective placental infection by R5 isolates, since there is evidence that trophoblasts are resistant to infection by free virus and can only be infected by direct inoculation or cell-to-cell transfer [123]. Another epithelial cell population, retinal pigmented epithelial cells of the eye, likewise are infected only by cell-associated virus in vitro [124]. Indeed, there is some controversy as to whether trophoblasts are infected at all by HIV in vivo [125], although most researchers have found evidence of infection of this placental layer both in vitro and in vivo [50, 116, 126, 127]. Placental cells from HIV+ women generally demonstrate upregulated production of antiviral interferons, yet interferon levels are not significantly different between placentas from transmitting and non-transmitting mothers [128]. Even the presence of placentitis does not reliably predict fetal infection risk. A carefully controlled study in Thailand demonstrated no increased risk of fetal infection associated with placental inflammation [129]. Thus the placenta may play a smaller role than originally believed in determining fetal infection outcomes.

Timing of vertical transmission

Elucidation of the timing of vertical lentivirus transmission is critical to the design of rational intervention strategies. In women who do not breast-feed their infants, the timing of vertical HIV-1 transmission can be narrowed to the intrauterine or intrapartum period. A widely accepted paradigm defines presumptive intrauterine HIV-1 infection by a positive culture or PCR assay from neonatal blood within the first 48 hours after birth. Presumptive intrapartum infection is defined by a negative culture or PCR assay in the first 48 hours, followed by positive conversion after 1 week [130]. Using this definition, rates of intrauterine HIV-1 infection in non-breastfed populations have been estimated at

between 25–50%, while intrapartum exposure has been stated to account for 50–75% of neonatal infections [6, 131-135] . Because infants may be infected late in utero and not have a positive blood test until after the first week of life, these assumptions are not based on firm knowledge. The FIV model is well suited to help resolve questions regarding the likely importance of in utero versus intrapartum HIV-1 transmission.

Another question amenable to study in the FIV system is when during gestation the fetus is most likely to be infected. The ACTG 076 protocol calls for institution of therapy in mid-pregnancy [22] . Because infant infection rates are greatly reduced, it seems likely that most fetal HIV-1 infections occur during the second half of pregnancy. Further evidence that most HIV transmission occurs in the third trimester comes from a study which demonstrated a total absence of HIV provirus in tissues from 21 elective second trimester abortions in Spain [136] . Rare studies addressing the issue of fetal HIV-1 infection timing and tissue distribution have reported dissimilar results [52, 53] . By exploring fetal infection rates at defined timepoints of gestation, the FIV system can be used to help elucidate the time window of most intrauterine lentivirus transmission.

In situ studies

Although much information can be gained from clinical observations and blood-based assays, the real strength of animal models is the ability to examine a full range of tissues at defined timepoints in disease progression. Information reported to date suggests that the tissue distribution of FIV is virtually identical to that of HIV-1 [81, 85, 113, 114, 137-140] . Therefore, the feline model provides an excellent opportunity to follow in vivo lentivirus distribution in the early days following infection.

Characterization of FIV infection in vivo has been limited by a shortage of reagents for identification of cell phenotype in the feline species, and by the lack of sensitive assays for viral proteins and DNA, in tissue sections. Several groups have identified FIV in tissue sections by means of in situ RNA hybridization [81, 83, 141-147] . However, the tissue digestion steps required for in situ RNA hybridization often destroy protease-sensitive cell-specific antigens, limiting the number of markers available to specifically identify the FIV+ cells. Identification of FIV-specific proteins by immunohistochemistry obviates the need for harsh digestion steps. However, few monoclonal antibodies proven to bind to FIV in fixed-tissue specimens are available; moreover, detection of a single epitope requires high copy numbers of virus. Thus, there are few reports of identification of FIV in tissue sections by immunohistochemistry [144, 148, 149] . Because the very few reports of HIV-1 dissemination in human fetuses are contradictory, elucidation of fetal cell targets in the FIV system may help resolve questions resolving the earliest targets of lentiviruses in fetuses. However, the full benefit of in vivo modeling using FIV will not be realized until there are improved methods for detecting viral proteins, RNA, and DNA in situ.

Cell tropism and the thymus

The thymus is an early target of HIV-1 [150, 151] . Because the fetal thymus is crucial to an individual's cell mediated immune development, this organ deserves special consideration in any discussion of maternal-fetal lentivirus transmission. HIV+ CD8+ T cells are found in experimentally infected $SCID_{hu}$ thymus tissue, but mature CD8+ T cells are not infected in vitro, suggesting that the CD8+ T cells become infected early in development when they bear both CD4 and CD8 molecules [152] . Like HIV-1, both

CD4+ and CD8+ T cells may harbor FIV [115]. FIV may infect CD8+ cells during early thymic maturation while the cells are in the CD4/CD8 double-positive stage, as is the case with HIV [152], though infection of mature CD8 single-positive cells may also occur [153]. Regardless of the exact nature of lentivirus/lymphocyte interaction, it seems clear that one of the keys to preventing mother-to-child HIV-1 infection is inhibiting establishment of virus within the thymic compartment.

Neurotropism

Because HIV-1, FIV, and SIV all exhibit fetal neurotropism [114, 154, 155], mention should be made of this potential life-long viral reservoir. In the brain both microglia and astrocytes may be infected; however, cellular receptor usage, likelihood of productive infection, and responses to cytokines and viral products can be highly variable [156-158]. In some cases, viral products themselves may upregulate vascular and leukocyte adhesion molecules and enhance spread of virus this otherwise protected body compartment [159]. For example, the HIV protein tat induces upregulation of intercellular adhesion molecules on brain endothelium [159]. A cycle of inflammation and further recruitment of HIV-infected leukocytes may operate in neurologic spread. Both α- and β-chemokines induce surface display of cell adhesion molecules in brain endothelium and expression of CXCR4 by astrocytes [159]. The FIV model provides an ideal opportunity to explore the mechanisms leading to viral spread through the fetal central nervous system.

Aims of this project

The goal of the research described in this dissertation was to use the FIV model of intrauterine HIV-1 infection to elucidate basic mechanisms of maternal-fetal lentivirus pathogenesis. The three specific aims were:

- Aim 1: To determine timing and placental/fetal tissue tropisms, and to evaluate potential surrogate maternal and viral markers, correlated with intrauterine FIV-B-2542 transmission.
- Aim 2: To develop assays for the *in situ* detection of FIV proteins and DNA in tissue sections, and to identify cells targeted by FIV during the acute phase of infection.
- Aim 3: To extend aim 1 studies to compare the intrauterine transmissibility of FIV isolates representing *env* clades A and C, and to use the assays developed in aim 2 to determine in vivo placental and fetal cell tropisms.

Future studies

The results described in this report help to answer questions about maternal-fetal lentivirus transmission not easily studied in humans. These issues include: (1) the relative contributions of in utero and intrapartum vertical lentivirus transmission, (2) the specific timing of fetal infection during gestation, (3) the correlation of maternal hematologic surrogate markers with the timing of fetal infection, (4) the in vivo correlation of placental virus localization with fetal infection outcome, and (5) the placental and fetal cells targeted by lentiviruses.

The FIV system can be used to explore answers to important questions which may lead to novel interventions for maternal-fetal HIV-1 transmission. These questions include:

- Are specific viral or maternal determinants associated with high risk of transplacental transmission? If so, can a practical test be devised to identify those mothers most at risk of transmitting virus to their infants? Such knowledge might allow targeted chemoprophylaxis in regions where universal intervention is impractical.
- What are the placental and/or fetal restrictions on the range of viruses permissive for fetal infection? Can these restrictions be exploited to prevent virus/cell interactions and protect the fetus from infection?
- Do all fetal virus exposures lead to established infections? What of the evidence that many infants exposed to virus in utero do not become infected? Are there unique viral dynamic and/or host responses operating in utero which tilt the balance in favor of the host? If so, might we adapt those principles towards prophylaxis or treatment for both vertical and horizontal HIV-1 infections?
- Is there hope for a vaccine which will reduce or eliminate maternal-fetal virus transmission?

Although great strides have been made in reducing mother-to-child HIV-1 transmission in industrialized countries, new strategies will be required to further reduce infant infection rates and to make available interventions in regions of scarce resources. Improved knowledge of the pathogenesis of maternal-fetal HIV-1 transmission could reveal hitherto unconsidered strategies for more effective interventions which will be more widely accepted than current regimens. The FIV model will be an important resource in our continued search for ways to better understand and reduce the tragedy of mother-to-child HIV-1 transmission.

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CHAPTER ONE

TIMING AND TISSUE TROPISMS OF MATERNAL-FETAL FELINE IMMUNODEFICIENCY VIRUS TRANSMISSION

INTRODUCTION

Mother-to-infant human immunodeficiency virus type 1 (HIV-1) transmission helps fuel the AIDS pandemic. Progress has been made towards preventing infant infections in industrialized nations, but globally 2 million new infants are infected each year [1] . HIV-1 prevalence exceeds 30% among women of childbearing age in parts of sub-Saharan Africa [2, 3].

HIV-1 can be transmitted in utero, during parturition, and post-natally via breastmilk [4] . Significant controversy remains as to the relative contribution of each of these exposure routes [5-8] . Antenatal infection has been documented by HIV-1 isolation from aborted fetal tissues and from cord blood of term babies [9-12] . Maternal antiviral chemotherapy is the only means presently available to reduce the risk of fetal infection. However, availability and cost of treatment severely limit the impact of chemoprophylaxis worldwide.

Little is known about the events which occur during intrauterine HIV-1 transmission. Most of our assumptions are extrapolated from in vitro, epidemiologic, and anecdotal observations. Primary cultures of human placental and fetal macrophages can

be infected with HIV-1 in vitro [13] . Likewise, primary cultures of fetal-derived syncytiotrophoblast support HIV-1 replication in vitro [14, 15] although cell-mediated viral entry may be required [16] . Reports on the timing and tissue tropisms of fetal HIV-1 infection are few and somewhat contradictory [17, 18] . Because of inherent limitations in human intragestational studies, mechanisms of intrauterine lentivirus transmission might best be explored in an animal model.

Feline immunodeficiency virus (FIV) infection in cats causes disease virtually indistinguishable from that caused by HIV-1 in humans and simian immunodeficiency virus in Asian non-human primates [19-21] . Our laboratory has identified FIV isolates vertically transmitted from pregnant cats to their offspring with high frequency [22, 23] . Herein are described studies utilizing one such isolate, FIV-B-2542, to identify the gestational timing and tissue targets during fetal infection. Evaluated also are maternal hematologic and viral kinetic parameters which might correlate with the timing of intrauterine lentivirus transmission.

MATERIALS AND METHODS

Animals and sampling

Twelve 8–12-month-old female cats (queens) were inoculated intravenously with 1000 weanling cat infectious doses of an acute-phase plasma pool of FIV-B-2542 [24] . FIV infection was confirmed in the queens by virus isolation and DNA polymerase chain reaction (PCR) on blood mononuclear cells as described below. The queens were bred with an FIV-naive male cat 4–12 months after infection. Pregnancy was diagnosed by abdominal palpation and correlated with observed breeding dates. Placental and fetal

tissues were harvested by cesarean at one of four intervals: 3, 5, 7, or 9 weeks (term) post-conception. Five queens provided two separate litters to this study while 7 queens provided only 1 litter each. We collected a total of 17 litters, 4–5 at each specified time point. As negative controls, term fetuses and placentas from a sham-inoculated queen were assayed.

Hematology.

Blood from queens and from 7 and 9 week gestation fetuses was collected at the time of cesarean. Heparinized whole maternal blood was analyzed for complete blood count and $CD4^+$ and $CD8^+$ T cell subsets as described by Dean et al [25] . Maternal and fetal blood mononuclear cells were isolated by ficoll-Hypaque separation (Histopaque-70, Sigma Corp., St. Louis, MO) and rinsed with phosphate-buffered saline (PBS, pH 7.4) for use in culture and PCR. Fetal and additional maternal blood samples were collected into tubes with acid citrate dextrose (Alsever's solution) at a 1:1 dilution for plasma viral RNA quantitation. Plasma was separated from cells by centrifugation and stored at -70° C until use. Statistical analysis of maternal hematologic and viral kinetic values was performed by one-way analysis of variance (ANOVA) using InStat software (GraphPad Software Inc., San Diego, CA).

Tissue Processing

Immediately upon surgical removal, amniotic fluid was collected and placentas were separated from fetuses. Care was taken to maintain the integrity of the amniotic sac to prevent maternal and placental contamination of fetal tissues. Viable 7 and 9 week gestation fetuses were euthanized by pentobarbital sodium injection following blood

collection. Fetuses of 3 and 5 weeks gestation were non-viable after surgical removal, and their cardiovascular systems were insufficiently developed for blood collection. In the laboratory, fetal data were recorded including sex, body weight, and thymic and splenic weights. To further eliminate the possibility of maternal contamination of fetal samples, fetuses were rinsed with sterile PBS and externally disinfected with 70% ethanol prior to dissection in a laboratory hood. After dissection individual organs were isolated, rinsed with PBS, and dissociated into cell suspensions through mesh sieves. Dissociated cells were quantitated with a hemacytometer and fractionated for dilutional coculture and PCR. From the 7 and 9 week gestation fetuses a spectrum of tissues was collected including brain, thymus, liver, spleen, mesenteric lymph node, bone marrow, and blood mononuclear cells. From the 5 week gestation fetuses brain and thymus were individually isolated, but hemotapoietic and lymphoid organs including liver, spleen, and bone marrow were pooled due to limitations in recoverable cell numbers. Due to very small size and lack of organ development, 3 week gestation fetuses were processed whole.

Cell-associated virus detection

Infectious cell-associated virus was isolated and amplified in vitro by dilutional cocultivation of 10^6-10^1 sample blood mononuclear cells or dissociated tissue cells with 10^6 naive feline blood mononuclear cells in 24-well plates according to methods described by O'Neil et al [22] . Productive in vitro infection was assessed by a capsid antigen (p26) capture ELISA described by Dreitz et al [26] performed on supernatants collected twice weekly over 4 weeks. Optical densities (OD), measured by absorbance at A_{450} (reference A_{570}), were recorded using a Dynatech 5000MR™ microplate reader

(Dynatech Corp., Chantilly, VA). Positive reactions were defined as those with a minimum OD of 0.1 and at least twice that of negative control supernatant run in tandem.

Provirus detection

Nested limiting-dilutional DNA PCR was used to semi-quantitate proviral burden. The protocol was described by O'Neil et al [22] . The primers used for first round amplification, gag1 (5'-GGGAATGGACAGGGGCGAGAT-3') and gag2 (5'-TTGCTGCACTTGATTCACTGG-3'), flanked a 1299 base pair sequence in the *gag* gene. Primers gag3 (5'-TTGACCCAAAAATGGTGTCCA-3') and gag4 (5'-TTCTGCTTGTTGTTCTTGAGT-3') were used in the second round to produce a 293 base pair sequence nested within the first round amplimer. Positive samples were log diluted and limiting dilutional PCR assays performed to identify negative endpoints. Amplimer specificity was confirmed by Southern blot analysis using a biotinylated internal *gag* oligoprobe (biotin-5'-GCTGCAGATAAAGAAATATTGGATGAAA-3') [22] and a streptavidin-alkaline phosphatase enzymatic chromagen detection system according to manufacturer's instructions (BluGene®, Gibco BRL, Gaithersburg, MD).

Viral RNA quantitation

Amniotic fluid, maternal plasma, and plasma from 7 and 9 week gestation fetuses were tested for viral RNA employing a semi-quantitative competitive reverse transcriptase PCR (sQC-RT PCR) assay based on a method described by Diehl et al [27, 28] . The primers used in the single round, 40 cycle, reaction were gag3 and gag4. Half-log serially diluted competitor RNA containing from $10^{1.5}$–10^{4} copies was added to viral

RNA elutes extracted from 200 µl of the 1:1 plasma:Alsever's samples. Amplimer products were visualized and specificity was confirmed as for DNA PCR.

Antibody ELISA

FIV-specific IgG, IgM, and IgA were assayed by a sandwich enzyme-linked immunosorbent assay (ELISA) modified from a method described by Dreitz et al [26] . Briefly, 2X dilutions of plasma or amniotic fluid were aliquoted across 98-well microtiter plates coated with whole pelleted FIV. Plasma dilutions ranged from 1:32-1:32,768. Plasma antibody binding was carried out at room temperature for 1 h. Plates were washed and wells overlaid with peroxidase-labeled secondary antibody solutions containing isotype-specific goat anti-cat antibodies to IgG-γ, IgM-µ, (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or IgA-α (Bethyl Laboratories, Montgomery, TX). Secondary antibody solutions were diluted 1:500. Optical densities and positive results were determined as for the p26 antigen-capture ELISA assay. Titers were recorded as the inverse of the highest dilution yielding a positive signal.

RESULTS

Litter size and timing of fetal infection

The mean litter size in the cohort of queens in the early chronic phase of FIV infection was 3.9 (+/- 1.3 SD) fetuses (full data summary in Table 1.1). This figure is virtually identical to that seen in our uninfected specific pathogen-free (SPF) cat colony (unpublished data). FIV infection was defined as a positive PCR result on extracted DNA from at least one tissue (excluding placenta) in a given individual. All animals with

detectable plasma viral RNA also had measurable provirus in blood mononuclear cell DNA (Table 1.1). Coculture was performed in tandem with PCR in order to confirm that the molecular-based assays were detecting infectious virus. At 3 weeks of gestation, 0 of 16 fetuses (0%) were infected with FIV as determined by DNA PCR. At 5 weeks, 1 of 19 fetuses (5%) was FIV^+, at 7 weeks 6 of 16 (39%) were FIV^+, and at 9 weeks 9 of 15 (60%) were FIV^+ (Fig. 1.1; Table 1.1). The sharp rise in fetal infection prevalence during the third gestational trimester was highly significant ($P < 0.001$). Fetuses with detectable FIV infection came from multiple litters, and most 7 and 9 week gestation litters contained both infected and uninfected individuals. In our hands DNA PCR detected ~20% more FIV-infected fetal tissues than did coculture (data not shown). No PCR-negative samples were positive by coculture, and no negative control samples yielded false positive results by either coculture or PCR.

Table 1.1: Maternal viral and hematologic parameters at time of cesarean, and FIV infection status of fetuses in litters.

Queen ID	Gestation stage (week)	PBMC proviral load*	Plasma viral RNA load[ƒ]	Hemato crit	WBC[¶] (cells/ul)	CD4 count (cells/ul)	CD8 count (cells/ul)	CD4/CD8 ratio	FIV+ fetuses/ total litter
ID4	3	1000	1000	30	4943	191	439	0.44	0/3
2549	3	100	<1000	34	6134	473	294	1.61	0/4
3459	3	100	<1000	36	5060	296	433	0.68	0/7
3450	3	10,000	1,000	28	4442	209	626	0.33	0/2
3 week mean	-	2800	700	32	5146	292	448	0.77	Total 0/16
3 week SEM**	-	2409	173	1.8	356	64	68	0.29	0% FIV+
HS3	5	100	<1000	32	4432	350	160	2.19	0/5
HO4	5	1000	1000	31	7433	568	350	1.62	1/4
3472	5	100	<1000	31	3200	317	276	1.15	0/6
3443	5	1000	5,000	22	7651	845	734	1.15	0/4
5 week mean	-	550	1850	29	5679	520	380	1.53	Total 1/19
5 week SEM	-	260	1060	2.3	1106	122	124	0.25	5% FIV+
HO4	7	1000	1000	29	5823	568	350	1.62	2/4
2885	7	1000	1000	27	6650	769	676	1.3	2/2
3449	7	1000	<1000	30	6171	705	781	0.90	0/4
3471	7	100	<1000	26	6761	218	515	0.42	2/4
2885	7	1000	1000	26	7045	994	723	1.37	0/2
7 week mean	-	820	760	28	6490	651	609	1.12	Total 6/16
7 week SEM	-	180	147	0.8	218	128	78	0.21	38% FIV+
2549	9	100	<1000	22	3924	422	136	3.10	2/4
ID4	9	1000	1000	18	5891	290	530	0.55	4/4
3448	9	1000	<1000	31	9409	790	489	1.62	0/3
3450	9	1000	1000	24	4629	385	619	0.62	3/4
9 week mean	-	775	700	24	5963	472	444	1.47	Total 9/15
9 week SEM	-	225	173	2.7	1219	110	106	0.60	60% FIV+

* Number provirus copies/microgram DNA determined by limiting dilutional PCR.

ƒ Number viral RNA copies/ml plasma determined by reverse transcriptase PCR; samples below test threshold assigned a value of 400 for calculations.

¶ White blood cells.

** Standard error of mean.

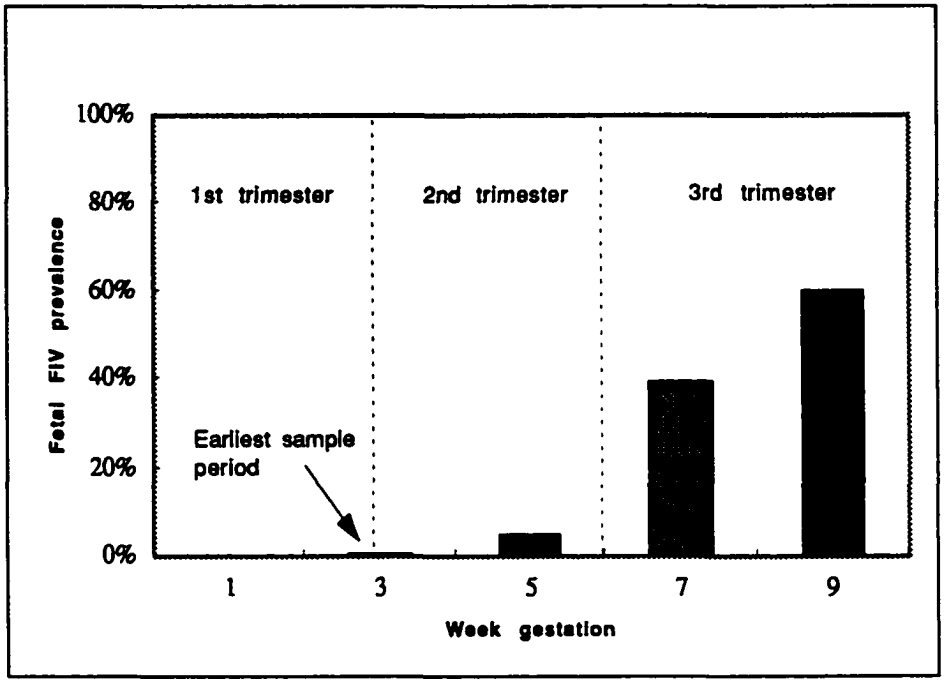

Figure 1.1: Fetal FIV infection prevalence at 3, 5, 7, and 9 weeks (term) gestation as determined by virus isolation/coculture and DNA PCR.

Fetal tissue tropisms

In the 15 FIV^+ fetuses of 7 and 9 week gestation, specific tissue infection proportions were: blood mononuclear cells 60% (9/15), brain 60% (9/15), thymus 47% (7/15), bone marrow 33% (5/15), mesenteric lymph node 33% (5/15), spleen 27% (4/15), and liver 7% (1/15) (Fig. 1.2). Twelve of the 15 fetuses were infected in more than one tissue (sample DNA PCR result shown in Fig. 1.3). Of the 3 fetuses from which only a single tissue was detectably infected, 1 exhibited virus in the brain, 1 in the thymus, and 1 in PBMC. One 5 week gestation fetus exhibited virus in brain tissue. In no 3 week fetuses (processed whole) was virus detected. We observed no grossly identifiable lesions or quantitative morphologic defects attributable to fetal FIV infection. Among the 7 and 9 week gestation-stage fetuses, body weight, thymus-to-body and spleen-to-body weight

proportions; and male:female ratios were virtually identical between FIV-negative and FIV-positive groups (Table 1.2).

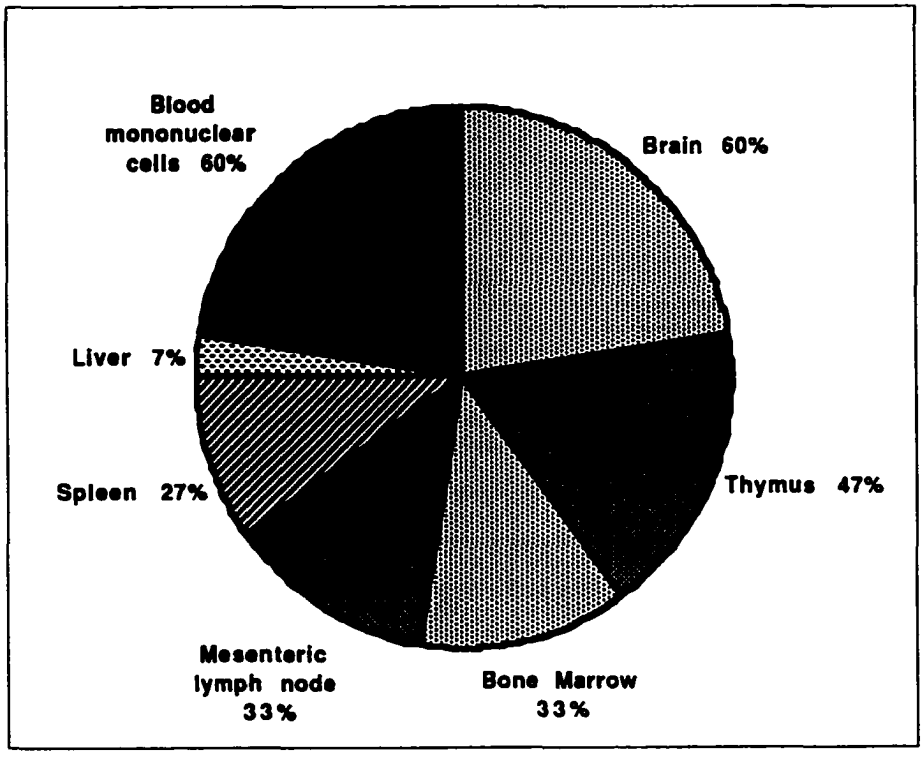

Figure 1.2: Tissue infection rates in 7- and 9-week-gestation FIV^+ fetuses (n=15) determined by virus isolation/coculture and DNA PCR.

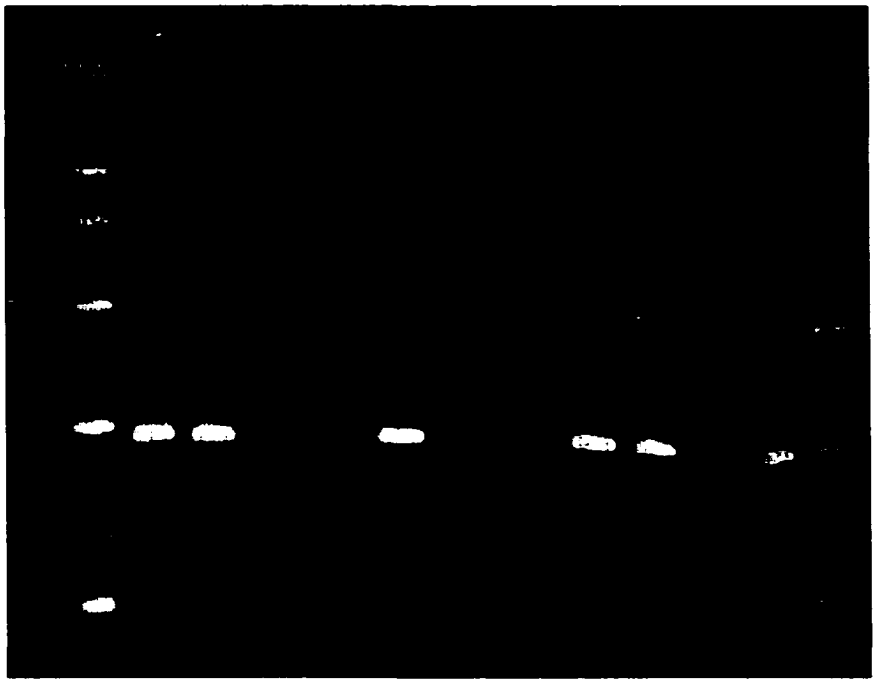

Figure 1.3: Nested DNA PCR demonstration of FIV infection in isolated fetal tissues. Results from term (9 week) fetus 1, queen 2549, are shown. Bands of 292 base pairs in lanes 1, 2, and 5 indicate infection of fetal brain, thymus, and mesenteric lymph node, respectively. Infection of placenta and maternal blood mononuclear cells is demonstrated by bands in lanes 8 and 9, respectively. Lane key: MW -molecular weight marker (base pairs); 1 - brain; 2 - thymus; 3 - liver; 4 - spleen; 5 - mesenteric lymph node; 6 - bone marrow; 7 - fetal blood mononuclear cells; 8 - placenta; 9 - maternal blood mononuclear cells; 10 - negative control (blood mononuclear cell DNA from sham-inoculated queen); 11 - positive control (blood mononuclear cell DNA from a queen previously demonstrated FIV-positive by coculture and PCR).

Table 1.2: Comparison of morphologic parameters between FIV-negative and FIV-positive late-gestation (7 and 9 week) fetus cohorts; standard deviation (SD) in parentheses.

Fetal morphologic parameter	FIV-negative (n=12)	FIV-positive (n=11)
Mean body weight g (SD)	98 (13)	94 (18)
Mean thymus/body weight ratio (x 10^{-3}; SD)	2.8 (1.0)	3.1 (1.8)
Mean spleen/body weight ratio (x 10^{-3}; SD)	1.8 (0.6)	2.0 (0.7)
Male:female ratio	7:5	7:4

Maternal hematology

Mean maternal $CD4^+$ T cell counts in the queens chronically infected with FIV were one-quarter those of uninfected cats in our SPF colony: 501 cells/ml (+/- 266 SD) in the infected group vs. 2215 cells/ml (+/- 675 SD) in uninfected SPF cats (unpublished data). Of the hematologic values compared between the 4 gestation-stage maternal cohorts, only a progressive decline in hematocrit approached statistical significance (P = 0.07). Maternal white blood cell count (P = 0.68), CD4 count (P = 0.20), CD8 count (P = 0.37), and CD4/CD8 ratio (P = 0.44) were all unassociated with advancing gestation (individual cat and gestation cohort data shown in Table 1). Some queens provided two litters to this study; however, there were no significant differences in maternal hematologic or viral kinetic markers at the time of the different cesarean collections (Table 1.1).

Viral RNA loads

Plasma viral RNA loads were relatively low in this group of chronically infected queens. Nearly half of the queens had plasma viral RNA loads below our test detection threshold (1000 copies/ml). Of plasma samples positive for viral RNA, most were at the lowest limit of detection (sample RT-PCR result shown in Fig. 1.4). Analyzed by gestation stage, 2/4 queens had detectable viral RNA at 3 weeks gestation, 2/4 at 5 weeks, 3/5 at 7 weeks, and 2/4 at 9 weeks (Table 1.1). As with hematologic values, there was no correlation between maternal plasma viral RNA load and gestation stage (P = 0.36; samples below test threshold were assigned a value of 400 copies/ml for statistical purposes). No viral RNA was detected in any amniotic fluid or 7 and 9 week gestation

fetal plasma samples (including samples from fetuses with detectable tissue infection by coculture and DNA PCR). In order to confirm test sensitivity, high-titer frozen archived plasma samples from a previous study in our laboratory were re-assayed for viral RNA. The samples again demonstrated viral burdens ranging from 10^6—10^8 copies/ml, matching the previously reported results [27, 28].

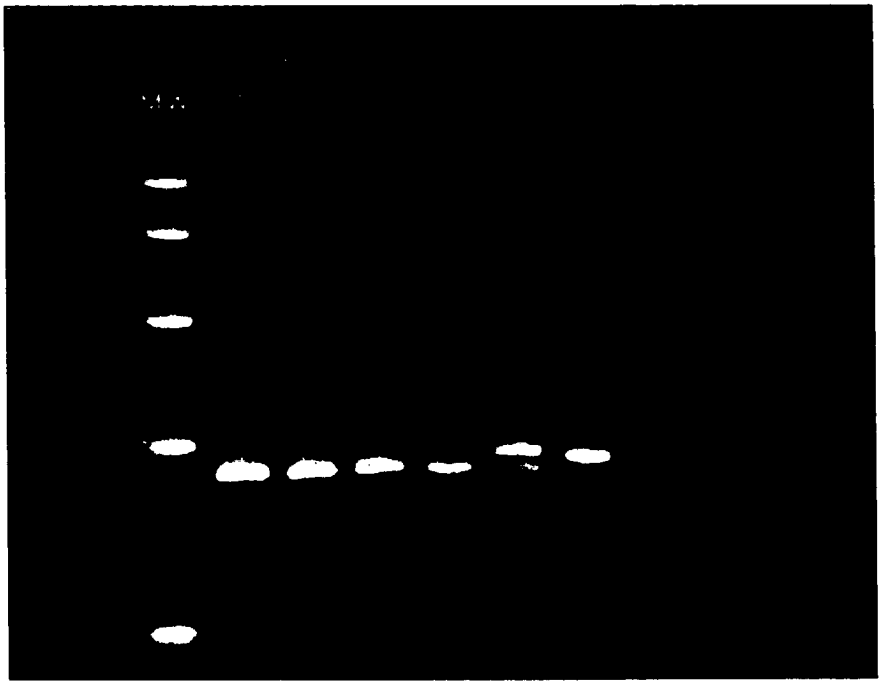

Figure 1.4: Semi-quantitative reverse transcriptase PCR (viral load) assay for queen 2549. Half-log dilutions of a synthesized 23 base pair deletion competitor molecule were co-amplified with RNA extracted from 100 μl sample plasma. In this figure a double band is seen in lane 5, which was loaded with 100 copies of competitor. The native viral RNA amplimer is 292 base pairs while the competitor is 269 base pairs (bands separated by electrophoresis through 3% Metaphor agarose). Because the input plasma sample of 0.1 ml co-amplified equivalently with 100 copies of competitor, the calculated viral RNA burden is 1000 copies per ml plasma. Lane key: MW - molecular weight marker (base pairs); 1 through 6 - half-log dilutions of competitor RNA from 10^4 to $10^{1.5}$ copies, respectively, co-amplified with RNA extracted from 100 μl sample plasma; 7 - negative control plasma alone; 8 - 100 copies of RNA competitor alone (to confirm assay sensitivity).

Cell-associated viral and proviral loads

Cell-associated virus and provirus were detected by coculture and DNA PCR in all infected queens prior to breeding and at cesarean. Blood mononuclear cells, placental, and fetal tissues derived from the sham-inoculated queen were all FIV-negative by coculture and PCR. Maternal blood mononuclear cell-associated viral and proviral burdens ranged from 100-10,000 copies per μg DNA (Table 1.1). Depending on the source, ~1–4 μg DNA was routinely recovered from 1 million manually quantitated frozen and thawed cells. Maternal cell-associated viral and proviral burdens were not associated with gestation interval ($P = 0.51$). Placental cell-associated viral and proviral loads were similar to maternal blood mononuclear cell loads by the third week of gestation, and remained equivalent throughout gestation (Fig. 1.5). In contrast to maternal blood mononuclear cells and placental tissue, fetal cell-associated viral and proviral loads were uniformly low. Regardless of gestational age, cell-associated viral and proviral loads in fetal tissues were almost always at the lowest detectable limit (~10 copies/μg DNA; data not shown).

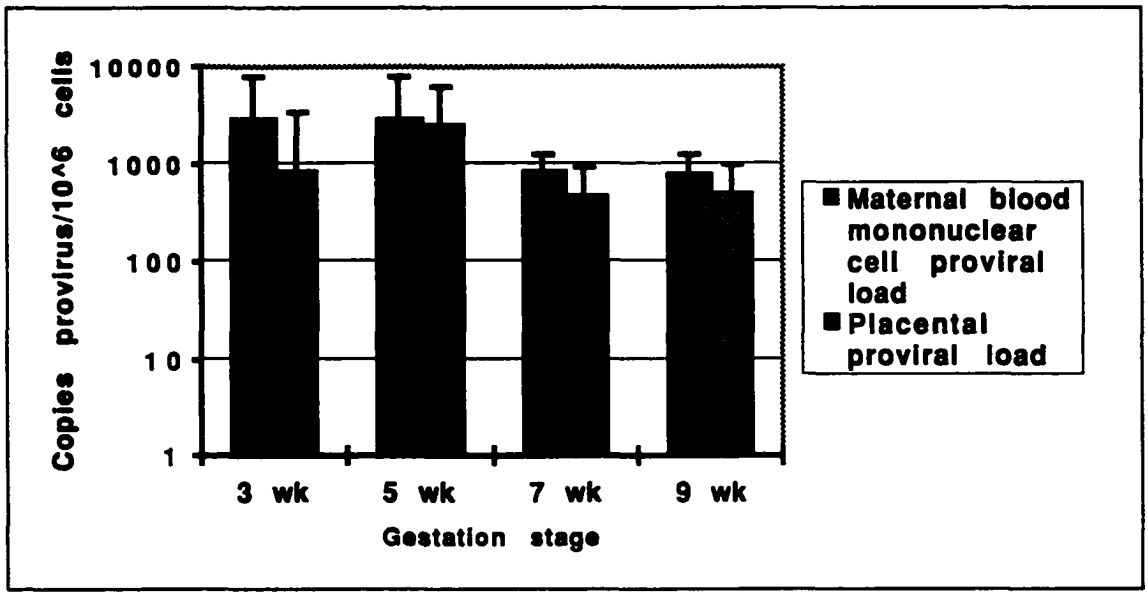

Figure 1.5: Maternal blood mononuclear cell proviral loads versus placental proviral loads at 3, 5, 7, and 9 weeks gestation determined by limiting dilutional DNA PCR.

Antibody responses

Queens had FIV-specific IgG antibody titers ranging from 512–16,384 (Fig. 1.6). There was no association between maternal plasma IgG titer and gestation stage (P = 0.80). Maternal circulating IgM and IgA titers were uniformly low or undetectable (Fig. 6). In normal feline pregnancies maternal antibody does not cross the placenta into fetal circulation. No antibody of any isotype was detected in fetal plasma or amniotic fluid samples (not shown).

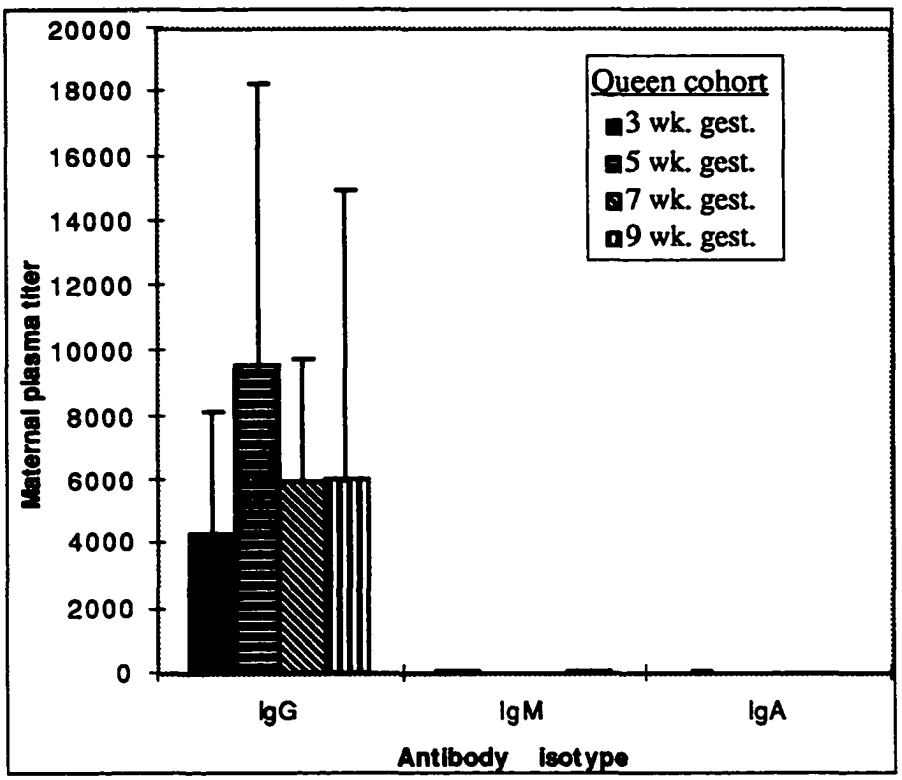

Figure 6. FIV-specific mean maternal plasma IgG, IgM, and IgA antibody titers at 3, 5, 7, and 9 weeks gestation determined by ELISA.

DISCUSSION

Nearly all maternal-fetal FIV transmission occured in the third trimester of gestation. This finding supports the speculated timing of intrauterine HIV-1 transmission [5-7] . The relatively late occurence of fetal infection could explain why zidovudine therapy instituted in mid-pregnancy reduces by two-thirds vertical HIV-1 transmission rates in non-breastfeeding populations [29-32] .

FIV localized to fetal brain and tissues rich in mononuclear cells. Microglial cells, endothelial cells, and astrocytes may all have been sources of provirus in the brain [33, 34] . However, specific cell sources of virus could not be determined from the whole-

brain tissue homogenates. The relatively low degree of viral detection in liver could suggest either that the primary site of fetal leukopoiesis had transferred to bone marrow by the time transplacental FIV transmission occured, or that potentially infected remnant fetal hematopoietic cells in the liver did not persist in sufficient numbers to be detected. The bone marrow becomes the primary hematopoietic organ at approximately day 45 in the 63 day gestation period of the cat [35] . The comparatively infrequent detection of virus in vascular organs such as liver and spleen suggested that presence of circulating blood mononuclear cells alone was insufficient to result in a positive tissue test using our assays. Taken together these observations imply that both fetal cell phenotype and stage of maturation may influence viral tissue tropism during in utero infection.

In our cohort of chronically infected queens, plasma viral RNA loads were relatively low. We did not find this surprising, as low plasma viral RNA burdens are characteristic of the chronic phase of FIV infection in our experience (unpublished data). Despite the low plasma viral RNA titers, cell-associated virus and provirus was detected in blood mononuclear cells of all virus-inoculated queens and 96% of placentas representing all stages of gestation. Because more than half of term fetuses were infected with FIV, it seems clear that high maternal plasma viral RNA titers were not required to effect intrauterine transmission of FIV-B-2542.

In humans, maternal and viral factors both appear to contribute to the risk of intrauterine HIV-1 transmission [36, 37] . Maternal plasma viral burden is considered the best surrogate marker of HIV-1 vertical transmission risk [38, 39] . However, viral load alone is an incomplete predictor of fetal infection. Although high maternal viral titers are associated with increased frequency of infant infections, there is no threshold below

which transmission does not occur [40, 41] . Moreover, maternal zidovudine therapy significantly reduces infant infections with only a modest reduction in maternal viral load, suggesting a different mechanism of protection. Unique metabolism and hormone production appear to enhance the pharamacologic efficacy of zidovudine in the placenta [42] . More study is required to elucidate the role of viral load in determining risk of both HIV-1 and FIV vertical transmission.

Apart from a gradual decline in hematocrit, we identified no correlation between maternal hematologic parameters and gestation stage. While the FIV-infected queen cohort in our study had significantly lower CD4 counts than did uninfected cats in our SPF colony, there was no consistent pattern of decline in $CD4^+$ T cell numbers or CD4/CD8 ratios associated with advancing gestation. Some studies in pregnant women have likewise demonstrated no significant correlation between gestation stage and hematologic parameters [43] . In our study, other potential surrogate markers such as maternal white blood cell counts, viral and proviral loads, placental proviral loads, and antibody titers were also unassociated with the rise of fetal infections in the third trimester. The role of antibody in transplacental HIV-1 versus FIV transmission cannot be directly correlated because maternal antibody ordinarily crosses the placenta in humans but does not in the cat. However, several studies involving women and infants have shown no correlation between maternal and fetal antibody levels or types (e.g. neutralizing vs. non-neutralizing) and risk of vertical HIV-1 transmission [44, 45] .

Because of the failure of maternal or viral markers to explain the timing of intrauterine FIV transmission, we postulate that fetal and/or placental factors arising in late gestation are primarily responsible for determining fetal susceptibility to lentivirus

infection. Such factors might include: (1) rapid late-phase fetal growth with a concomitant increase in exposure to maternal circulating virus flowing through the placenta; (2) gestation-specific hormonal changes which induce viral activation and/or release across the placenta; and (3) cellular maturation in the fetus. Regarding the third scenario, it is possible that key cellular receptors or intracellular pathways required for viral entry, integration, replication, and assembly are not expressed in the fetus until terminal stages of development. We can only speculate as to why some fetuses in a litter became infected while others did not. Possible contributing factors include fetal genetic makeup, immunodevelopment, variation in local uterine or placental viral concentrations, or simple random chance.

Elucidation of the timing of vertical lentivirus transmission is critical to the design of rational intervention strategies. In women who do not breastfeed their infants, the timing of vertical HIV-1 transmission can be narrowed to the intrauterine or intrapartum period. A widely accepted paradigm defines presumptive intrauterine HIV-1 infection by a positive culture or PCR assay from neonatal blood within the first 48 hours after birth. Presumptive intrapartum infection is defined by a negative culture or PCR assay in the first 48 hours, followed by positive conversion after 1 week [46] . Using this definition, rates of intrauterine HIV-1 infection in non-breastfed populations have been estimated at between 25–50%, while intrapartum exposure has been stated to account for 50–75% of neonatal infections [7, 10, 47-50] .

The prevalence of FIV infection in third trimester fetuses in this study was similar to that documented in vaginally delivered kittens up to 8 weeks of age who were not exposed to infected milk in previous studies from our laboratory [22, 23] . Taken

together, our results suggest that the intrauterine route of vertical FIV transmission is the primary source of neonatal infections. Of note is the finding that 40% of fetuses with detectable FIV in one or more body tissues did not contain detectable virus in blood mononuclear cells. Such animals might have been incorrectly interpreted as having been infected intrapartum had they been vaginally delivered and subsequently developed a detectable viremia post-natally. If fetal HIV-1 dissemination parallels that of FIV, current clinical definitions may underestimate the number of late in utero fetal infections while overstating the importance of intrapartum virus exposure. Human neonates with demonstrable HIV-1 infection at birth progress to AIDS and death faster than do infants whose first positive test occurs later in infancy [51] . However, this finding may have more to do with the phenotype of the infecting HIV-1 isolate than the timing of transmission. Intrauterine-transmitted HIV-1 isolates which replicate to detectable levels at birth may be inherently more pathogenic than intrauterine-transmitted isolates which do not reach such levels until days or weeks after birth.

In conclusion, the present study demonstrates that: (1) fetal FIV infections occur primarily in the final trimester of gestation, (2) virus targets the fetal brain and tissues rich in mononuclear cells, and (3) commonly monitored surrogate maternal hematologic and viral load markers do not help explain the late timing of intrauterine virus transmission. Questions remain as to why some virus isolates cross the placenta while others do not, which specific cells are initially involved in transplacental transmission and fetal virus dissemination, and which circumstances arising in late gestation are responsible for susceptibility to infection. The FIV model can be used to address these questions and enhance our understanding of maternal-fetal lentivirus transmission.

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CHAPTER TWO

CELLULAR TROPISM OF FELINE IMMUNODEFICIENCY VIRUS IN TISSUES OF ACUTELY INFECTED CATS

INTRODUCTION

Elucidating host-virus interactions in vivo depends in large part on the localization of virus to specific cells in tissues. In situ identification of HIV-1 RNA in human lymphoid tissues provides proof that continuous viral replication occurs in spite of declining or undetectable viral loads in plasma [1-4] . However, systematic study of early HIV pathogenesis is limited by uncertainty surrounding the exact time of an individual's infection, and paucity of tissues available for examination. For these reasons, animal models are best suited to track early lentiviral disease progression in vivo.

Feline immunodeficiency virus (FIV) infection in cats causes disease virtually indistinguishable from that caused by HIV-1 in humans and simian immunodeficiency virus (SIV) in Asian macaques [5, 6] . The known distribution of FIV is similar to that of HIV-1 and SIV, including mucosal interfaces, lymphoid organs, sites of hematopoiesis, circulating mononuclear cells, and the central nervous system [7-13] . In vitro FIV cell targets include, but are not limited to, T lymphocytes [14, 15] , monocyte/macrophages [12, 16] , dendritic cells [10, 17-19] , and central nervous system microglia and astrocytes [20, 21] . Therefore, the feline model provides an excellent opportunity to trace likely HIV-1 dissemination pathways in the early days following infection

Characterization of FIV pathogenesis in vivo has been limited by a shortage of reagents for identification of cell phenotypes in feline tissue sections, and by the small number of described assays for detecting virus in situ. FIV is most often revealed in tissue sections by in situ RNA hybridization [10, 17, 18, 22-27] . However, tissue digestion steps required for in situ RNA hybridization often destroy protease-sensitive cell-specific antigens, limiting the number of markers available to identify the cells infected. Moreover, the special precautions required to prevent target and probe degradation by RNases, and the relatively high cost of developing or purchasing FIV RNA probes has limited the application of this technique. Identification of FIV-specific proteins by immunohistochemistry obviates the need for protease digestion steps and RNase-free protocols. However, few monoclonal antibodies proven to sensitively and specifically bind FIV in tissue sections are available. Additionally, because monoclonal antibodies bind only a single epitope, high virus copy number may be required for detection. Thus, there are few reports of FIV identification of in tissue sections by immunostaining [19, 26] .

As with HIV-1, inactive FIV provirus may be integrated in cell chromosomes. (Unintegrated intracellular lentiviral DNA may also be present transiently.) Identification of latently infected cells requires DNA in situ PCR, in situ DNA hybridization, or a combination of the two [28] . Identification of latently infected cells is paramount to understand disease pathogenesis because under certain conditions there may be little demonstrable viral replication in spite of high proviral burdens [29-32] . Fluid-phase PCR is routinely employed to sensitively detect FIV provirus in cell suspensions [33-37] . However, to our knowledge there is only one report describing in situ DNA PCR

(coupled with in situ hybridization) to identify provirus in tissues from a single FIV-infected cat [13].

In this study we employed in situ assays developed to detect FIV proteins and DNA in tissues from cats acutely infected with clade B or clade C FIV isolates [38, 39]. FIV-B-2542 and FIV-C-PaddyGammer (FIV-C-Pgmr) replicate to high titer during acute phase infection and can be transmitted mucosally [10, 25, 40-42]. Our results contribute further insight on the cell targets of FIV during acute infection. We discuss problems encountered during assay development and strategies employed to resolve them. Improved in situ assays leading to more precise mapping of in vivo FIV pathways should provide valuable insight into the course of early HIV dissemination.

MATERIALS & METHODS

Animals and tissue processing

Two groups (five cats per group) of eight-week-old cats from a specific pathogen-free (SPF) breeding colony maintained at Colorado State University (Fort Collins, CO) were inoculated intravenously with 100 tissue culture infectious doses (TCID) of acute-phase plasma pools of FIV [43]. Cats were inoculated with FIV-B-2542 [44] or FIV-C-Pgmr [45]. The cats were observed daily for signs of illness following virus inoculation. Three weeks post-inoculation, the cats were anesthetized with ketamine/acepromazine and blood was collected into heparinized and EDTA tubes. Following blood collection, the animals were euthanized by intravenous pentobarbital sodium injection. Full necropsies were performed. Blood was collected primarily to establish acute-phase virus stock for future studies in our laboratory. Tissues were gathered to develop the histologic assays described in this report. At necropsy we collected brain, peripheral and internal

lymph nodes, thymus, liver, spleen, small and large intestine, pancreas, kidney, and bone marrow. Blood and tissues from an age-matched uninfected SPF cat were used as negative controls. Tissues were preserved in a variety of fixatives including 10% neutral-buffered formalin, 4% paraformaldehyde, absolute ethanol, and Histochoice-MB (Amresco, Solon, OH). Tissues were fixed overnight and processed the following morning into paraffin-embedded blocks by a "short-run" method which avoided the use of formalin and which minimized the length of time tissues were immersed in liquid paraffin (Colorado State University Histology Laboratory, Fort Collins, CO). Routine five uM paraffin sections were placed on silanized slides without heat treatment and allowed to air dry at least one day prior to staining.

DNA PCR

Nested DNA polymerase chain reaction (PCR) assays adapted from a method by O'Neil et al [44] were performed on isolated blood mononuclear cells to confirm FIV infection. Blood mononuclear cells from anticoagulated whole blood were isolated by ficoll-Hypaque density centrifugation separation (Histopaque-70, Sigma Corp., St. Louis, MO) and rinsed with phosphate-buffered saline (PBS, pH 7.4). DNA was extracted from the cells with the Qiamp DNA Blood Mini Kit (Qiagen, Carpinteria, CA) according to the manufacturer's instructions. Purified DNA was quantitated with a Beckman DU-70 spectrophotometer (Beckman Coulter, Brea, CA). One microgram of sample DNA was amplified by PCR. PCR solutions were comprised of 2.5 mM MgCl2, 1X PCR buffer, 200 uM each dNTP (all P-E Applied Biosystems, Foster City, CA), 100 nM each primer (Sigma Genosys, The Woodlands, TX), 2.5 U Taq polymerase (Sigma, St. Louis, MO),

and deionized H_2O qs 100 uL. The first round primers were gag129 (5'-CGTAACTA-CAGGACGAGAACCTG-3') and gag 802 (5'-CCAACTTTCCCAATGCTTCAAG-3'). Primers gag3 (5'-TTGACCCAAAAATGGTGTCCA-3') and gag4 (5'-TTCTGC-TTGTTGTTCTTGAGT-3') were used in the second round to produce a 293 base pair sequence nested within the first round product. PCR reactions were carried out in a Perkin-Elmer 480 thermocycler (P-E Applied Biosystems) with an initial 94° C denaturation for 1 min, followed by cycles of 1 min each 94° C denaturation, 55° C (1st round) or 60° C (2nd round) primer annealing, and 72° C extension. After 35 cycles, a final extension at 72° C for 7 min was performed. One uL of the first round product was placed in the second round reaction. Ten percent of the final PCR product was electrophoresed on a 1.5% Seakem LE agarose gel containing Gelstar DNA fluorescence solution (both FMC Bioproducts, Rockland, ME) according to manufacturer's directions, and visualized under UV illumination.

Viral RNA load

Semi-quantitative viral RNA loads were determined based on an assay described by Diehl et al [39] . A 21-base deletion competitor RNA molecule based on the gag 3/gag 4 gene product was synthesized by employing a 5' primer consisting of gag3 and ten complementary bases located 21 nucleotides 3' to gag3 (5'-TTGATCCAAAAA-TGGTGTCCAGAAGGGTTAG-3'). This resulted in an amplified product which looped out the 21 bases immediately 3' to gag3. The 21-base deletion DNA PCR product was cloned using the Topo TA System (Invitrogen, San Diego, CA). Plasmid DNA was purified using the QiaFilter Plasmid Midi Kit (Qiagen) according to instructions. Plasmid DNA was linearized with the NotI restriction endonuclease (New England Biolabs,

Beverly, MA). Run-off RNA transcription was performed with the Ampliscribe T7 High Yield Transcription Kit (Epicentre, Madison, WI) and residual DNA was digested with RQ1 RNase-free DNase (Promega, Madison, WI). RNA was purified with the Qiamp Viral RNA Mini Kit (Qiagen) and quantitated on the DU-70 spectrophotometer. RNase-free 1X tris-EDTA buffer (pH 7.0; Sigma) was added to the purified RNA to a final concentration of 10^{10} molecules/uL. Individual aliquots were frozen at –70° C; they were thawed and serially diluted immediately prior to PCR.

To determine plasma viral RNA load, RNA was purified from multiple 200 uL EDTA-plasma volumes using the Qiamp Viral RNA Mini Kit (Qiagen). One-half of the purified RNA (equivalent to the amount in 100 uL plasma) and 1 µl of freshly thawed, serially diluted competitor RNA containing from 10^9 to 10^2 copies of FIV RNA was added to each PCR reaction. PCR solutions were derived from the GeneAmp EZrTth RNA PCR Kit (PE Applied Biosystems). Reverse transcriptase PCR (RT-PCR) was performed in a P-E 9600 thermocycler (P-E Applied Biosystems) using primers gag3 and gag4. Following a 30 minute reverse transcription step at 65° C, the samples were cycled 40 times at 94° C for 10 sec, 60°C for 20 sec, and 72° C for 30 sec, with a final 72° C extension for 4 min. Products were electrophoresed on 3% Metaphor agarose gels containing Gelstar (FMC Bioproducts, Rockland, ME) and visualized under UV illumination. The 21-base size separation allowed differentiation of competitor and wild-type products. Plasma viral RNA load was calculated based on the point of band intensity equivalence between competitor and wild type PCR products.

Tissue culture infectious dose

Pooled plasma samples from the FIV-inoculated cats were placed into coculture with primary naïve feline blood mononuclear cells to create amplified acute-phase viral stocks for future studies in our laboratory. Plasma samples were inoculated into wells containing $2x10^6$ naïve feline blood mononuclear cells pre-stimulated with 5 ug/mL concanavalin A (Sigma) and maintained with 100 U/mL recombinant human IL-2 (Roche Molecular Biochemicals, Indianapolis, IN) in RPMI medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 2% glutamine, and 1% penicillin/streptomycin (all Gibco BRL, Rockville, MD). Cell cultures were maintained in 24-well Falcon plates (Becton Dickinson, Franklin Lakes, NJ) in humidified 37° C incubators with 5% added CO2. One-half of the culture medium was replaced twice weekly. Productive in vitro infection was assessed by testing supernatants with a capsid antigen (p26) capture ELISA employing monoclonal antibody 51G11.1 as described by Dreitz et al [46] . Optical density (OD), measured by absorbance at A_{450} (reference A_{570}), was recorded using a Dynatech 5000MR™ microplate reader (Dynatech Corp., Chantilly, VA). Positive reactions were defined as those with a minimum OD of 0.1 and at least twice that of negative control supernatant run in tandem. Tissue culture infectious dose (TCID) of primary coculture supernatants was determined by in vitro inoculation of a dilutional series of the primary coculture supernatants with additional naïve feline blood mononuclear cells. Cultures were maintained and assayed as described above. Supernatant tissue culture infectious dose was determined from the highest dilution yielding two consecutive positive ELISA results in secondary coculture within 4 weeks.

FIV chromogenic (single label) immunohistochemistry

Tissue sections on silanized glass slides were deparaffinized by brief heat treatment and rehydration through xylene and a series of graded alcohols, and then washed in TENT solution (0.05 M Tris, pH 7.4, 0.001 M EDTA sodium, 0.15 M NaCl, 0.05% Tween,). Endogenous peroxidases were inactivated by immersing the slides in a Coplin jar with 3% H_2O_2 in phosphate buffered saline (PBS, pH 7.4) for 5 minutes on an orbital rocker with agitation. Formalin- and paraformaldehyde-fixed sections were subjected to antigen retrieval by placing the slides in an uncovered plastic Coplin jar in citrate Antigen Unmasking Solution (Vector). The slides were heated in a microwave oven on high power for 1 min, low power for an additional 9 min, and allowed to cool uncovered on a benchtop for 20 min. No antigen retrieval was performed on ethanol or Histochoice-fixed tissues. Sections were blocked for 15 minutes in 1% each naïve goat and cat serum in TNB blocking buffer (TSA™ Systems, NEN, Boston, MA). A second block was performed with 1 ug/ml unconjugated protein A (Sigma) in TNB for 5 minutes. While the tissue sections were being blocked, equal parts high titer FIV antibody-positive cat plasma and 1 mg/ml stock biotin-conjugated protein A (Sigma) were diluted 1:100 in TNB and allowed to bind at room temperature in solution. The sources of FIV-specific antibodies were cats 2103 and 2104, both chronically infected with FIV-A-Petaluma. Plasma from these animals is routinely used as an antibody source for FIV immunoassays in our laboratory [44, 46] . After 20 minutes of protein A-biotin binding to antibody at the Fc fragment, the solution was further diluted as determined empirically for a given collection of plasma. Final dilutions typically ranged from 1:1000—1:10,000. (We used the lowest dilution for a given plasma source which

consistently produced no staining in naïve control tissues.) Tissue sections were washed with TENT after blocking and the biotin-labeled antibody solution was applied. Sections were incubated in a humidified chamber at 37° C for 2 hours. Slides were washed and signal was amplified with tyramide using the TSA Biotin System (NEN) according to the manufacturer's instructions. Positive signal was detected with 3,3'-diaminobenzidine tetrahydrochloride (DAB), Vector VIP, or Nova Red chromogens (Vector Laboratories, Burlingame, CA) and counterstained with Gill's hematoxylin, methyl green (Vector), or 0.5% Evan's blue dye (Sigma). Sections were dehydrated through graded alcohols to xylene, mounted with Cytoseal XYL (Stephens Scientific, Kalamazoo, MI) and routinely coverslipped. Equivalent specimens from FIV-unexposed SPF cats were included in each run as negative controls. Histologic sections were examined with an Olympus BX60 microscope (Olympus America, Lake Success, NY).

Quantitative image analysis

Digital images of immunostained tissues were captured using the 3-CCD 1140 x 1520 detail CoolSNAP camera and software system (Roper Scientific GmbH, Bergkirchen, Germany). Images were analyzed with Metamorph™ software (Universal Imaging Corp., West Chester, PA). Using a stage micrometer, field area visualized by the CCD camera through the 20X objective was determined to be 0.13 mm^2. Positively stained cells were enumerated using Metamorph's thresholding (color calibration) and cell counting functions. Average pixel area per positive cell was determined by summing the area of chromogen stain in 50 individual FIV^+ cells and dividing the total by 50. Positive cell counts were determined by measuring the total pixel area of chromogen within the field and dividing the total positive pixel area by the average value per

positively stained cell for that region. Ten fields per tissue were counted. Field cell counts were logged into an Excel spreadsheet (Microsoft Corp, Redmond, WA) from which mean values and standard deviations were calculated. Because there were no significant differences between the FIV-B-2542 and FIV-C-Pgmr source tissues in mean number of infected cells per unit area in comparable regions, reported values are for all the FIV-infected cats as a group. In order to convert number of positive cells per 20X field field to an estimate of FIV^+ cells per gram of tissue, we made the assumption that the examined field was representative of the compartment as a whole. We then determined the number ov FIV^+ cells per cm2 tissue using the formula: $\frac{FivPosCells}{0.13mm^2} * \frac{100mm^2}{cm^2} = \frac{FivPosCells}{cm^2}$. Assuming that 1 g of tissue =~1 cm^3, we used the following formula to estimate the number of FIV^+ cells per g tissue: $\sqrt{\frac{FivPosCells}{cm^2}^3} = \frac{FivPosCells}{g}$.

FIV RNA in situ hybridization

Slides were rehydrated and endogenous peroxidases quenched as described above. Formalin, paraformaldehyde, and Histochoice-fixed sections were treated with 10 ug/mL proteinase K (Sigma), prewarmed to 37° C, for 1—20 minutes as determined empirically for a given section. Sections were prehybridized with a commercial hybridization solution containing 45% formamide (Amresco) and 10 ug/mL sheared denatured salmon sperm DNA and 100 ng/mL tRNA (both Sigma) at 53° C for 10 min. Following prehybridization, coverslips were shaken off and hybridization solution containing 50 ng digoxigenin-labeled whole FIV antisense RNA probe/slide (Lofstrand Labs, Lofstrand, VA) was applied and slides were recoverslipped. Slides were heated on a 70° C block for 10 minutes and then placed in a 53° C incubator overnight. The next day slides were

rinsed in ethanol and air dried. Sections were rehydrated through a graded alcohol series and washed in a solution of 0.2% BSA and 0.2X SSC at 53° C for 10 minutes. After washing in TENT, 1:100 peroxidase-labeled anti-digoxigenin FAb (Roche Molecular Biochemicals) and 6 ng/mL RNase A (Sigma) were applied to the slides and incubated at 37° C for 20 minutes. Signal was amplified with tyramide and detected with chromogen or fluorescence and counterstained as described above. Negative controls consisted of tissues from FIV-unexposed cats, as well as tissues from FIV^+ cats which were hybridized with sense rather than antisense probe.

DNA in situ hybridization

Tissue sections were processed essentially as described above for RNA in situ hybridization. Ethanol-fixed sections were not digested with proteinase K. Following prehybridization, hybridization solution containing 50 ng/slide digoxigenin-labeled sense RNA probe was applied and sections coverslipped. Slides were heated at 90° C on a heat block for 30 seconds (ethanol-fixed tissues) or 3 minutes (formalin-fixed tissues) to denature chromosomal DNA and allow probe hybridization. Slides were incubated at 37° C overnight and probe was detected and slides counterstained as described above.

FIV and cell phenotype fluorescence immunohistochemistry

For two- or three-color fluorescence immunohistochemistry, protein A-biotin-conjugated feline anti-FIV antibodies were detected with Fluorolink™ Cy3-streptavidin (Amersham Pharmacia Biotech, Piscataway, NJ) following biotin-tyramide amplification, or directly with Cy3-conjugated tyramide. Cell phenotype antibodies were labeled with fluorescein isothiocyanate (FITC) or coumarin, with or without tyramide amplification as

needed. To idenitfy the FIV-infected cells, mouse and/or rabbit-origin phenotype-specific monoclonal antibodies were added to the cat antibody/biotin-protein A solution immediately prior to placement on tissue sections. Following primary antibody binding, FITC-conjugated anti-mouse or anti-rabbit IgG (Sigma) diluted 1:100 was applied to slides for 20 minutes. In some cases, tyramide amplification was required to visualize phenotype labels (TSA Fluorescence Systems, NEN). In such cases, slides were immersed in 3% H_2O_2 for 1 minute between successive amplifications to quench any unreacted horseradish peroxidase from the previous tyramide amplification.

T cells were labeled with polyclonal rabbit anti-CD3 antibody (Sigma). Monocytes bearing lipopolysaccharide (LPS) receptors were detected with anti-CD14 (Dako), or Alexa 488-conjugated LPS (Molecular Probes, Eugene, OR). Four antibodies were used to identify subsets of tissue macrophages: AM-3K (a gift from M. Takeya, Osaka Prefecture University, Sakai, Japan) [47-49] , FeMy, which recognizes a feline myeloid cell antigen [50] , Mac 387, which labels a subset of macrophages and polymorphonuclear cells (Serotec), and anti-CD 74 (Sigma), a histiocyte marker. Dendritic cells were labeled with anti-FDC monoclonal CNA.42 (Dako), anti-fascin (anti-p55; Dako), or rabbit polyclonal anti-S-100 (Serotec). Mesenchymal cells (including leukocytes) were differentiated from epithelial cells by labeling for the intermediate filaments vimentin and cytokeratin, respectively (Serotec). (Note: unless otherwise stated all of the cell phenotype markers listed above are mouse anti-human monoclonal antibodies found to cross-react with homologous feline antigens.)

Following fluorescent antibody labeling, cell nuclei were stained with 1 ug/mL 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) for 1 minute. If two cell

phenotype markers were simultaneously applied with FIV staining, one phenotype marker was labeled with coumarin and the other with FITC. No nuclear stain was performed on such sections so as not to confuse DAPI with coumarin fluorescence through the blue channel. Hydrated sections were allowed to partially air dry and were mounted with Vectashield anti-fade medium (Vector). Digital images were captured using the CoolSnap™ camera and software system (Roper Scientific, Munich, Germany). Quantitation of the cell phenotypes containing FIV was performed by direct visualization of multiple tissue sections representing all of the FIV-infected animals. The cell phenotype of 50 FIV^+ cells per tissue, or all FIV^+ cells if fewer than 50 were present in a given tissue, was determined and manually recorded. Serial digital images of multi-stained fluorescent sections, captured with the CoolSNAP™ system described above, were overlaid using Adobe Photoshop software (Adobe Systems, San Jose, CA) on a Power Macintosh G3 computer (Apple Computer, Cupertino, CA).

RESULTS

FIV acute infection kinetics

All cats inoculated with FIV were positive by DNA PCR on isolated blood mononuclear cells collected at the time of necropsy. Plasma viral RNA loads ranged from 10^5—10^6 copies/mL in cats inoculated with FIV-B-2542, and 10^6—10^8 copies/mL in cats inoculated with FIV-C-Pgmr. Pooled plasma-inoculated supernatant tissue culture infectious dose (TCID) means were $5.1x10^3$ TCID/mL for the FIV-B-2542 group and $2.5x10^4$ TCID/mL for the FIV-C-Pgmr cohort. Blood mononuclear cells and plasma from the FIV-naïve cat were negative for FIV by PCR and coculture.

FIV immunohistochemistry

FIV antigens were detected in the tissues of infected cats, and no FIV was detected in naïve cat specimens (Figs. 2.1 and 2.2). For detecting FIV proteins, ethanol- and Histochoice-fixed tissues were superior to antigen-retrieved formalin and paraformaldehyde-fixed specimens. No FIV staining was observed in formalin and paraformaldehyde-fixed tissues without microwave antigen retrieval. Lymphoid tissues from FIV-inoculated cats contained many immunopositive cells, while equivalent tissues from naïve cats did not. In lymph nodes, most FIV^+ cells were found in in germinal centers and in cells in paracortical regions (Table 2.1). Fewer positive cells were present in medullary regions. In germinal centers, FIV was detected in or on the surface of large cells (>30 uM diameter) with arborizing extensions, suggesting antigen trapping by the follicular dendritic cell network (Fig. 2.3). In other areas, FIV antigens were located within cell cytoplasm, as expected (Fig. 2.4). Most FIV^+ cells in the paracortex were morphologically compatible with lymphocytes, and their anatomic location was consistent with T-cell rich regions. Few FIV^+ cells were seen within B cell-rich zones of follicles. Fewer FIV+ cells were seen in lymph node medullary regions than in the cortex (Table 2.1).

Within the thymus, a rim of positive cells was sometimes concentrated in the inner cortex near mid-lobule (Fig. 2.5). In general, FIV^+ immature thymocytes in the peripheral cortex outnumbered infected mature thymocytes in the medulla (Table 2.1; Fig. 2.5). Most FIV expression in the thymic medulla was near or within Hassal's corpuscles (Fig. 2.6). In contrast to what has been reported for HIV, we did not detect FIV antigens in $cytokeratin^+$ thymic epithelial cells.

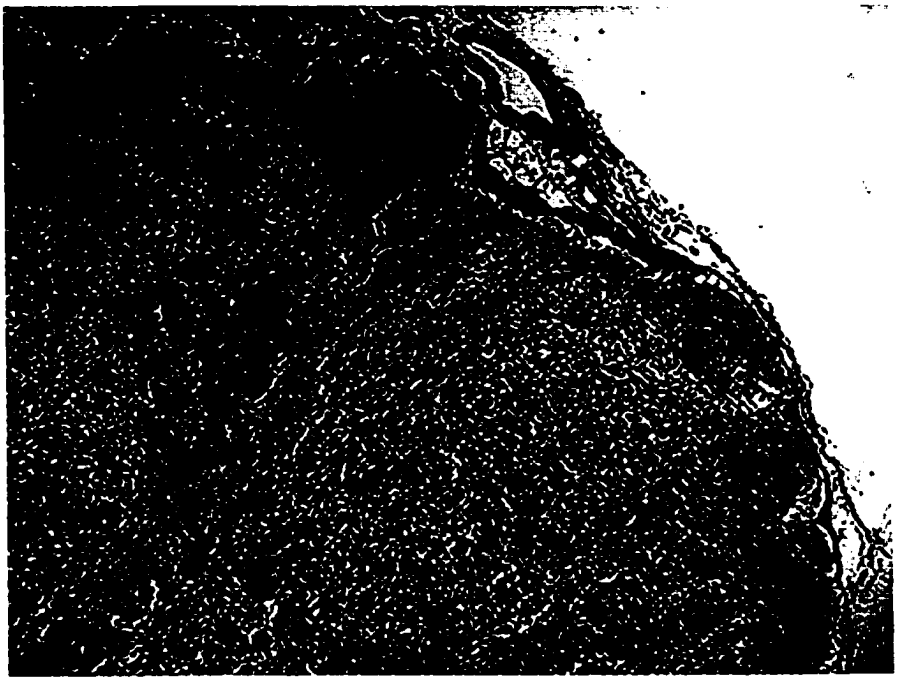

Figure 2.1: Immunohistochemistry, lymph node: FIV-naïve cat. Note paucity of follicles and absence of FIV antigens (purple). Vector VIP, Evan's blue counterstain; bar = 400 uM.

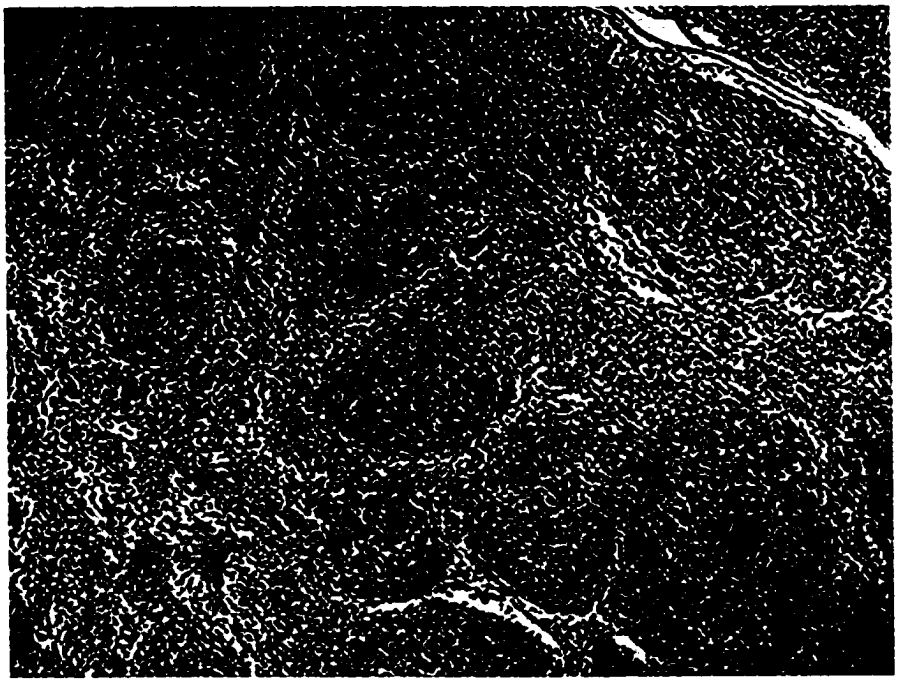

Figure 2.2: Immunohistochemistry, lymph node: Cat acutely infected with FIV-B-2542. Note well formed primary and secondary follicles. FIV (purple) is in germinal centers and in cells distributed throughout the parafollicular cortex. Vector VIP, Evan's blue counterstain; bar = 400 uM.

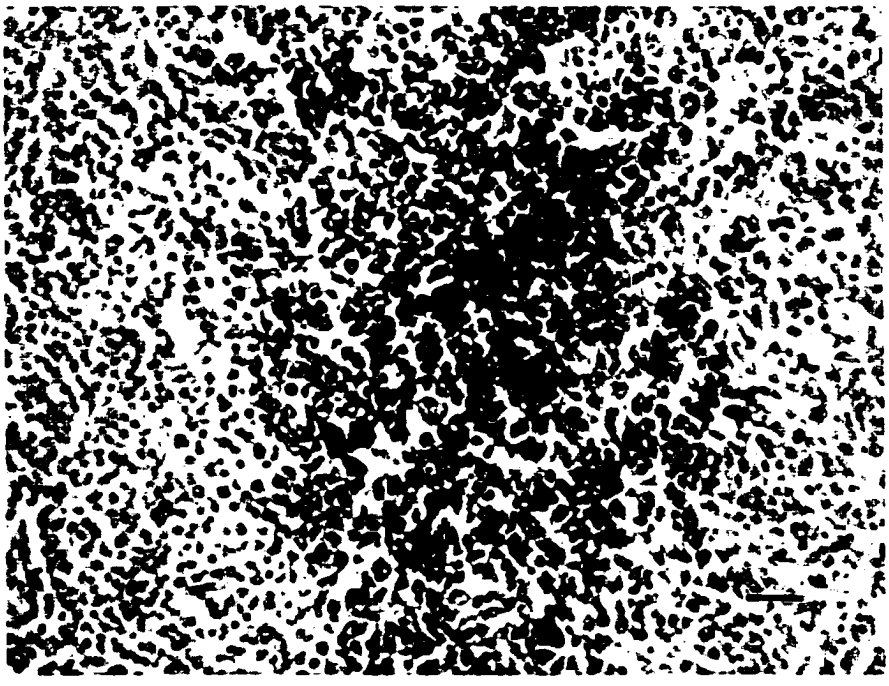

Figure 2.3: Lymph node, immunohistochemistry: Note diffuse faint staining and dark extended cell processes (purple) suggestive of follicular dendritic cell FIV antigen trapping in the germinal center. Vector VIP, Evan's blue counterstain; bar = 90 uM.

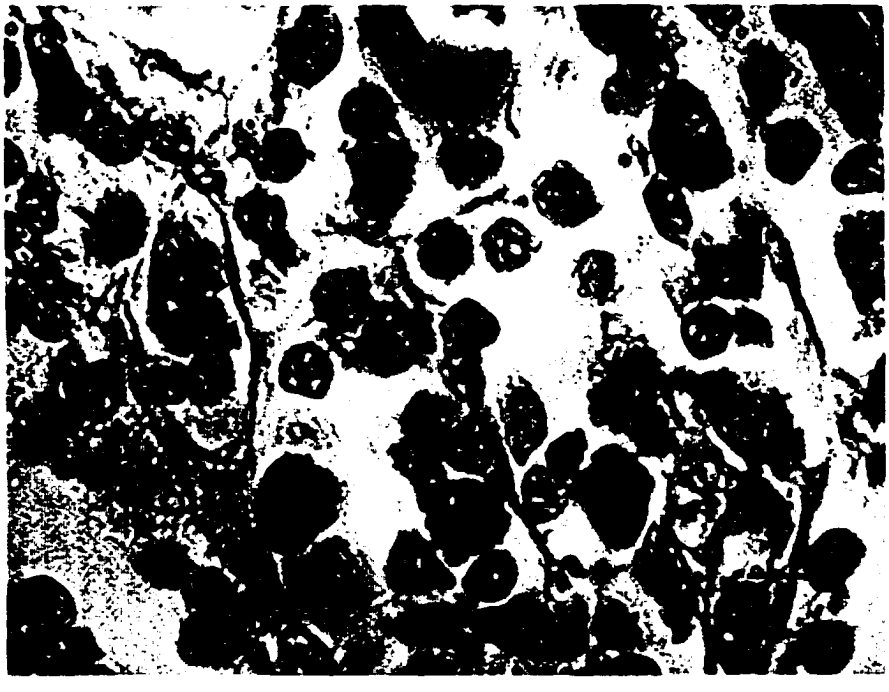

Figure 2.4: Immunohistochemistry, lymph node. Note cytoplasmic localization of FIV antigens (dark purple). Vector VIP, Evan's blue countersain; bar = 15 uM.

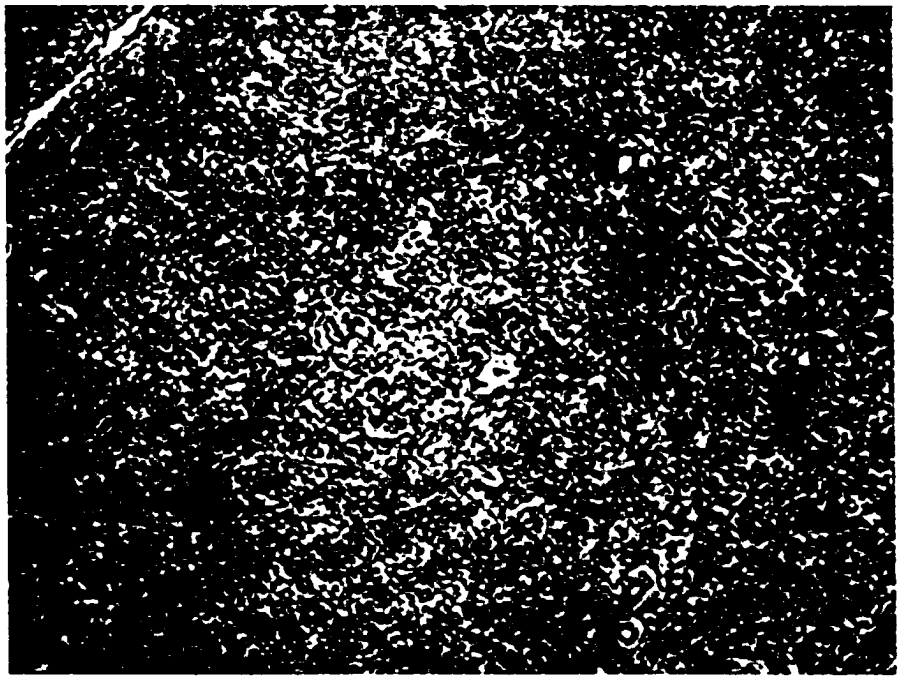

Figure 2.5: Immunohistochemistry, thymus. Note rim of FIV+ cells in mid-lobule. FIV-positive cells (dark purple) in cortex outnumber positive cells in medulla. Vector VIP, Evan's blue counterstain; bar = 200 uM.

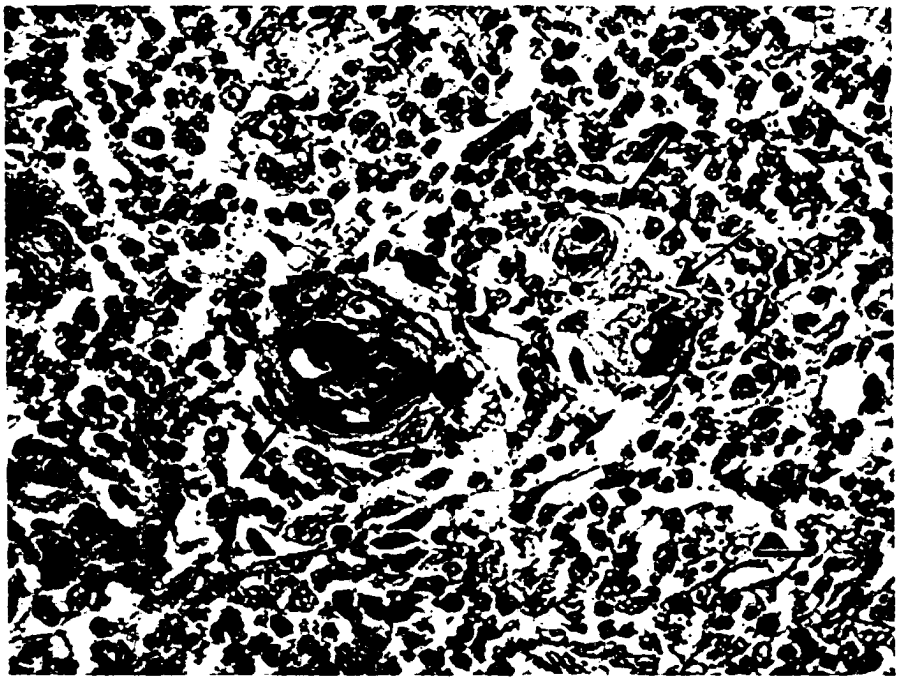

Figure 2.6: Immunohistochemistry, thymus. Note FIV (purple) in Hassal's corpuscles (arrows) and in scattered smaller round cells (presumably mature thymocytes). Vector VIP, Evan's blue counterstain; bar = 40 uM.

Table 2.1: Prevalence of cells exhibiting active FIV replication in tissues from cats acutely infected with FIV-B-2542 and FIV-C-Pgmr, as determined by immunohistochemistry and software-assisted quantitative analysis.

Tissue	FIV+ cells/20X field*	FIV+ cells/g tissue
Brain	0	0
Thymus	15.3	1,278,713
Lymph node	18.5	1,700,175
Liver	0.6	9,930
Spleen	8.0	483,472
Intestine§	12.1	899,320
Kidney	0	0
Bone marrow	14.3	1,155,421

* Mean for tissues examined from all FIV^+ cats; 20X field = 0.13 mm^2.
§ Mucosa and submucosa only.

There were abundant FIV^+ cells morphologically resembling leukocytes in the lamina propria of all intestinal sections examined (Fig. 2.7; Table 2.1). Indeed, intestinal sections proved to be one of the most reliable sample sources for detecting FIV from a given animal. Surprisingly, FIV antigens were also detected in cells of the mucosal epithelium. Staining was not observed when FIV-naïve serum was substituted for FIV Ab^+ serum at the same concentration (Fig. 2.8). In order to confirm that the staining of intestinal epithelial cells was not an antibody-binding artifact, sections were probed for viral RNA by in situ hybridization. Viral RNA was detected in cells of the mucosal epithelium, as well as leukocytes in the lamina propria and GALT (Fig. 2.9). Peyer's patches in the ileum demonstrated reactive and hyperplastic changes in FIV-infected cats, with abundant FIV antigens (Fig. 2.10). Submucosal GALT from other intestinal segments was histologically unremarkable and usually contained fewer FIV^+ cells than did the overlying lamina propria (Table 2.1).

Within the spleen, most FIV^+ cells were located in expanded periarteriolar lymphatic sheaths (PALS) which sometimes took on the appearance of follicles (Fig.

2.11; Table 2.1). These structures were common in FIV-infected cat tissues but not in naïve controls. The hyperplastic PALS in FIV^+ cat tissues were adjacent to arterioles and were rich in T cells, as confirmed by anti-CD3 staining. Very few FIV^+ cells were identified in non-PALS areas. Liver contained scattered rare FIV^+ cells (Table 2.1). Where present, intrahepatic FIV^+ cells had an appearance and location consistent with Kupffer cells or circulating mononuclear cells (Fig 2.12). FIV demonstrated no tropism for kidney. Although many FIV isolates, including FIV-B-2542, have a known tropism for the central nervous system [9, 21, 51] , we identified no FIV antigens in the brains of these acutely infected animals (Table 2.1).

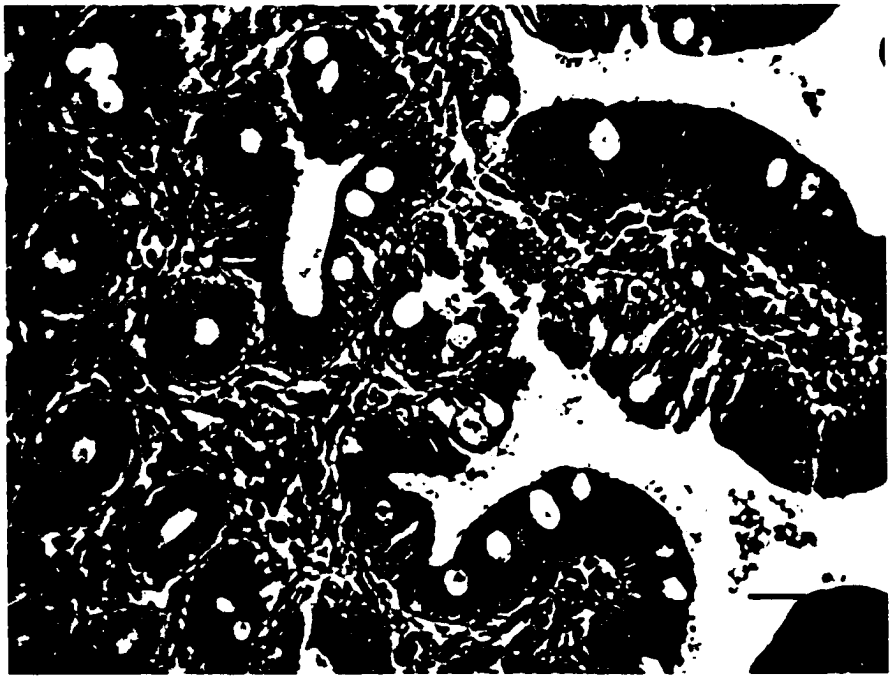

Figure 2.7: Immunohistochemistry, small intestine. FIV (dark purple) is found within leukocytes in the lamina propria, especially between crypts. Surprisingly, FIV antigens are also appear to be located in mucosal epithelial cells. Vector VIP, Evan's blue counterstain; bar = 90 uM.

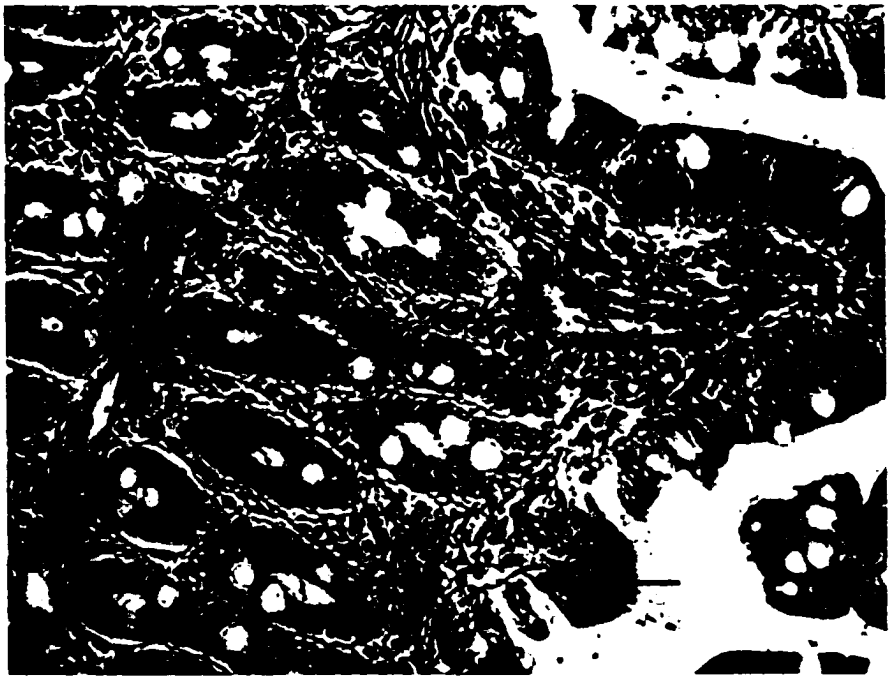

Figure 2.8: Immunohistochemistry, small intestine. Same specimen as shown above (Fig. 2.7), but here labeled with FIV-naïve serum instead of FIV Ab^+ serum. Vector VIP, Evan's blue counterstain; bar = 90 uM.

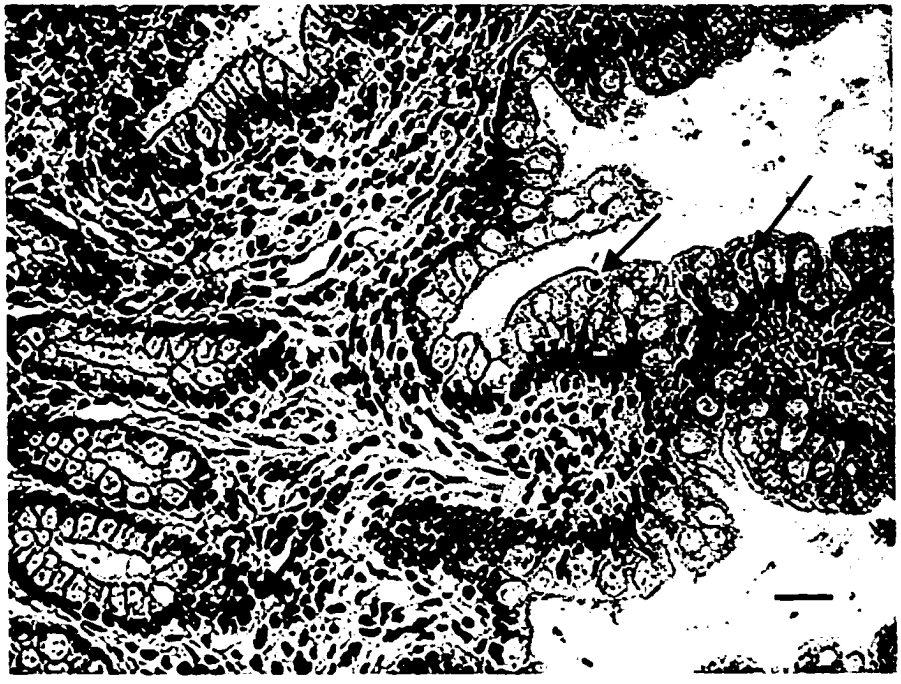

Figure 2.9: RNA in situ hybridization, small intestine. Note viral RNA in cells within the lamina propria (thick arrows) and mucosal epithelium (thin arrows). FIV RNA—DAB, hematoxylin counterstain; bar = 90 uM.

Figure 2.10: Immunohistochemistry, Peyer's patch, ileum. Note reactive hyperplasia and abundant FIV antigens (brown) centrally. DAB, hematoxylin counterstain; bar = 200 uM.

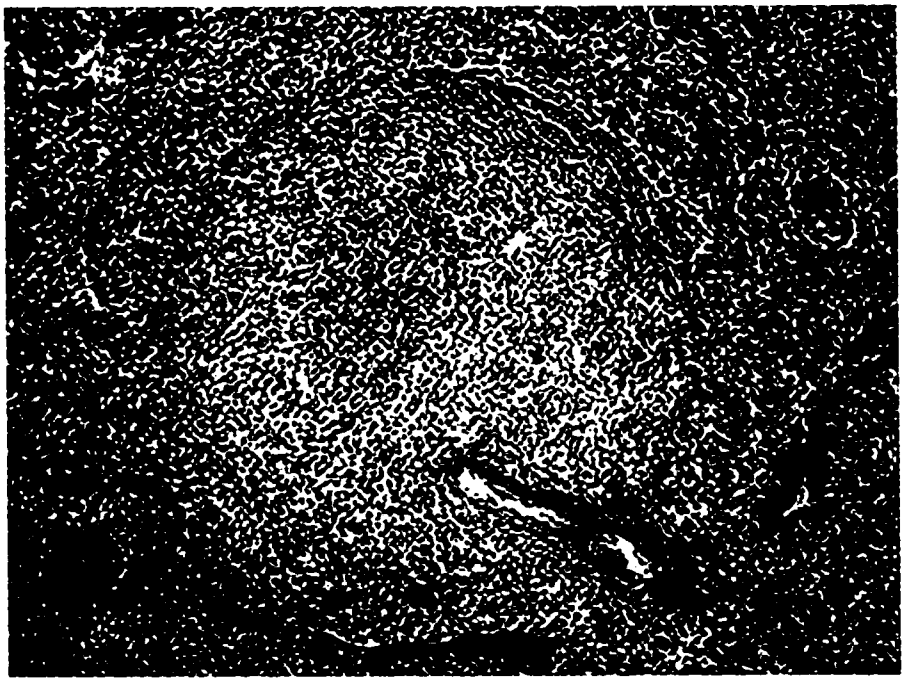

Figure 2.11: Immunohistochemistry, spleen. Note expanded periarteriolar sheath with many FIV^+ cells (purple). FIV^+ cells also line the adventitia of the arteriole at lower right. Vector VIP, Evan's blue counterstain; bar = 200 uM.

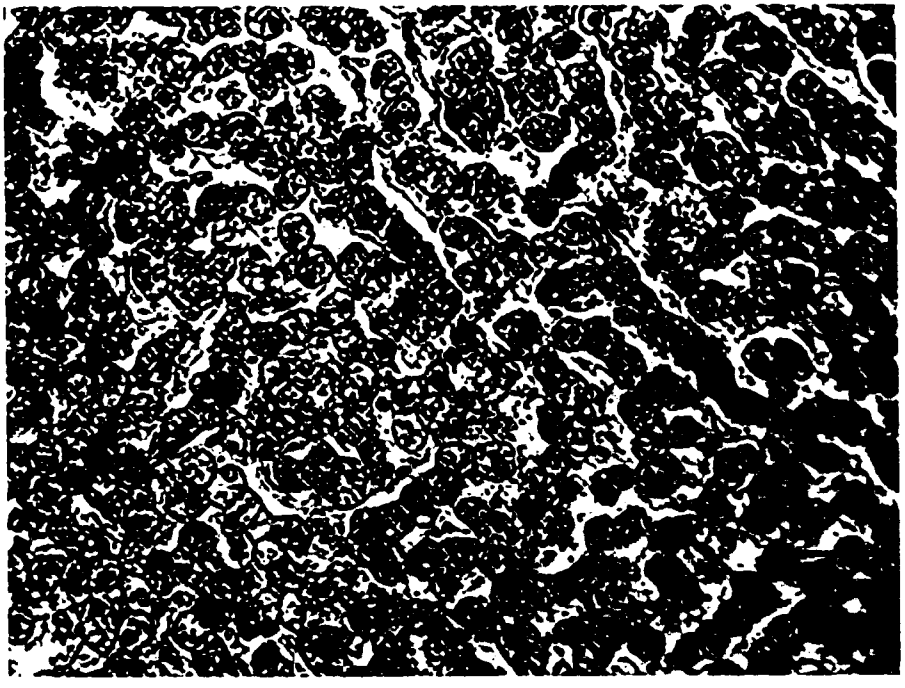

Figure 2.12: Immunohistochemistry, liver. A rare FIV^+ (arrow) Kuppfer cell or intrasinusoidal mononuclear cell is located in the center of this photomicrograph. Vector VIP, Evan's blue counterstain; bar = 40 uM.

RNA in situ hybridization

We detected FIV^+ cells by RNA in situ hybridization in tissues from inoculated cats, and saw no staining in FIV-naïve cat tissue (Figs. 2.13 and 2.14) However, in general, we detected more FIV^+ cells by immunohistochemistry than by RNA in situ hybridization. Possible explanations for this are considered in the Discussion. Moreover, tissue digestion steps required to make viral nucleic acids probe-accessible caused some loss of morphologic detail. We performed RNA in situ hybridization to serve as the baseline by which other assays were compared, and to confirm infection of cells identified as positive by immunohistochemistry. Because in our hands immunohistochemistry demonstrated equal or superior sensitivity to RNA in situ hybridization, we employed immunohistochemistry for detecting FIV in the cell co-labeling studies described below.

DNA in situ hybridization

The same tissues positive for FIV by immunohistochemistry and RNA in situ hybridization also contained demonstrable provirus by DNA in situ hybridization,; tissues from naïve animals were appropriately negative (Figs. 2.15 and 2.16). However, as with RNA in situ hybridization, we detected fewer FIV-infected cells by DNA in situ hybridization than by immunohistochemistry (Figs. 2.17 and 2.18).

Figure 2.13: RNA in situ hybridization, lymph node. There is no probe hybridization in tissue from this naïve cat. Vector NovaRed, Evan's blue counterstain; bar = 200 uM.

Figure 2.14: RNA in situ hybridization, lymph node. Note FIV RNA^+ cells (red/brown) in the peripheral cortex and subcapsular sinus from this cat with acute FIV infection. Vector NovaRed, Evan's blue counterstain; bar = 200 uM.

Figure 2.15: DNA in situ hybridization, lymph node. Naïve cat with no FIV provirus. FIV—DAB, hematoxylin counterstain, bar = 120 uM.

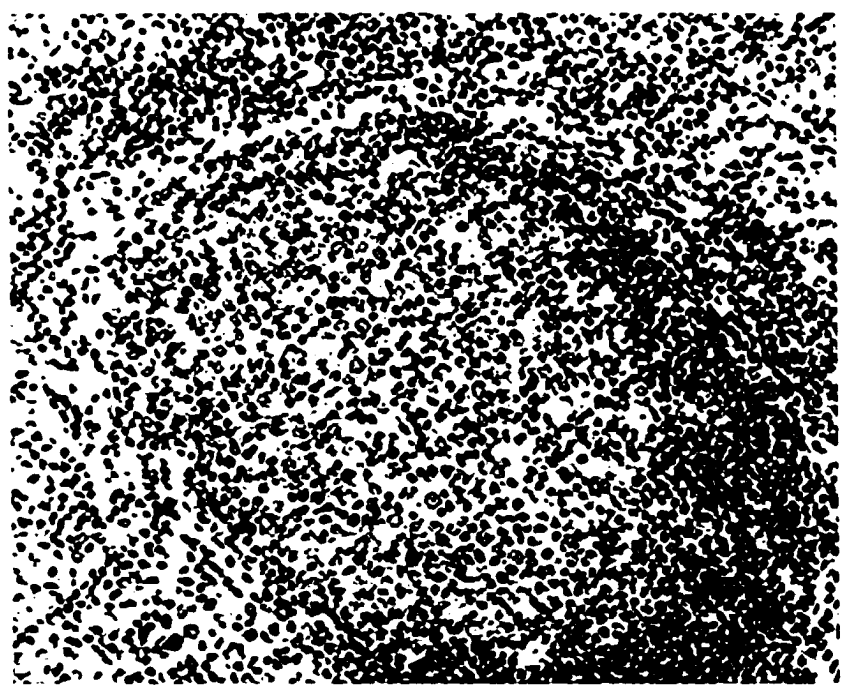

Figure 2.16: DNA in situ hybridization, lymph node. Most provirus^{+} cells (brown) in lymph node from this FIV^{+} cat are located in the parafollicular cortex. FIV—DAB, hematoxylin counterstain, bar = 120 uM.

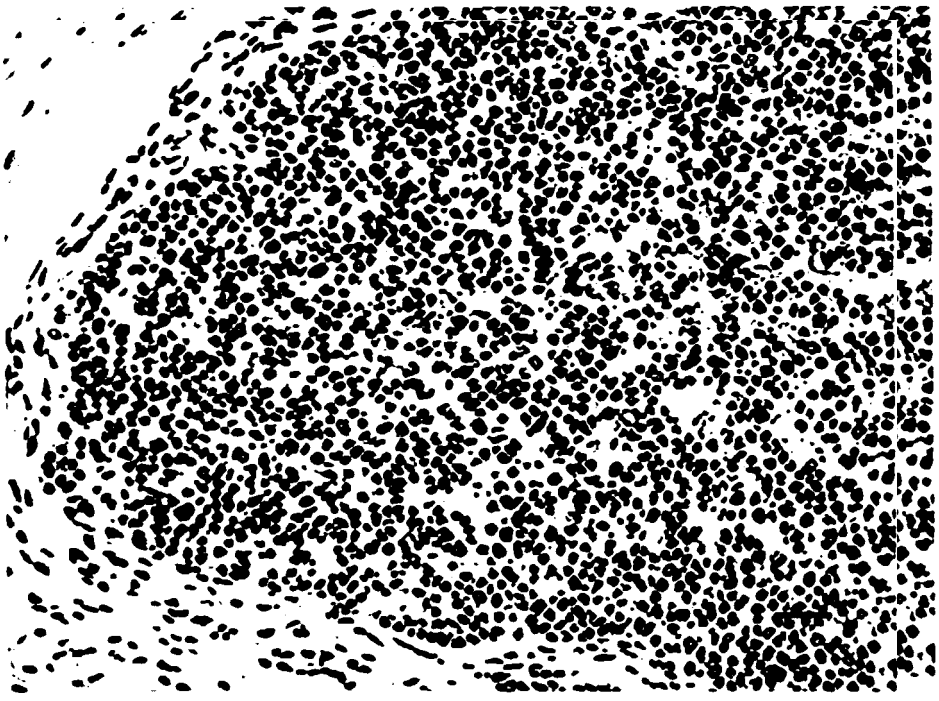

Figure 2.17: Immunohistochemistry, Peyer's patch, ileum. Note FIV^+ cells (brown) amid diffuse staining, suggesting both intracytoplasmic and surface viral localization. FIV—DAB, hematoxylin counterstain; bar = 90 uM.

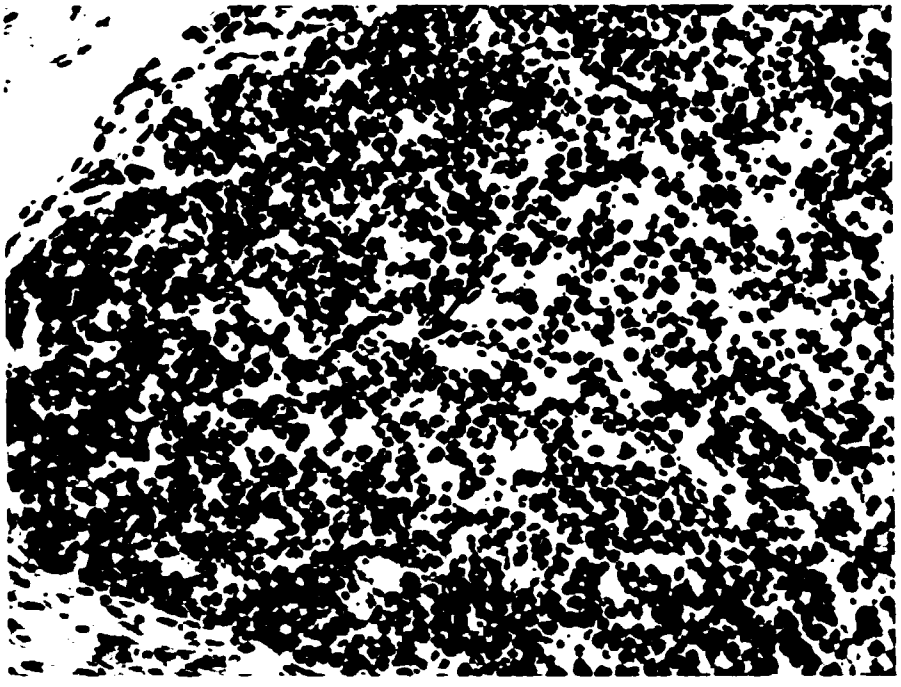

Figure 2.18: DNA in situ hybridization, Peyer's patch, ileum. Relatively few cells contain detectable provirus (arrows), suggesting a lower sensitivity of DNA in situ hybridization for detecting FIV than immunohistochemistry. FIV—DAB, hematoxylin counterstain; bar = 90 uM.

FIV/cell phenotype dual fluorescence immunohistochemistry

Most phenotype-identified cells containing detectable FIV antigens were $CD3^+$ T cells (Fig. 2.19; Table 2.1). In the thymus, many unlabeled FIV^+ cells in the cortex were of the same size and shape as $CD3^+$ T cells in the medulla, suggesting that they were immature thymocytes.

In most tissues, ~10—15% of FIV^+ cells co-labeled with the anti-macrophage monoclonal antibody AM-3K (Fig. 2.20; Table 2.1). AM-3K labeled many more tissue macrophages than did the other histiocyte markers we employed. In co-labeling assays, $FeMy^+$ and Mac 387^+ cells were also positive for AM-3K, but $FeMy^+$ and Mac 387^+ cells were generally exclusive of one other (except in lymph node cortical parafollicular regions). $FeMy^+$ cells sometimes contained FIV antigens (Fig. 2.21), but Mac 387^+ macrophages were almost never FIV^+ (Fig. 2.22). $CD74^+$ histiocytes were likewise devoid of detectable FIV antigens. Monocytes bearing LPS receptors, as determined by positive staining with anti-CD14 antibody or Alexa 488-conjugated LPS, were virtually always negative for FIV. Monoclonal antibodies which demonstrated inconsistent or complete absence of labeling of feline monocyte/macrophages in tissue sections by our methods included those directed against human and murine CD11b (Sigma), human CD11c (Sigma), human CD68 (Dako), and human lysozyme (Dako).

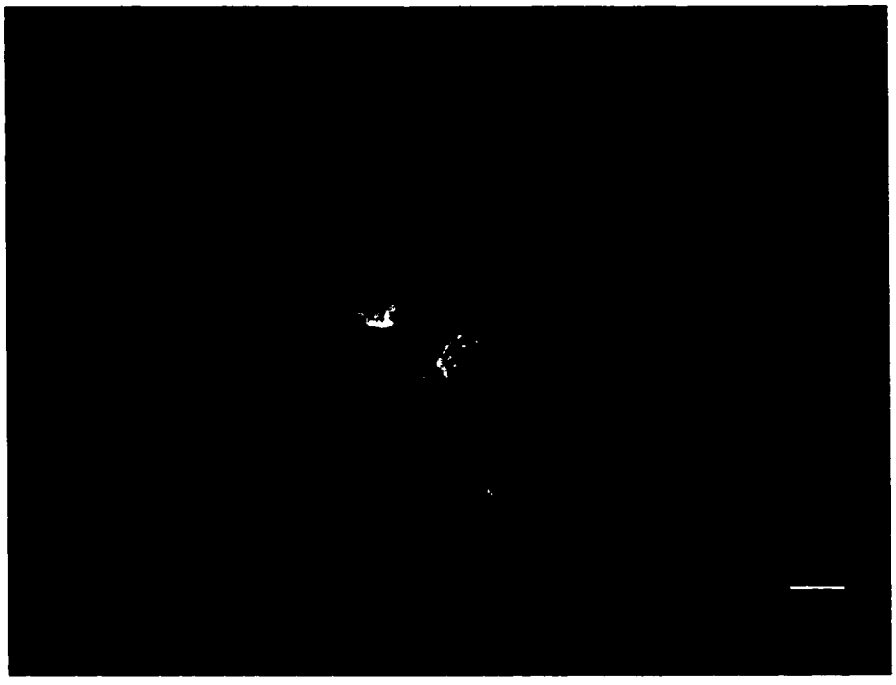

Figure 2.19: Fluorescence immunohistochemistry, lymph node. FIV antigen (red) is mostly within T cells (green). FIV—Cy3, CD3—FITC, nuclei—DAPI; Bar = 15 uM.

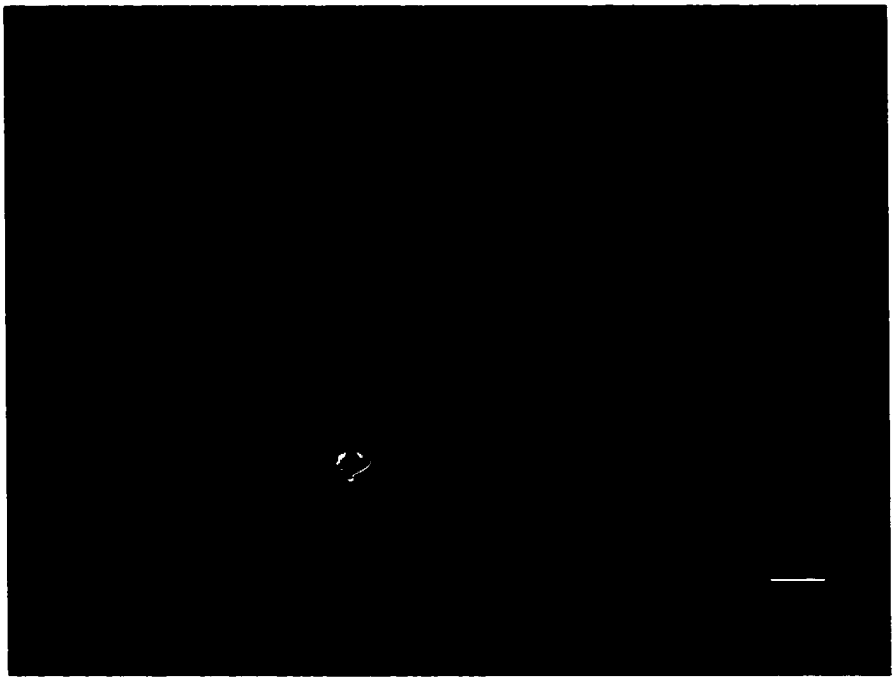

Figure 2.20: Fluorescence immunohistochemistry, lymph node. An uninfected AM-3K$^+$ macrophage (green) is at the center. An infected macrophage (orange/yellow) is to the lower left. FIV—Cy3, CD3—AM-3K, nuclei—DAPI; bar = 40 uM.

Figure 2.21: Fluorescence immunohistochemistry, lymph node. A FeMy^{+} macrophage without FIV (green) and two with FIV (yellow) are evident. FeMy antibody also bound collagen or reticulin (wavy strands) in this assay. FIV—Cy3, FeMy—FITC, nuclei—DAPI; bar = 40 uM.

Figure 2.22: Fluorescence immunohistochemistry, lymph node. Note the absence of FIV co-localization (red) with Mac 387^{+} macrophages (green). FIV—Cy3, Mac 387—FITC, nuclei—DAPI; bar = 15 uM.

Approximately 5—10% of FIV^+ cells in the thymus contained protein S-100 (Fig. 2.23). These cells, located primarily in mid-lobule, had a location and size consistent with dendritic cells, although in other tissues S-100 protein is also found in cells of the nervous system and in melanocytes. Anti-S-100 antibody recognized only a small subset of dendritic cells in lymph nodes, which rarely contained FIV antigens. Thymic medullary dendritic cells labeled with antibody CNA.42 (Dako). CNA.42 is an antibody derived from a human follicular dendritic cell (FDC) antigen. Feline thymic $CNA.42^+$ dendritic cells rarely contained detectable FIV antigens. Moreover, in feline lymph nodes CNA.42 recognized parafollicular interdigitating dendritic cells but not germinal center FDC. In all tissues FIV antigens were rarely detected in $CNA.42^+$ cells. Unlike what has been reported for HIV [52] , we did not detect FIV in thymic epithelial cells (Fig. 2.24).

FIV rarely co-localized with the interdigitating follicular cell marker fascin (p55; Dako) in lymph nodes (Fig. 2.25). Follicular dendritic cells in lymph nodes were not well identified by any of the antibodies we employed. Although labeling of lymph node follicular dendritic cells was poor, FIV antigens were detected in large $MHCII^{hi}$ antigen presenting cells in lymph node germinal centers (Fig. 2.26). The anti-human HLA antibodies we employed only faintly labeled $MHCII^{lo}$ cells, such as B cells (Fig. 2.26).

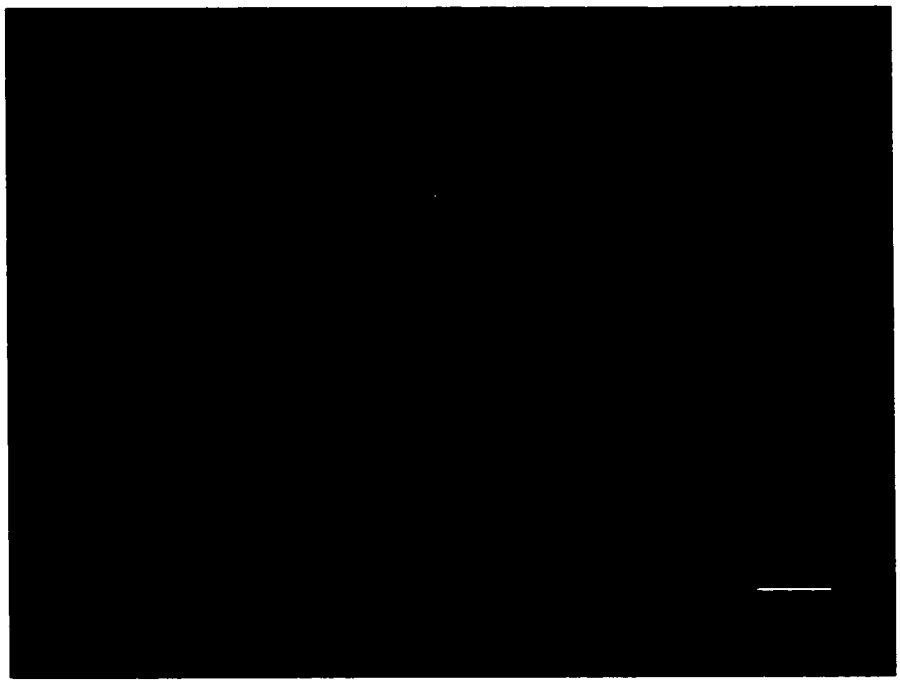

Figure 2.23: Fluorescence immunohistochemistry, thymus. Two S-100^{+} dendritic cells (green) are evident; the lower of the two contains FIV antigens (red/yellow). FIV—Cy3, S-100—FITC, nuclei—DAPI; bar = 15 uM.

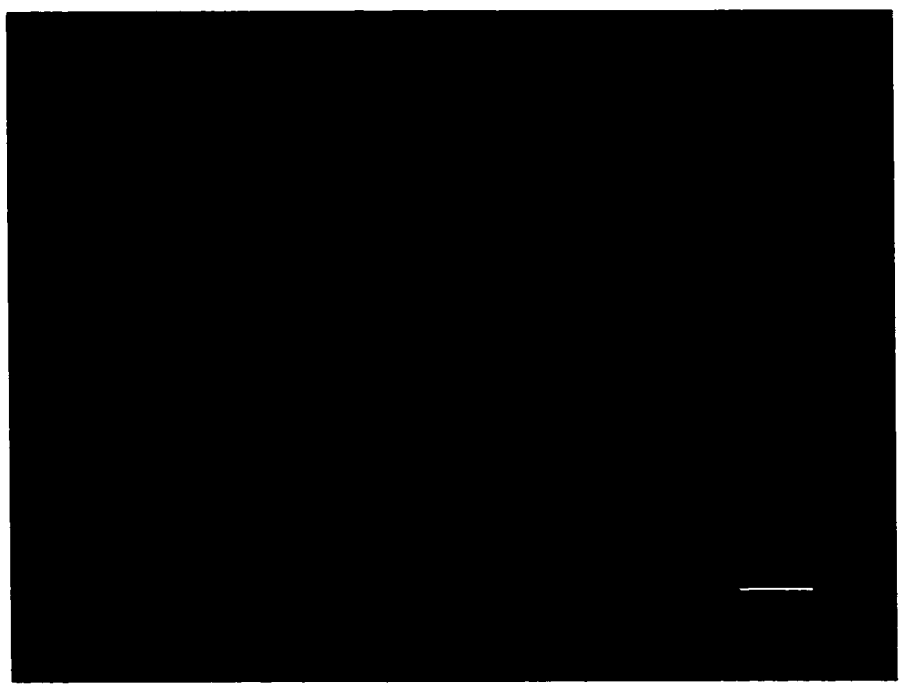

Figure 2.24: Fluorescence immunohistochemistry, thymus. FIV antigens (red) do not co-localize with cytokeratin^{+} thymic epithelial cells (green). FIV—Cy3, cytokeratin—FITC, nuclei—DAPI; bar = 15 uM.

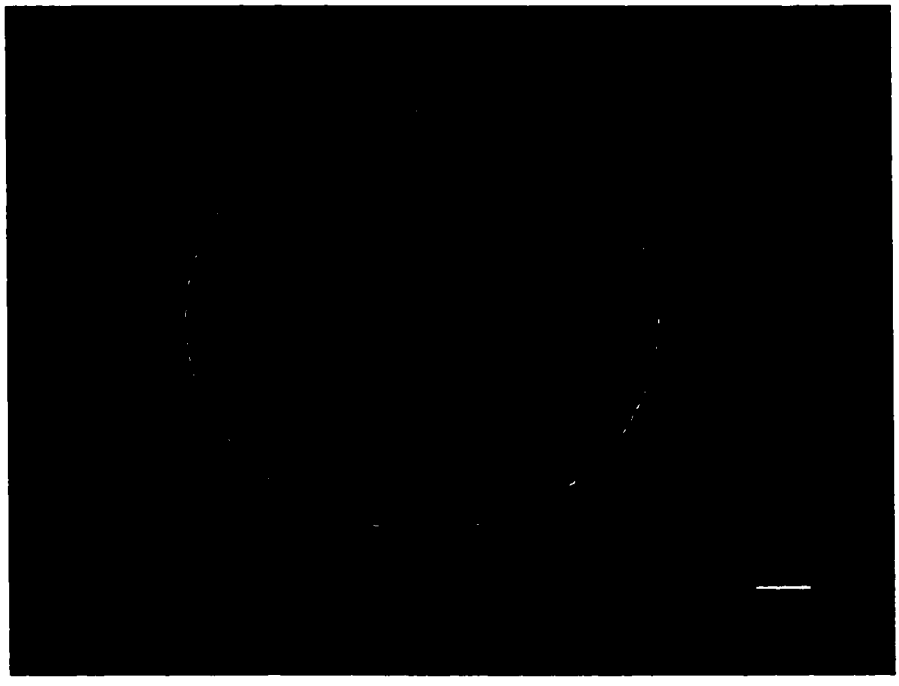

Figure 2.25: Fluorescence immunohistochemistry, lymph node. Most FIV^+ cells (red/yellow) are within a follicle (hatched). FIV is not associated with interdigitating dendritic cells (blue). FIV—Cy3, CD3—FITC, fascin (p55)—coumarin; bar = 200 uM.

Figure 2.26: Flourescence immunohistochemistry, lymph node follicle. FIV (red/yellow) co-localizes with $MHCII^{hi}$ cells (patchy green staining). Punctate FIV (red) in non-$MHCII^+$ cells is likely associated with T cells. Very faint staining of $MHCII^{lo}$ cells (B cells) is present centrally. FIV—Cy3, HLA-DQ,R—FITC, nuclei—DAPI; bar = 90 uM.

We identified B cells in feline tissue sections with anti-murine CD45R/B220 and/or anti-human CD 21 (Pharmingen). B cell infiltrates, which occasionally formed follicle-like structures, were prominent in thymuses of FIV-infected cats (Fig. 2.27), but absent in FIV-naïve cat thymus specimens. Similar thymic B cell infiltrates have been observed by others in animals infected with FIV [23, 53] and SIV [54] . B cells in all tissues contained no detectable FIV antigens.

In sections of intestine, leukocytes in the lamina propria and GALT contained FIV antigens. FIV was also detected in mucosal epithelium; some viral antigens appeared to be present within cytokeratin^{+} epithelial cells (Fig. 2.28).

FIV^{+} cells were positive for vimentin, the intermediate filament expressed by leukocytes and other mesenchymal cells (Fig. 2.29). Bone marrow was a rich source of FIV-infected cells. However, these FIV^{+} cells rarely stained with any of the mature leukocyte markers in our study (Fig. 2.28; Table 2.1). These were most likely primordial leukocytes, although in this study we lacked an anti-CD34 antibody proven to work in feline tissues in order to confirm that hypothesis.

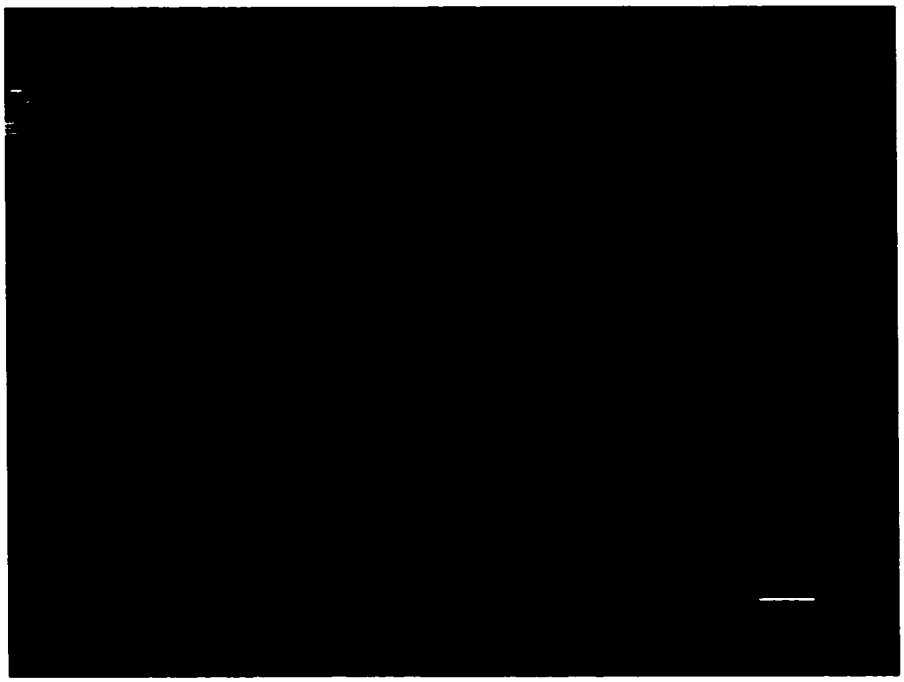

Figure 2.27: Flourescence immunohistochemistry, thymus. Note follicle-like infiltrates of B cells (green) in a lobule void of FIV antigens (red). FIV was not found in B cells in any tissue. FIV—Cy3, CD45R/B220—FITC, nuclei—DAPI; Bar = 90 uM.

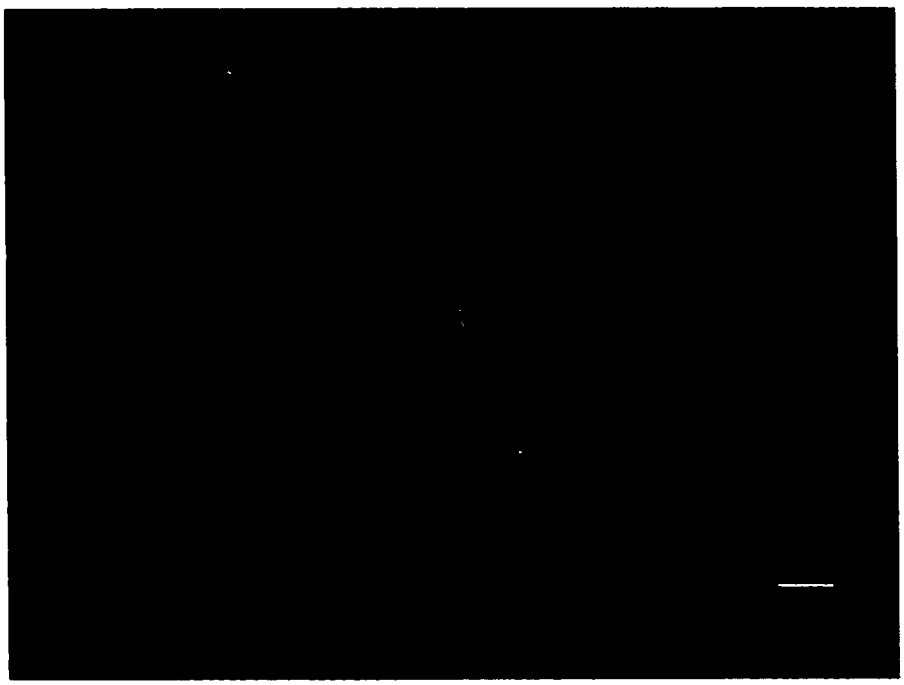

Figure 2.28: Fluorescence immunohistochemistry, duodenum. FIV (red) is most prominent in cytokeratin$^-$ cells of the lamina propria (leukocytes). However, some viral antigen appears to co-localize with cytokeratin$^+$ (blue) epithelial cells. FIV—Cy3, cytokeratin—coumarin; bar = 200 uM.

Figure 2.29: Flourescence immunohistochemistry, lymph node. Note co-localization of FIV (red) with a round cell (probably a lymphocyte) expressing vimentin (green). Fusiform mesenchymal cells also express vimentin. FIV—Cy3, vimentin—FITC, nuclei—DAPI; bar = 15 uM.

Figure 2.30: Fluorescence immunohistochemistry, bone marrow. Many cells are productively infected with FIV (red), although no T cells (green) are evident. FIV—Cy3, CD3-FITC, nuclei—DAPI; bar = 40 uM.

Table 2.2: Proportion of FIV^+ cells in lymphoid tissues identified as mature ($CD3^+$) T cells, macrophages, or dendritic cells. Numbers are percent (standard deviation).

Tissue	$CD3^+$ T cells	Macrophages	Dendritic cells	Unidentified
Lymph node	74.5 (11.6)	17.3 (13.2)	9.6 (5.4)	8.4 (4.2)
Thymus*	47.3 (28.2)	9.8 (4.4)	11.4 (7.6)	33.9 (13.6)
Spleen	81.7 (10.9)	5.3 (3.3)	11.4 (4.8)	4.7 (3.2)
Intestine§	71.8 (18.4)	12.8 (4.6)	18.3 (9.0)	13.0 (8.5)
Bone marrow¶	7.5 (6.4)	9.5 (7.9)	2.3 (1.9)	82.3 (5.2)

* Most of the unidentified cells in the thymus were in the cortex and appeared to be immature thymocytes.
§ FIV^+ cells in intestinal sections were located in the mucosa and submucosa only.
¶ Unidentified FIV^+ cells in bone marrow were probably leukocyte progenitors.

Due to the relative insensitivity of the DNA in situ hybridization assay, we could not draw quantitative conclusions about the phenotypic spectrum of cells containing FIV provirus. Most cells with detectable provirus were $CD3^+$ T cells (Fig. 2.31). However, the total number of cells detected by fluorescence in situ hybridization was too small for meaningful comparison of different cell phenotypes containing FIV DNA.

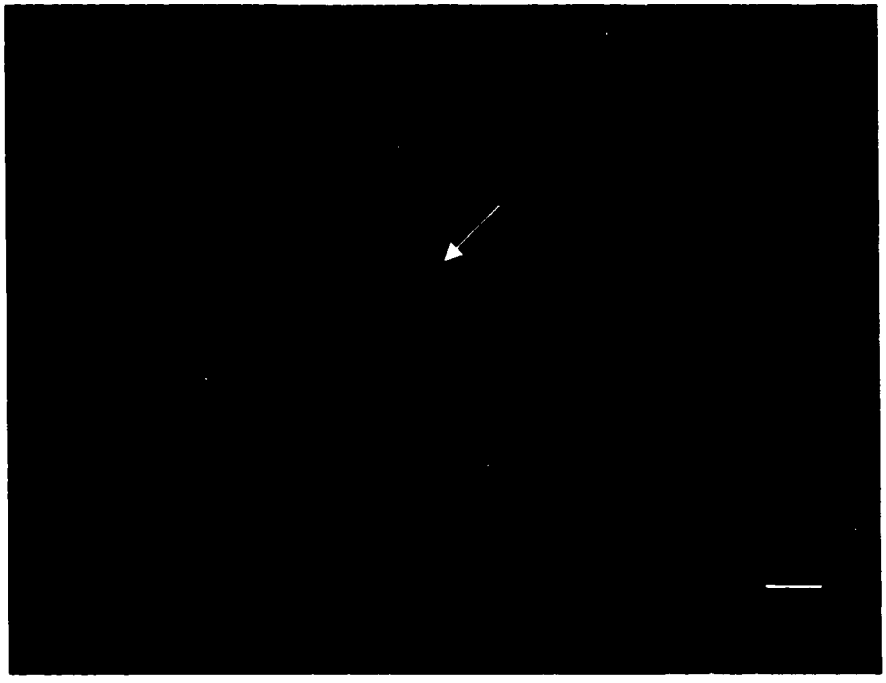

Figure 2.31: Fluorescence DNA in situ hybridization, lymph node. FIV provirus (red; arrow) is within the nucleus of a T cell (green). Nuclei are stained with DAPI (blue). FIV probe—Cy3, CD3—FITC, nuclei—DAPI; Bar = 30 uM.

DISCUSSION

As in previous studies from our laboratory, FIV-B-2542 and FIV-C-Pgmr reached high plasma viral RNA and blood mononuclear cell proviral titers during the acute phase of infection [10, 40, 55, 56] . Plasma and mononuclear cells from infected cats readily infected naïve feline blood mononuclear cells in culture and resulted in high tissue culture infectious doses in the supernatants. These laboratory data complemented our finding of widespread and productive infection in vivo.

FIV-B-2542 and FIV-C-Pgmr infected the same range of cells targeted by HIV-1 and SIV, including but not limited to T-lymphocytes, macrophages, and dendritic cells [57, 58] . In our group of cats in the acute-phase of infection, 70—85% of cells producing

detectable levels of FIV were T cells. This finding is in agreement with recent studies which demonstrate that lentiviral expansion occurs largely in T cells from the earliest phases of infection onward [59] . For example, transmucosal infection by HIV-1 and SIV appears to involve T cells directly at the site of exposure, at least in the tonsil and intestinal tract [60-62] . Macaques vaginally inoculated with $SIV_{mac}251$ demonstrate infection of intraepithelial dendritic cells (Langerhans cells) within 1 hour, but in <24 hours the majority of cells infected are T cells, both at the site of inoculation and in regional lymph nodes [63] . As early as day 2 following vaginal inoculation, simian/human immunodeficiency virus $(SHIV)^+$ cells are detected in cortical parafollicular cells of lymph nodes in pig-tailed macaques [64] . Our in vivo studies agree with prior studies of FIV-C-Pgmr which demonstrated early thymic tropism following transmucosal infection [10] . Regardless of the cell types first targeted by lentiviruses, it is becoming clear that $CD4^+$ T cells rapidly constitute the majority of cells productively infected following initial exposure [65] .

In most tissues we examined 15% or fewer FIV^+ cells were identified as macrophages. Based on the assumption that macrophages represent the major targets of R5 or "M-tropic" viruses, we might have anticipated a greater percentage of FIV-containing cells to be macrophages in our acutely infected cats. Until recently it was widely believed that HIV-1 and SIV first targeted monocyte/macrophages (and/or dendritic cells) and later spread to T cells [66, 67] . However, there is increasing evidence that T cells are among the very first cells infected by HIV-1 and SIV, even by so-called "macrophage- or M-tropic" viruses [4, 65] . At least one of the isolates we inoculated, FIV-B-2542, has a proven in vitro macrophage tropism [12] . It is possible that by

inoculating the virus intravenously, we bypassed selection of "M-tropic" phenotypes imposed by transmucosal passage, or that such selection does not in fact occur. Regardless of exposure route, labeling a lentiviral isolate as "macrophage-tropic" needs not conflict with the finding of marked T cell involvement in the earliest phases of in vivo infection.

The virulent FIV isolates we employed in this study resulted in a large number of FIV^+ cells per gram of lymphoid tissue during acute-phase infection as estimated from our quantitative methods. These values are in agreement with equivalent studies demonstrating high lymphoid tissue viral RNA burdens in people during acute-phase HIV-1 infection [4] . One surprising finding in the FIV-infected cats was the apparent detection of FIV antigens and RNA in intestinal epithelial cells. Further study will be required to determine whether this finding was indicative of productive epithelial cell infection , viral transmigration through epithelial cells, FIV^+ transmigrating/intraepithelial leukocytes, or some combination of the above.

In our hands, immunohistochemistry proved more sensitive than RNA in situ hybridization for FIV detection in tissue sections. We used a polyclonal antibody source, which may have been able to label more viral elements than could be labeled by the RNA probe. Moreover, in situ hybridization is wholly dependent upon individually optimized tissue protease digestion [28] . In situ hybridization sensitivity is also influenced by the amount of homology between virus and probe sequences. Our whole-genome RNA probe series was derived from an *env* clade A isolate, FIV-Petaluma. Even under the best of circumstances, RNA in situ hybridization may require radiolabeled probes coupled with many days of hybridization and radiographic development to detect viral nucleic acids [1,

2, 68] . In some instances, computer analysis is required to distinguish between significant dot clusters (indicating probe hybridization) and background. It has been postulated that a minimum of 10 RNA copies are required for detection of specific RNA sequences in tissue sections [28] . Thus, in cases of low copy number, immunohistochemistry may reveal virus in tissues below the detection threshold of in situ hybridization. Further study and experience should permit a convergence of results by in situ hybridization and immunohistochemistry, increasing the validity of both approaches.

As with RNA in situ hybridization, we identified fewer FIV^+ cells by DNA in situ hybridization than we did by immunohistochemistry. This was due to a difference in assay sensitivities, although in some tissues there may have been FIV attached to the surface of non-infected cells, such as follicular dendritic cells. The same variables influencing the success of RNA in situ hybridization applied to DNA in situ hybridization, with the exception that DNA in situ hybridization could be performed on ethanol-fixed specimens. RNA in situ hybridization failed to consistently detect FIV in ethanol-fixed specimens. Since double-stranded DNA is tightly wound and is closely associated with histones and other nuclear proteins, DNA sequences may be more difficult to access by nucleic acid probes than are RNA sequences. Thus, it is possible that even more copies of DNA target are required for detection than the 10-copy minimum proposed for RNA detection. It seems unlikely that 10 or more copies of lentiviral DNA genome are found within most host cells. However, because the genome is ~9 kilobases in length, and because we used whole FIV genome RNA as probe, it is possible that there was sufficient signal to detect <10 FIV priviral genome copies. The ability of a probe to hybridize with a DNA target may also be influenced by the

replication state of the cell. Cells undergoing high rates of transcription, or chromosomes preparing for division, may have less tightly coiled DNA which is more accessible to probes. We have experimented with in situ DNA PCR $^{+}$/- in situ hybridization as a means of more sensitively detecting provirus in tissues, but to date we have not achieved acceptable levels of specificity (unpublished observations). It is unclear whether our ability to detect FIV DNA by in situ hybridization was limited by the number of DNA copies per cell, the transcriptional and/or replicative status of chromosomal DNA, or some combination of these and other factors.

As with many other tissue-based assays, the greatest determinant of success in our histologic studies was choice of tissue preservatives. Although we could detect FIV via immunohistochemistry in formalin- and paraformaldehyde-fixed tissues subjected to microwave antigen retrieval, we found that sensitivity was invariably lower than that seen in the same tissues fixed in precipitating agents such as ethanol and Histochoice. Moreover, we found that ethanol-fixed tissues more reliably produced positive signal by DNA in situ hybridization than did tissues fixed by other means. We attribute the superiority of ethanol-fixed tissues for DNA in situ hybridization to reduced cross-linking of histones and other nuclear proteins, permitting readier denaturation and separation of DNA strands. Protease-treated formalin-fixed tissues could yield satisfactory DNA in situ hybridization results, but the time of digestion had to be individually optimized for each tissue section, a cumbersome source of assay variation. The chief drawback of DNA in situ hybridization in ethanol-fixed tissues was heat-induced loss of nuclear membrane integrity in certain cell types, most notably thymocytes. In contrast to DNA in situ hybridization, we did not achieve satisfactory results with RNA in situ hybridization in

ethanol-fixed tissues, possibly because the absence of protective cross-linked proteins allowed viral RNA degradation.

In our hands, light microscopic chromogenic immunohistochemistry proved the most sensitive and specific means of identifying FIV in tissue sections. For example, the hazy diffuse pattern of FIV staining in lymph node follicle germinal centers, presumably the result of dendritic cell surface antigen trapping, could only be demonstrated by light microscopy and not by immunofluorescence. Sensitivity by light microscopy was enhanced by the deposition of relatively large amounts of chromogen at sites of enzyme-labeled antibody adherence. Specificity was high when compared to immunofluorescence because light microscopy circumvented the problem of separating endogenous tissue autofluorescence from true signal. For these reasons, we reserved the use of fluorescence immunohistochemistry for co-labeling studies. We found light microscopy generally unsuitable for co-labeling studies due to inconsistent color layering within the cytoplasm of co-labeled cells, and overly heavy accumulation of chromogen deposits which obscured cell morphology.

The application of fluorescence techniques to immunohistology is hindered by endogenous tissue autofluorescence. In tissues we examined, significant autofluorescence was produced by red blood cells, hepatocytes, and other cells with endogenous phycobilirubin-like molecules, and by certain collagens, reticulins, and elastins. Autofluorescence was particularly troublesome in formalin-fixed tissues. Indeed, we sometimes found it impossible to identify fluorochrome-labeled cells in formalin-fixed tissues with high autofluorescent backgrounds such as spleen, liver, and blood vessels. In order to aid the differentiation of true fluorescent signal from autofluorescent

background, we sometimes employed long-pass emission filters which allow a broad range of light wavelengths to be viewed following specific wavelength excitation. Although the long pass filters did not decrease the intensity of background, they usually permitted us to distinguish by color between true fluorochrome signal and autofluorescence. For example, when viewed through the long pass filter, FITC-stained cells maintained their dark green color while autofluorescent elements appeared yellow or yellow-green because of emission by the latter of a broader spectrum of wavelengths. When viewed through a "cut" or narrow band emission filter, FITC staining was often overwhelmed by autofluorescence of the same color due to confinement of all emitted light to the same wavelength. For fluorescence immunohistochemistry we did not counterstain with dyes such as Evan's blue, which quench autofluorescence through some color channels, because they invariably led to increased background in other color channels. We encountered increased autofluorescence through the red (rhodamine/TRITC) channel when tissues were counterstained with Evan's blue, nuclear fast red, or methyl green. The only light microscopic counterstain we employed which had no effect on endogenous tissue fluorescence was hematoxylin.

We adopted the FIV immunohistochemistry protocol employing polyclonal antibody from chronically infected cats only after exploring other antibody sources. We found no single murine-origin FIV-specific monoclonal antibody, or combination of such antibodies, sufficiently sensitive to detect FIV in tissue sections even with tyramide amplification. We immunized a rabbit with concentrated FIV and adjuvant to generate non-feline polyclonal antibodies, but this source was ultimately abandoned due to high non-specific background staining. In contrast to the murine and rabbit antibody sources,

we discovered that appropriately diluted plasma antibodies from cats chronically infected with FIV could be used to sensitively and specifically label FIV in tissue sections. The broad array of FIV-recognizing antibodies yielded more label per viral unit than could be achieved with monoclonals, and the absence of adjuvant-induced non-specific antibodies, such as those present in the immunized rabbit serum, allowed for greater specificity. Protein A binds feline antibodies at the Fc fragment, leaving the antigen recognition site free for epitope binding. By binding anti-FIV antibody Fc fragments with biotin-protein A prior to placement on the tissues, spurious binding to endogenous antibodies was circumvented. Even using a feline polyclonal antibody source, tyramide amplification was essential for detecting FIV at the primary antibody dilutions required to prevent non-specific labeling of naïve control tissues. Our experience was that increasing levels of naïve serum during blocking steps did not reduce non-specific tissue staining. In many cases, high concentrations of naïve blocking sera simply increased background. Only by increasing the dilution of primary antibody did we eliminate non-specific tissue labeling. The drawbacks of using a polyclonal antibody source from FIV-infected cats include: (1) the need for standardization of every plasma collection due to titer variation within and between individual cats, (2) presence of potentially confounding non-FIV-specific antibodies in serum, and (3) difficulty in standardization of primary antibody reagent between laboratories. However, despite its shortcomings, the immunostaining protocol described here represents an advance permitting investigators to more readily detect FIV in tissue sections.

In summary, we describe methods for detecting FIV proteins and DNA in tissues from acutely infected cats. Most FIV-positive cells in cats acutely infected with FIV-B-

2542 and FIV-C-Pgmr are T cells. To a lesser extent FIV antigens also co-localize with macrophages and dendritic cells. FIV^+ bone marrow cells, probably leukocyte progenitors, are common. Further work is needed to classify the full range of cells infected by FIV in vivo. In our studies, RNA and DNA in situ hybridization labeled fewer FIV^+ cells than did immunohistochemistry. A simple and specific in situ PCR assay might significantly increase the sensitivity of FIV nucleic acid detection in tissues without the need for radioactive reagents. Our studies reinforce the value of collecting tissues in both cross-linking and precipitating fixatives, in order to maximize the amount of information which might be derived through subsequent in situ assays. Improved methods for detecting FIV in tissues will enhance our understanding of lentiviral pathogenesis, and may point to new targets for prevention and intervention of HIV/AIDS.

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CHAPTER THREE

MATERNAL-FETAL FELINE IMMUNODEFICIENCY VIRUS TRANSMISSION IS UNRELIABLY DIAGNOSED BY PLACENTAL AND BLOOD ASSAYS

INTRODUCTION

Improved knowledge of the pathogenesis of maternal-fetal HIV-1 transmission will help identify new strategies for preventing infant infections. Chemotherapy has reduced the rate of vertical HIV-1 transmission in industrialized nations, but has largely failed to have an impact in the developing world because of limited supplies and inconsistent compliance [1] . Even in the United States, where chemotherapeutic intervention of vertical HIV-1 transmission is widely available, compliance rates are frequently poor [2] . Moreover, increasing evidence from both human and animal studies demonstrates potential toxicities of antiviral drugs on the developing fetus and infant [3-6] . Development of new modes of intervention depends on an improved knowledge of the basic mechanisms of vertical HIV-1 transmission. Because studies of intragestational virus transmission in humans are limited by practical and ethical considerations, animal models are useful to characterize basic lentiviral pathogenesis during pregnancy.

A widely accepted animal model of human AIDS is simian immunodeficiency virus (SIV) infection of Asian-origin nonhuman primates [7] . However, a significant limitation of the SIV model is the extremely low rate of maternal-fetal transmission. Indeed, the rate of fetal infection is so low that maternal-fetal SIV transmission studies

have been largely abandoned. Most researchers investigating antenatal SIV infection rely on direct virus inoculation into fetuses [8, 9] . Direct inoculation assures fetal infection, but bypasses normal maternal, placental, and viral determinants of transmission.

Feline immunodeficiency virus (FIV) infection of domestic cats causes disease which is virtually identical to human AIDS [10] . Both diseases are caused by lentiviruses with similar genomic organization, structure, life cycle, and cell targets. The human and feline viruses are infectious mucosally, and contain members capable of utilizing the highly conserved chemokine receptor CXCR4 for cell entry [11, 12] . Like HIV-1, FIV infection in its natural host results in an acute flu-like illness, seroconversion, a long chronic phase characterized by viral down-regulation, and a terminal AIDS-like disease with immune decompensation, opportunistic infections, increased risk of neoplasias, and neurologic derangement [13] .

The FIV model affords the unique opportunity to study naturally occurring maternal-fetal lentivirus transmission. We have performed previous work on vertical FIV transmission which focused primarily on clade B isolates [14-16] . In this report we describe extension of those studies to include isolates representing two other clades: FIV-A-Petaluma (FIV-A-Pet) and FIV-C-PaddyGammer (FIV-C-Pgmr). Expanding the range of evaluated FIV isolates is important to further validate the model because HIV-1 subtypes differ in their rates of vertical transmission [17] . The results we describe here, some expected and others surprising, help to shed light on mechanistic questions difficult to study in humans. These include timing of vertical transmission, fetal tissue virus distribution and tropism, placental and fetal viral cell targets, and the role of the placenta in fetal infections.

MATERIALS AND METHODS

Animals and viral inocula

Six 8-week-old cats from a specific pathogen-free (SPF) breeding colony maintained at Colorado State University (Fort Collins, CO) were inoculated intravenously with FIV. Three cats (ID# 3659, 3664, and 3666) were inoculated with 100 weanling cat infectious doses of acute-phase FIV-C-Pgmr contained in supernatant from primary blood mononuclear cell coculture. Three cats were inoculated with FIV-A-Pet. The first of these three (3866) was inoculated with 1 mL plasma from an acutely infected cat (a gift from E Sparger, University of California—Davis). The other two cats (3395, 3867) were inoculated six weeks later with 2 mL whole blood from the cat 3866. Animals were observed daily for signs of illness following virus inoculation. FIV infection was confirmed in the queens by DNA polymerase chain reaction (PCR) on blood mononuclear cells, and by quantitative competitive reverse transcriptiase PCR (qcRT-PCR) on plasma. The queens were bred with an FIV-naive male cat 4–24 months after infection. Pregnancy was diagnosed by abdominal palpation and correlated with observed breeding dates. Placental and fetal tissues were surgically harvested by cesarean section at 9 weeks (term) post-conception. In both the FIV-A-Pet and FIV-C-Pgmr cohorts, one queen provided two sequential litters to this study while the other two queens in the group provided one litter each. We collected a total of 4 litters, 11—12 fetuses, from each group. As negative controls, term fetuses and placentas from a sham-inoculated queen were assayed.

Tissue Processing

Immediately upon surgical removal, amniotic fluid was collected and placentas were separated from fetuses. Care was taken to maintain the integrity of the amniotic sac to prevent maternal and placental contamination of fetal tissues. The fetuses were euthanized by pentobarbital sodium injection following blood collection. To further eliminate the possibility of maternal contamination of fetal samples, fetuses were rinsed with sterile phosphate-buffered saline (PBS) and externally disinfected with 70% ethanol prior to dissection in a laboratory hood. After dissection, individual organs were isolated and rinsed with PBS. We collected placenta and fetal brain, thymus, liver, spleen, mesenteric lymph node, bone marrow, and blood mononuclear cells. Duplicate samples of each tissue were collected for DNA polymerase chain reaction (PCR) and histologic analyses. Sections designated for fluid-phase PCR were homogenized, dissociated into cell suspensions through mesh sieves, washed, and pelleted by centrifugation. The portions of each tissue targeted for in situ assays were saved in one or more of the following fixatives: 10% neutral buffered formalin, 4% paraformaldehyde, absolute ethanol, Histochoice (Amresco, Solon, OH) and Streck's Tissue Fixative (Streck Laboratories, Omaha, NE). Because of small fetal tissue sizes, we could not save all samples in all fixatives. Tissues were fixed overnight and processed the following morning into paraffin-embedded blocks by a "short-run" method which avoided the use of formalin and which minimized the length of time tissues were immersed in liquid paraffin (Colorado State University Histology Laboratory, Ft. Collins, CO). Routine five μm paraffin sections were placed on silanized glass slides without heat treatment and allowed to air dry at least one day prior to staining.

Maternal hematology.

Blood from queens was collected into EDTA tubes at the time of cesarean and analyzed for complete blood count and $CD4^+$ and $CD8^+$ T cell subsets by flow cytometry as described by Dean et al [18] . Blood mononuclear cells were isolated and evaluated for proviral load as described below. Plasma and amniotic fluid were separated from cells by centrifugation and examined for viral RNA load (described below). Statistical comparisons were performed by the unpaired t-test using InStat software (GraphPad Software Inc., San Diego, CA).

DNA PCR and microplate hybridization assay

Maternal and fetal blood mononuclear cells were isolated by ficoll-hypaque density gradient separation (Histopaque-70, Sigma Corp., St. Louis, MO) and rinsed with PBS for use in DNA PCR. Nested DNA PCR was performed as described in Chapter 2, except that second round primer gag3 was conjugated with 5'-digoxigenin (Sigma Genosys, The Woodlands, TX). In these studies, we used a microplate hybridization assay to detect PCR products. To prepare plates for the microplate hybridization assay, 96-well microtiter plates were coated overnight at 4° C with 100 uL of a 10 ug/mL solution Avidin DX (Vector Laboratories, Burlingame, CA). Plates were washed 5X with 0.2% Tween (polyoxyethylene-sorbitan monooleate; Sigma, St. Louis, MO) in TEN (0.05 M Tris HCl, pH 7.4, 0.001 M EDTA sodium, 0.15 M NaCl) on an EL_X50 AutoStrip Washer (Bio-Tek Instruments, Winooski, Vermont). Wells were blocked with 200 uL TEN supplemented with 5% v:v donkey serum (Sigma) and 2% w:v bovine serum albumin (Boehringer-Mannheim, Indianapolis, IN) for 48 hr at 4° C. Following another wash, plates were air dried and sealed with Dynex plate sealer (Dynex Technologies,

Chantilly, VA). Plates were stored long-term dessicated at –20° C. Once thawed, plates were maintained at 4° C for up to several weeks. The seal covering the wells to be used in a given microplate hybridization assay was removed, saving the unused portion of the plate for future assays.

Ten percent of each second round PCR product was placed in an individual microcentrifuge tube with MHA buffer qs 50 uL. MHA buffer was comprised of pH 7.4 PBS supplemented with 2% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 0.5% sheared denatured salmon sperm DNA (Sigma). PCR products were denatured in a 95° C heat block for 5 minutes, and chilled on ice for 5 minutes. While the PCR products were denatured and chilled, biotinylated probe was bound to the avidin-coated plates. The probe, CB gag 503 (biotin-5'- TCACCTCCTAACCCTTCTCTTGC-3'), is complementary to bases 481—503 of the FIV *gag* gene sense strand. Fifty uL/well of probe, diluted to a final concentration of 50 pg/mL in MHA buffer, was incubated at 37° C for ~10 minutes. After the PCR products had chilled, excess probe was washed from the plates 5X with 0.2% Tween/TEN. The PCR product solutions were added to the wells and hybridized at 37° C for 30 minutes. Plates were washed and 50 uL/well of 1:1000 horseradish peroxidase-conjugated anti-digoxigenin FAb (Roche Molecular Biochemicals, Indianapolis, IN) was bound for 20 minutes at 37° C. After washing, peroxidase was detected with 3,3', 5,5'-tetramethylbenzidine (TMB)/H_2O_2 (Kirkegaard & Perry, Gaithersburg, MD) for 2—5 minutes. Reactions were stopped with 2.5 M H_2SO_4. Optical densities (OD), measured by absorbance at A_{450} (reference A_{570}), were recorded using a Dynatech 5000MR™ microplate reader (Dynatech Technologies). Positive reactions were defined as those with a minimum OD of 0.1. The optical reader was

blanked on the naïve DNA negative control well, which was <0.02 OD or the assay was repeated. By testing dilutional series of known FIV *gag* plasmid copy numbers, we determined that our PCR protocol approached a sensitivity of 1 provirus copy/ug DNA (unpublished data). Therefore, semi-quantitative DNA PCR results (provirus copies/ug DNA) were recorded in factors of 10 based on the highest log dilution yielding a positive result (1 copy, 10 copies, 100 copies, etc.).

Confirmation of virus isolates

In order to be certain that the virus isolates derived from the fetuses were of the expected genotype, second round *gag* PCR products were cloned into plasmid pCR2.1-TOPO (Invitrogen) and sequenced (Macromolecular Resources Laboratory, Fort Collins, CO). As a further confirmation of fetal FIV identity, *env* primers were used in PCR which amplify FIV-A-Pet but not FIV-C-Pgmr. These primers were env 961 (5'-ACA-GACCCATTACAAATCCCACTG-3') and env 1977 (5'-GGCTTCAGTCACCTT-TTCTACAGC-3'). Cycling conditions were performed as described for first round *gag* PCR. *Env* PCR products were detected by agarose gel electrophoresis as described in Chapter 2.

Viral RNA quantitation

Amniotic fluid and maternal and fetal plasma samples were tested for viral RNA load with a quantitative competitive reverse transcriptase PCR (qcRT-PCR) assay. We employed substitutional PCR to generate the competitor RNA molecule: wild-type second-round sense *gag* PCR product with a 23 base substitution. The substituted foreign sequence (FeLV 1776; 5'-TGACGAAGCTTTTCCCTTGACCC-3') was derived from

bases 1754—1776 of the feline leukemia virus (FeLV) *gag* gene. Software-assisted (MacVector, Oxford Molecular Group, Madison, WI) analysis of the FeLV sequence demonstrated no significant homology with FIV or endogenous cat genome sequences in the GenBank database. The substitutional PCR process involved a series of internal amplifications of the second-round *gag* product beginning at base 507 and working 5' back to the gag3 primer origin (base 446), trailing 5' sequences from the ends of primers in successive steps. The process is outlined in Figure 3.1. We first amplified a subunit of the second-round *gag* PCR product by substituting primer gag 507 (5'-AGTTCAAT-TATGGTTTACAGCCTTT-3') for gag 3. The next round of PCR employed primer gag 507 trailing at the 5' end the foreign (FeLV 1776) 23-base sequence (5'-CCCAGTTCC-CTTTTCGAAGCAGTAGTTCAATTATGGTTTACAGCCTTT-3'). By trailing the FeLV 1776 sequence from the gag 507 primer, we incorporated the foreign sequence into all amplified products (Fig. 3.1). We then "added back" the original gag3 primer binding sequence by trailing it from the 5' end of FeLV 1776 (5'-TTGACCCAAAAATGG-TGTCCACCCAGTTCCCTTTTCGAAGCAGT-3'). In the last step, we trailed a T7 RNA polymerase promoter sequence with a 5' CGC cap and 3' ten-base linker (5'-GCGTAATACGACTCACTATAGCGTGTCGAGTTGACCCAAAAATGGTGT-3') to the 5' end of gag 3. This allowed us to perform direct in vitro transcription of the final PCR product. RNA transcription was performed with the Ampliscribe T7 High Yield Transcription Kit (Epicentre, Madison, WI). Residual DNA was digested with RQ1 RNase-free DNase (Promega, Madison, WI). RNA was purified with the Qiamp Viral RNA Minutesi Kit (Qiagen) and quantitated on the DU-70 spectrophotometer. RNase-free 1X tris-EDTA buffer (pH 7.0; Sigma) was added to the purified RNA for a final

concentration of 10^{10} molecules/uL. Individual aliquots were frozen at –70° C for long-term storage. Aliquots were thawed and diluted to 100 copies/uL in RNase-free water immediately prior to qcRT-PCR.

To determine viral RNA load in fluids, RNA was purified from 200 uL amniotic fluid or EDTA-plasma samples using the Qiamp Viral RNA Minutesi Kit (Qiagen). One-half of the purified RNA (equivalent to the amount in 100 uL sample) and 100 copies of competitor RNA were added to each PCR reaction. PCR solutions were derived from the GeneAmp EZrTth RNA PCR Kit (P-E Applied Biosystems). RT-PCR was performed in a P-E 9600 thermocycler (P-E Applied Biosystems) using primers digoxigenin-gag 3 and gag 4 (Sigma Genosys, The Woodlands, TX). Following a 30 minute reverse transcription step at 65° C, the samples were cycled 40 times at 94° C for 10 sec, 60°C for 20 sec, and 72° C for 30 sec, with a final 72° C extension for 4 minutes.

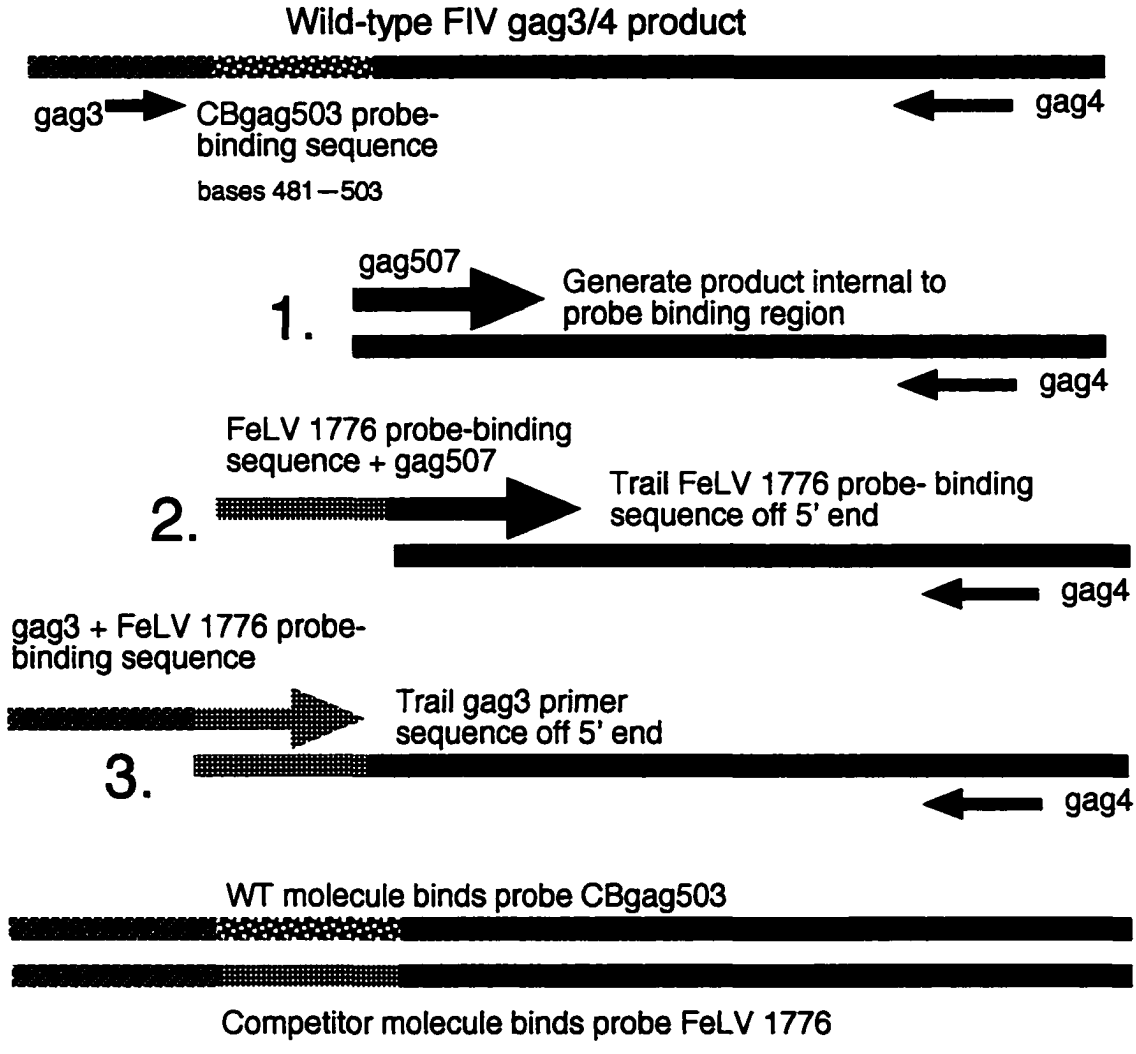

Figure 3.1: Substitutional PCR to generate RT-PCR competitor RNA molecule template. A second-round FIV *gag* PCR product with truncation of the 5' end is generated using an internal primer, gag 507, and gag 4. In the next step, a foreign DNA sequence (FeLV 1776) is included at the 5' and of the gag 507 primer. The foreign sequence is incorporated in all new PCR products. Next, the original gag 3 primer sequence is added at the 5' end of the FeLV 1776 primer, to generate the competitor DNA template with incorporation of the FeLV 1776 probe-binding sequence. In a final PCR step, a T7 RNA polymerase promoter with a linker sequence was trailed from gag , resulting in a DNA template with a built-in T7 promoter for in vitro RNA transcription (not shown).

PCR products were quantitated in 96-well microplate hybridizaton assay plates, as described above, with the following modifications: CB gag 503 probe (biotin-5'-TCACCTCCTAACCCTTCTCTTGC-3') was placed in the first 5 wells of an 8-well

column. FeLV 1776 probe (biotin-5'-ACTGCTTCGAAAAGGGAACTGGG-3'), which is complementary to the substituted FeLV sequence in the RNA competitor molecule, was placed in lanes 6 and 7. A mixture of wild-type and competitor probe was placed in lane 8, which was used as the negative control. Ten percent of each PCR product was placed in a microcentrifuge tube as described above. Fifty uL of undiluted PCR solution was placed in lanes 1 and 6. Serial 5X dilutions were carried out from lanes 1—5, and from lane 6—7. PCR product from the negative control tube was placed in lane 8. Hybridization and detection were carried out as described above. To calculate viral RNA copies/mL, we first calculated a quantitation standard (QS) average using the following formula: $QS = \frac{ODlane6 + (5 * ODlane7)}{2}$. To determine wild type RNA copy number, we took the highest dilution lane of wild type PCR product (lanes 1—5) with an OD ≥ 0.1, multiplied the OD by the dilution factor (e.g. 5X, 25X, 125X, etc.), and divided that figure by the quantitation standard average. The final figure was multiplied by 1000 (100 copies competitor/0.1 mL input plasma RNA) to determine RNA viral load in copies/mL. Samples below the lower detection threshold of 100 copies/mL were assigned a value of 40 copies/mL for statistical analysis.

Immunohistochemistry and DNA in situ hybridization

Light microscopic and fluorescent FIV immunohistochemistry and DNA in situ hybridization, with and without cell phenotype co-labeling, were performed as described in Chapter 2. The only modification was that the hybridization temperature for DNA in situ hybridization was increased from 37° to 70° C to increase sensitivity and specificity.

RESULTS

Fetal infection

Four litters each were collected from queens infected with FIV-A-Pet or FIV-C-Pgmr. A fetal FIV infection case was defined by a positive DNA PCR result on any tissue excluding placenta. PCR results for queen blood, placentas, and fetuses are summarized in Table 3.1. From the FIV-A-Pet queens 8/12 fetuses (67%) were FIV^+. From the FIV-C-Pgmr queens 10/11 fetuses (92%) were FIV^+. No viral RNA was detected in fetal plasma or amniotic fluid from either group. Unlike the FIV-C-Pgmr group, virus was never detected in brain or liver of FIV-A-Pet fetuses. In FIV^+ tissues, FIV-A-Pet fetuses had a mean of 1.8 provirus copies/ug DNA, while FIV-C-Pgmr fetuses averaged 8.7 copies/ug. There were more $provirus^+$ tissues per infected fetus in the FIV-C-Pgmr group than in the FIV-A-Pet cohort. In the FIV-A-Pet group there were 14% positive tissues per $provirus^+$ fetus while FIV-C-Pgmr fetuses averaged 49%. Mindful of differences between weights of different organs as well as other variables, we used the above data to calculate crude estimates of total body proviral burden. Based on a standard 100 g birth weight, the FIV-A-Pet cohort averaged 25×10^6 copies of provirus per FIV^+ fetus while the FIV-C-Pgmr fetuses averaged 426×10^6 copies. Thus, virus prevalence and tissue distribution, and proviral load were higher in FIV-C-Pgmr infected fetuses than in FIV-A-Pet infected fetuses.

Because FIV-A-Pet has not previously been documented to be transmitted vertically, we performed assays to confirm that the virus infecting the fetuses was indeed the FIV-A-Pet strain. After nested *gag* PCR, we cloned the products and performed

homology between FIV *gag* sequences, we could not be confident that the sequence data definitively distinguished the two isolates. Therefore, we designed primers specific for FIV-A-Pet which flank the third-through-fifth hypervariable loops (V3—V5) of the *env* gene. After a single round of PCR, DNA from FIV-A-Pet-positive fetuses yielded bright bands of the appropriate size on agarasoe gels, while no product was amplified from FIV-C-Pgmr fetal DNA (Fig. 3.2).

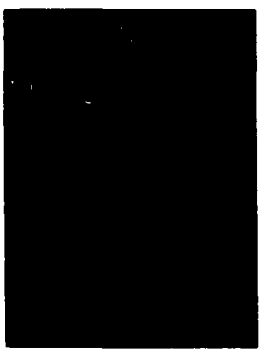

Figure 3.2: Results of PCR on DNA extracted from naïve and FIV *gag*-positive fetal thymuses using *env* primers designed specifically for FIV-A-Petaluma. Final product of single-round PCR reaction is 1016 base pairs. MW = molecular weight markers (base pairs); N = naïve, C = FIV-C-Pgmr, A = FIV-A-Pet.

Table 3.1: Summary of PCR results for queens, placentas, and fetuses.*

Queen (Q) or fetus (F)	Placenta	Brain	Thymus	Liver	Spleen	Mesen-teric LN	Bone marrow	PBMC	Plasma§	Amniotic fluid§
FIV-A-Pet										
Q3867								10	1239	
F1	-	-	1	-	-	-	-	-	<100	<100
F2	10	-	-	-	-	-	-	-	<100	<100
Q3395								10	<100	
F1	-	-	-	-	-	1	1	-	<100	<100
F2	1	-	-	-	1	-	-	-	<100	<100
F3	-	-	-	-	-	1	-	1	<100	<100
Q3866								10	1045	
F1	-	1	-	-	-	-	1	-	<100	<100
F2	10	-	-	-	1	-	-	-	<100	<100
F3	-	-	-	-	-	10	-	-	<100	<100
F4	10	-	-	-	-	-	-	-	<100	<100
Q3395								1	<100	
F1	-	-	-	-	-	-	-	-	<100	<100
F2	-	-	-	-	-	-	-	-	<100	<100
F3	1	-	-	-	1	-	-	-	<100	<100
FIV-C-Pgmr										
Q3659								1000	1265	
F1	1000	1	100	10	-	-	1	10	<100	<100
F2	1000	-	-	-	-	1	1	-	<100	<100
F3	100	-	-	-	-	-	-	-	<100	<100
Q3666								1000	1567	
F1	1000	10	-	-	10	-	-	-	<100	<100
F2	1000	-	-	-	10	-	-	10	<100	<100
F3	1000	10	10	10	-	1	-	10	<100	<100
F4	100	-	-	10	10	10	10	10	<100	<100
Q3659								100	<100	
F1	100	10	1	1	1	-	10	-	<100	<100
F2	100	-	1	10	1	1	1	10	<100	<100
Q3664								1000	4126	
F1	100	-	-	-	-	-	1	-	<100	<100
F2	1000	-	1	-	-	10	1	1	<100	<100

* Semi-quantitative DNA PCR results for tissues are proviral copies per ug DNA.
§ Plasma and amniotic fluid results are RNA copies/mL. The lower limit of detection for our quantitative RT-PCR assay was 100 copies/mL

Maternal hematology and viral loads

Mean hematocrit and serum total protein values at the time of cesarean were virtually identical between the FIV-A-Pet and FIV-C-Pgmr queens (all mean values,

standard deviations, and P-values shown in Table 3.2). Mean white blood cell count was higher in the FIV-C-Pgmr queens, but not significantly so. FIV-A-Pet queens had a higher mean $CD4^+$ T cell count and a lower $CD8^+$ count, but these values individually were not significant. However, there was a highly significant difference in CD4/CD8 T cell ratios between the groups. The mean ratio for the FIV-A-Pet queens was 1.3 while the mean for the FIV-C-Pgmr queens was 0.6 (p=0.003). Although the FIV-C-Pgmr queens had been infected longer than the FIV-A-Pet queens at the time of cesarean (mean 21 months post-infection for the FIV-C-Pgmr group versus 8 months for the FIV-A-Pet cohort), the FIV-C-Pgmr queens had higher plasma viral RNA loads than did the FIV-A-Pet queens; however, this difference was not statistically significant. FIV-C-Pgmr queen mean blood mononuclear cell proviral load, by contrast, was significantly higher (two log-fold) than the FIV-A-Pet group (p=0.01).

Table 3.2: Comparison of hematologic and viral kinetic parameters between FIV-A-Pet- and FIV-C-Pgmr-infected queens at the time of cesarean.

Value	FIV-A-Pet (SD*)	FIV-C-Pgmr (SD)	P-value
Hematocrit (%)	29 (2.4)	29 (5.6)	0.99
Total protein (g/dL)	6.4 (0.7)	6.5 (0.5)	0.82
WBC (cells/uL)	4782 (2103)	6131 (2938)	0.48
$CD4^+$ T cells/uL	501 (237)	422 (358)	0.73
$CD8^+$ T cells/uL	376 (120)	691 (597)	0.34
CD4/CD8 ratio	1.3 (0.2)	0.6 (0.2)	0.003
Viral RNA load (copies/mL plasma)	616 (667)	1750 (1716)	0.26
PBMC proviral load (copies/ug DNA)	78 (45)	7750 (4500)	0.01

* SD = standard deviation.

Placental FIV

All placentas from FIV-C-Pgmr queens were DNA PCR$^+$ and carried a mean proviral burden equivalent to that of purified maternal blood mononuclear cells (Table 3.3). By contrast, fewer than half of placentas from FIV-A-Pet queens were DNA PCR$^+$, and the mean placental proviral burden was ~1 log lower than that in maternal blood mononuclear cells. In the FIV-A-Pet cohort, only 60% of provirus$^+$ placentas were associated with an FIV$^+$ fetus.

Table 3.3: Placental FIV localization, correlation with fetal infection, and placental versus maternal blood mononuclear cell provirus burdens.

	FIV-A-Pet	FIV-C-Pgmr
FIV$^+$ placentas/total placentas	5/12	11/11
% FIV$^+$ placentas	42%	100%
FIV$^+$ placentas with FIV$^+$ fetus/total FIV$^+$ placentas	3/5	10/11
% FIV$^+$ placentas with corresponding FIV$^+$ fetus	60%	92%
Mean FIV$^+$ placental provirus burden, copies/ug DNA (SD*)	6.4 (4.9)	5909 (4700)
Mean maternal PBMC provirus burden, copies/ug DNA (SD)	78 (45)	7750 (4500)

* SD = standard deviation.

In situ FIV detection

No viral antigens were detected by immunohistochemistry in placental (Fig. 3.3) or fetal tissue sections. Control tissues from acutely infected cats, run in tandem with placental and fetal samples, were appropriately positive.

Although we detected no FIV antigens in placental tissue sections, we identified FIV provirus$^+$ cells by DNA in situ hybridization (Fig. 3.4). We found occasional FIV provirus$^+$ mononuclear cells in maternal blood vessels (Fig. 3.5), but most placental provirus-bearing cells were located in subepithelial stroma.

Figure 3.3: Immunohistochemistry, placenta. No FIV antigens are evident. FIV—Vector VIP, Evan's blue counterstain; bar = 200 uM.

Figure 3.4: DNA in situ hybridization, placenta. FIV provirus^{+} cells (arrows) in stroma. Also note generalized loss of tissue architectural detail resulting from the heat DNA denaturation step required for probe hybridization. FIV—DAB, Evan's blue counterstain; bar = 200 uM.

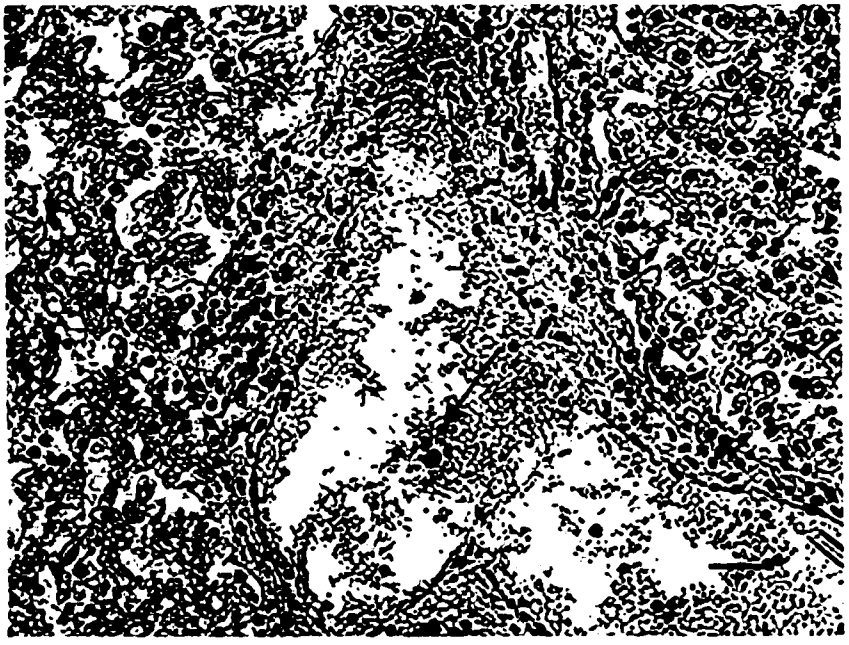

Figure 3.5: DNA in situ hybridization, placenta. Note the FIV provirus$^+$ mononuclear cell (arrow) in the central lumen of this vessel on the maternal side of the placenta. (Retained maternal vessels are normal in the feline endotheliochorial placenta, unlike the primate hemochorial placenta.) FIV probe—DAB, Evan's blue counterstain; bar = 90 uM.

Within fetuses, provirus was most readily detected by DNA in situ hybridization in the thymus (Fig. 3.6). Most cells with detectable provirus were located in the cortex. When present, medullary provirus was usually located within cells in or near Hassal's corpuscles. Because the relative insensitivity of the DNA in situ hybridization assay resulted in very little demonstration of provirus in tissue sections, correlations of viral integration with specific cell phenotypes could not be completed. Thymic medullary FIV DNA$^+$ cells were commonly CD3$^+$ T cells (Fig. 3.7). In the cortex, most FIV provirus$^+$ cells were CD3$^-$; however, these cells generally had a size and shape consistent with immature thymocytes. They did not label with antibodies directed against the B cell

surface antigen CD45R/B220 (Pharmingen), histiocyte marker Mac 387 (Serotec), or dendritic cell protein S-100 (Serotec).

Fetal lymph nodes were not available for histologic analysis because their small size provided volume sufficient only for DNA PCR. Provirus was not demonstrated by DNA in situ hybridization in fetal liver or spleen. This was probably due to very low levels of provirus, as determined by fluid-phase DNA PCR, coupled with limitations in the sensitivity of our in situ hybridization assay. Provirus^{+} cells were detected in brain sections from FIV-C-Pgmr-infected fetuses, most commonly in cortical grey matter or in structures surrounding the third ventricle such as hippocampus (Fig. 3.8). The identity of the FIV^{+} brain cells was not determined by co-labeling studies, although morphologically they had the appearance of glial or microglial cells.

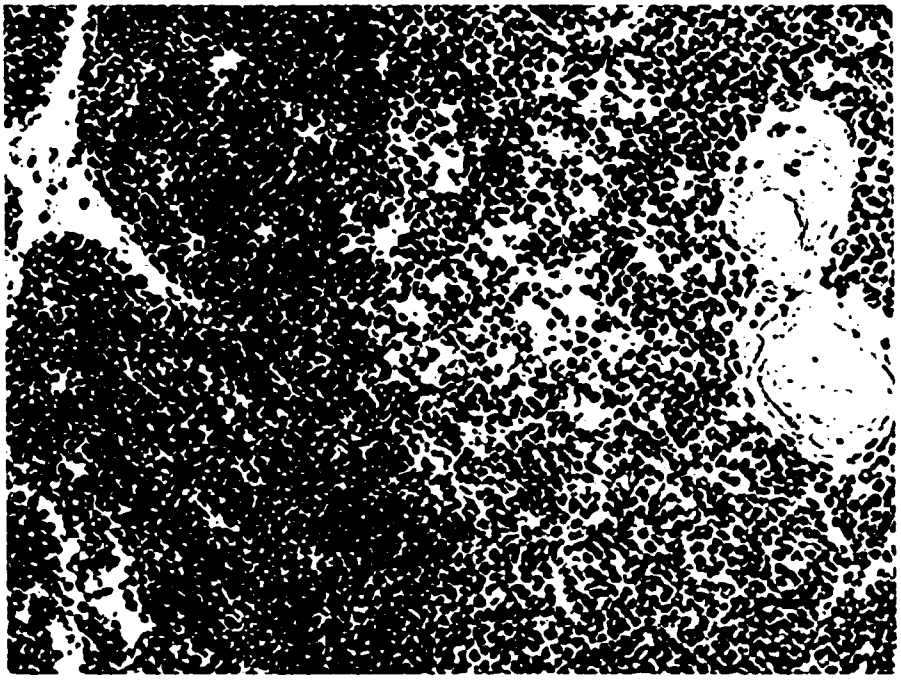

Figure 3.6: DNA in situ hybridization, fetal thymus. A few FIV DNA$^+$ cells are evident in the cortex and medulla (arrows). Round pale-staining structures to the right are Hassal's corpuscles..FIV probe—Vector NovaRed, hematoxylin counterstain; bar = 150 uM.

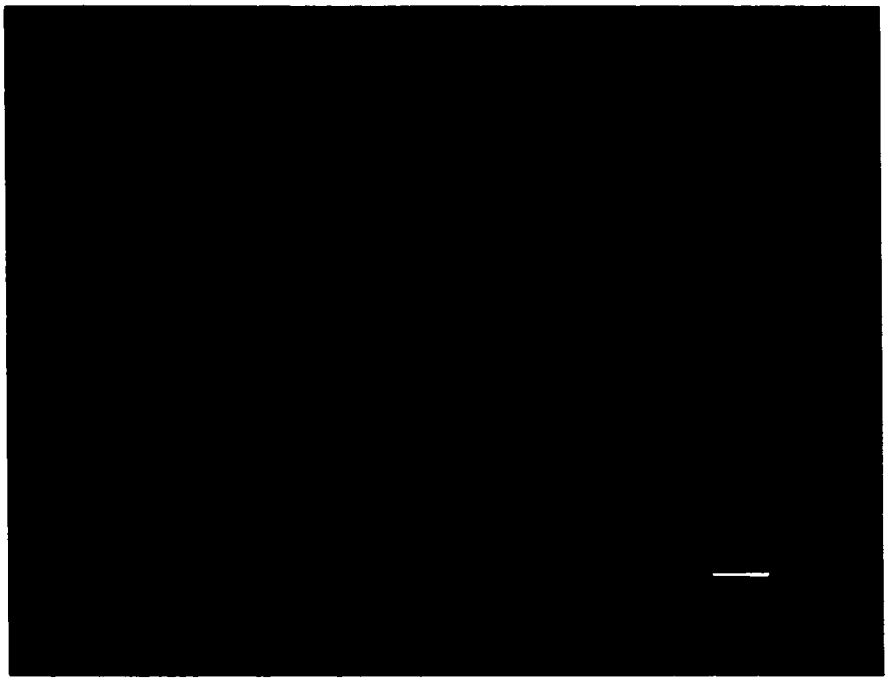

Figure 3.7: DNA in situ hybridization, fetal thymus. One of the CD3-expressing T lymphocytes (green) contains detectable FIV provirus (red). FIV probe—Cy3, CD3—FITC, nuclei—DAPI; bar = 90 uM.

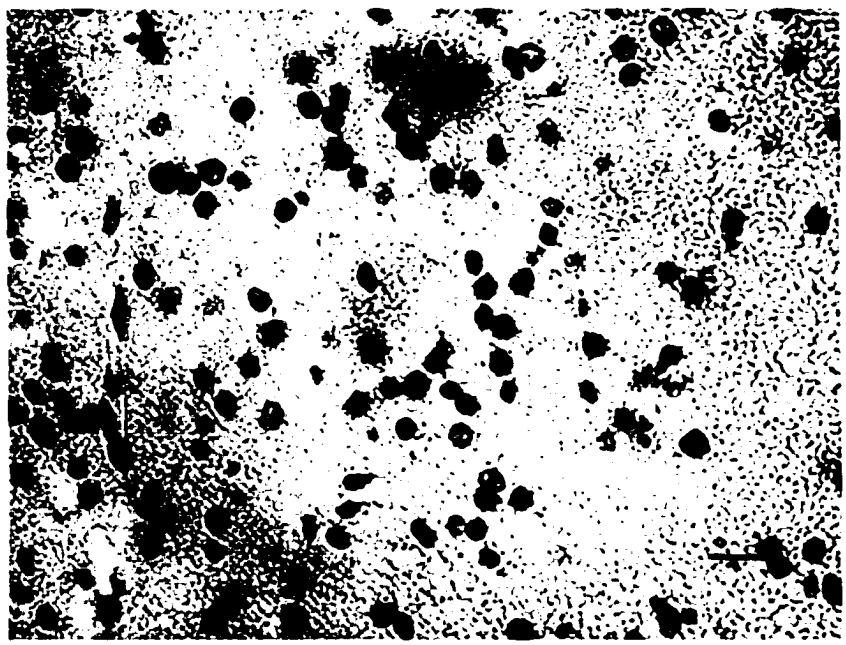

Figure 3.8: DNA in situ hybridization, fetal brain. Two FIV DNA$^+$ cells are seen at the upper left of this field, taken in the area of the hippocampus. FIV probe—Vector NovaRed, Evan's blue counterstain; bar = 40 uM.

DISCUSSION

In this report we describe maternal-fetal transmission of FIV isolates representing *env* clades A and C. These studies extend previous work characterizing in utero transmission and fetal tissue tropisms of FIV-B-2542 [14-16] . Taken together, our results show that intrauterine FIV transmission is not limited to a single isolate or clade, and suggest that vertical FIV transmission may be more common than formerly believed [19-21] . However, these findings also indicate that the kinetics and distribution of fetal virus can be highly variable among isolates. Graphical demonstration of the relative prevalence of FIV provirus in different tissues of fetuses born to queens infected with FIV-A-Pet, FIV-B-2542, and FIV-C-Pgmr is shown in Figure 3.9 (FIV-B-2542 fetal data derived from a previous study) [16] . Although FIV-A-Pet has not previously been shown to

transmitted transplacentally, PCR results using clade-specific *env* primers confirmed that in the present study FIV-A-Pet was transmitted in utero.

Because of the extensive similarities between FIV and HIV-1, it seems reasonable to presume that many features of maternal-fetal FIV transmission also apply to intrauterine HIV-1 transmission. We have shown in current and previous studies [16] that term fetuses frequently sequester FIV in tissues even in the absence of detectable virus in blood. Indeed, depending on the isolate, 40-84% of term fetuses with demonstrable tissue provirus lack detectable FIV in blood or plasma. Thus, blood tests alone at or near the time of birth are inadequate to document in utero infection. This is significant because prevailing definitions on the timing of vertical HIV-1 transmission rely solely on blood assays [22, 23] . If fetal HIV-1 sequestration parallels that of FIV, current models underestimate the number of late in utero HIV-1 infections and attribute too much weight to virus transmission during birth. Until more is known about virus sequestration in humans, definitions of vertical HIV-1 transmission timing based solely on blood assays during the first few weeks of infancy should be considered speculative.

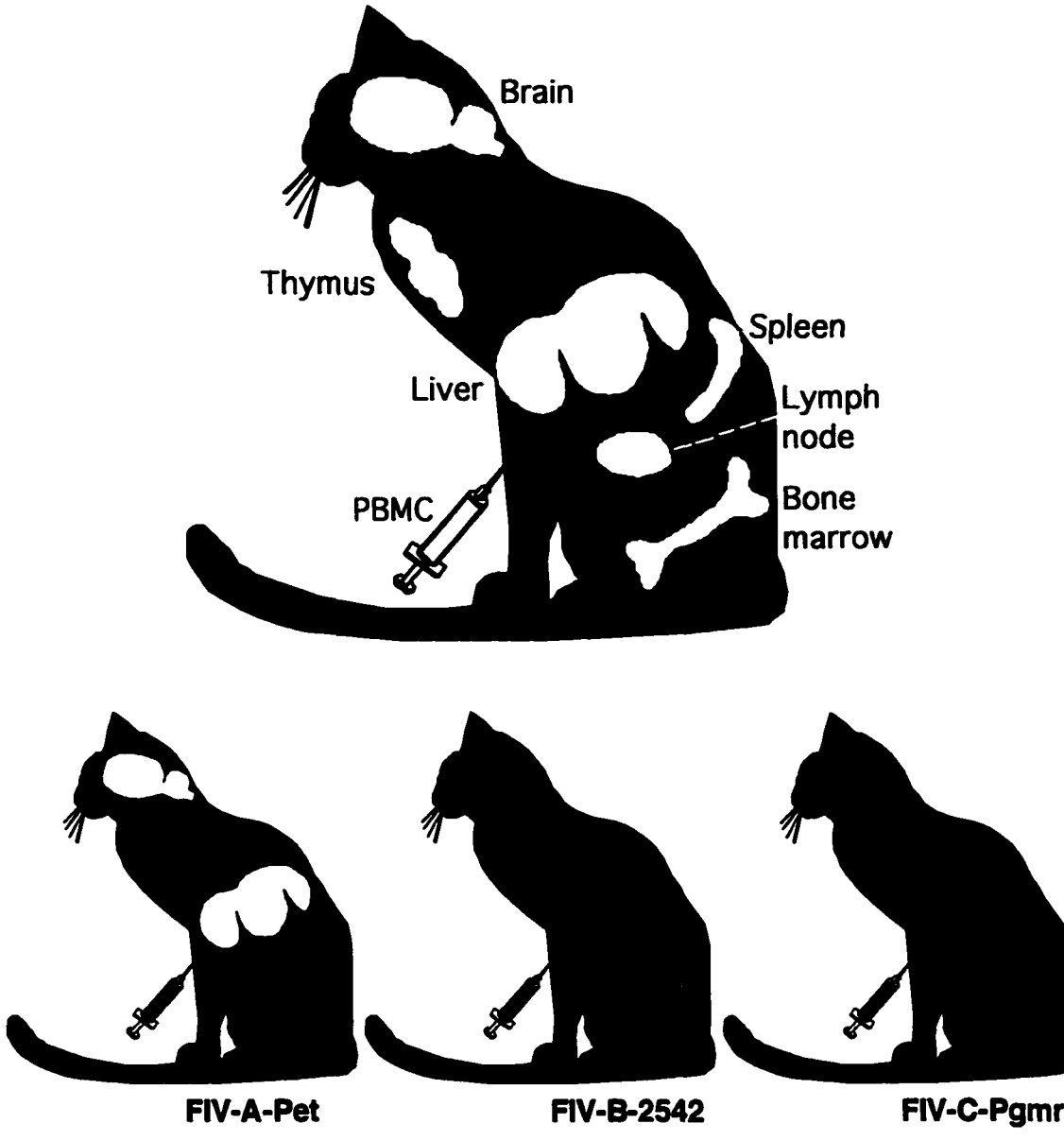

Figure 3.9: Comparison of provirus prevalence (DNA PCR$^+$) in different tissues of FIV$^+$ fetuses derived from FIV-A-Pet, FIV-B-2542, and FIV-C-Pgmr queens. At top is the anatomic key. Colors indicate the following prevalence percentages: white = 0%, green = 1—25%, blue = 26—50%, pink = >50%.

A great enigma surrounding vertical HIV-1 transmission is why, even in the absence of chemoprophylaxis, most infants do not become infected. One answer may lie in the nature of fetal virus exposure. Unlike horizontal HIV-1 transmission, in cases of fetal exposure the viral inoculum may be very low and intermittent, allowing a host even

with an immature immune system to mount an early and effective response. Many HIV^- infants of HIV^+ women show evidence of exposure to virus, or at least viral antigens, in utero. This might be expected given that maternal leukocytes are commonly found in fetal circulation [24] . Mononuclear cells of HIV^- neonates from HIV^+ women mount strong in vitro β-chemokine responses in response to challenge with HIV envelope peptides, while cells from HIV^+ infants do not [25] . Cytotoxic T-lymphocytes (CTL) do not appear to be required for this protection [25] . High levels of certain β-chemokines prevent in vitro infection of brain microglia [26] , possibly involving a calcium-regulated pathway [27] . The chemokine RANTES may help protect fetuses from de novo HIV infection even though cord blood leukocytes of fetuses, regardless of HIV status, produce less RANTES than do normal adult leukocytes [28] . Unfortunately, HIV-specific β-chemokine responses are lost early in life, dampening hopes that HIV-exposed but uninfected infants might be protected from subsequent infection [25] . The ability of a fetus to generate protective CTL in response to HIV-1 exposure remains controversial. Some investigators have have seen no HIV-specific CTL in HIV^- children of HIV^+ women [29] while others have identified HIV-1-specific CTL in infants diagnosed as uninfected [30-32] . Thus, there is increasing evidence that protective fetal responses to virus exposure in utero may in part account for the relatively low rate of observed vertical HIV-1 transmission.

If most vertical HIV-1 transmission occurs in utero, how does one explain recent clinical results demonstrating signficant reductions in infant infections even when chemoprophylaxis is not begun until near the time of birth [33, 34] ? The answer could lie in the dynamics of virus replication in the fetus. Although we identified FIV provirus

in fetal tissues by DNA PCR and in situ hybridization, our assays did not detect viral antigens by immunohistochemistry. Although FIV dissemination occured in the fetus, the rate of active replication was low. By extension, we might expect little HIV-1 replication in utero. Moreover, there is evidence that human fetal responses may contain or eliminate HIV-1 infection via inhibitory cytokines and/or cytotoxic T lymphocytes, as described above. Thus, institution of antiviral chemotherapy at the time of birth, even though the fetus may have been exposed to HIV-1 previously, could be sufficient for preventing establishment of infection. There is precedent for post-exposure protection from lentivirus infection. Initiation of anti-viral therapy up to 24 hours after virus inoculation can prevent experimental SIV infection in both adult and neonatal macaques [35-37]. Encouragingly, from the practical standpoint of clinical intervention, initiation of therapy prior to first virus exposure may not be imperative.

Although maternal blood cells may confound assays of placental infection, it is becoming increasingly clear that infection of fetal-derived placental elements by HIV-1 and FIV is common. Indirect evidence in the FIV system stems from the fact that placental proviral loads may approach those of purified maternal blood mononuclear cells, implying spread of virus through the placenta. Since nucleated maternal cells likely comprise <5% of the total placental cell population, it is hard to envision how placental provirus burdens could rival those of purified maternal blood mononuclear cells without viral expansion in fetal-derived elements. More convincingly, in situ demonstration of provirus in placentas from FIV-C-Pgmr infected queens corroborates findings with HIV-1 demonstrating viral integration in fetal-derived placental elements [38-40]. However, we detected very little provirus in placental sections from FIV-A-Pet infected queens.

Likewise, investigators in some settings have not identified placental HIV-1 in situ [41]. Thus, placental dissemination of FIV and HIV-1 may vary among isolates.

Our results suggest that induction of high placental proviral burdens may be an indicator of the viral phenotypes most capable of productively infecting fetuses. However, placental infection per sé does not guarantee fetal infection, nor does placental infection necessarily precede fetal infection. For example, high levels of FIV-B-2542 provirus are found in placentas by the end of the first trimester of pregnancy, yet only ~60% of term fetuses are infected [16]. In the current study, FIV-C-Pgmr was detected in all placentas and in most fetuses. Yet FIV-A-Pet was detected in fewer than half of placentas. Moreover, only 60% of FIV provirus^{+} placentas were associated with an infected fetus. In some instances, FIV-A-Pet was found in the fetus without detectable virus in the corresponding placenta. Unlike FIV-C-Pgmr, the mean proviral burden of FIV-A-Pet placentas was ten-fold lower than that of maternal blood mononuclear cells, and provirus was not detected in placental tissue sections by DNA in situ hybridization. Thus, infection of placentas by FIV-A-Pet occurred inconsistently and at a low level. Maternal leukocytes may have been the source of some or all provirus detected by fluid-phase PCR in the FIV-A-Pet placentas. In spite of the infrequent finding of provirus in placental tissues, FIV-A-Pet was detected in more than half of term fetuses. A plausible explanation for the lack of concordance between placental and fetal proviral integration might be direct transmigration of maternal leukocytes into fetal circulation. However, the possibility of breaches of placental integrity with passage of free virus into fetal circulation cannot be excluded.

The role of the placenta in maternal-fetal HIV-1 transmission remains ambiguous. Some researchers believe that the placenta is the pivotal player in determining fetal infection outcomes [39] . Placental cells are preferentially infected by R5 HIV-1 phenotypes [42] . Likewise, NSI HIV-1 isolates are usually, though not always, the predominant phenotype found in infants [43, 44] . Thus, selection at the level of the placenta may determine the likelihood of certain virus isolates reaching the fetus. However, as with FIV, placental infection alone does not accurately foretell fetal HIV-1 infection. Placentas of non-transmitting mothers are as likely to test positive for HIV-1 as are placentas from transmitting mothers [45] . Small breaks in placental epithelium are ubiquitous at the time of birth and are unassociated with vertical transmission risk [Byers, 1998 #1824]. Neonates may be infected with multiple HIV-1 genotypes, arguing against placental selection of a single isolate [44] . Placental immunity has been proposed as a first-line defense against fetal infection. However, although placental cells from HIV^+ women exhibit increased levels of antiviral interferons, placental interferon levels are not different between transmitting and non-transmitting mothers [46] . Thus, the dubious predictive value of placental FIV localization with fetal infection outcome is in agreement with results supporting a diminished role for the human placenta in preventing or facilitating intrauterine HIV-1 transmission.

This is the first report of vertical transmission of FIV-A-Petaluma. Indeed, we originally included this isolate in our studies as a potential negative control for vertical transmission. In early reports other investigators did not observe infection of kittens born to queens infected with FIV-A-Pet, although only blood was examined in those neonates [19] . As with HIV-1, it is possible that fetuses exposed to FIV in utero may eliminate the

virus prior to establishment of permanent infection. Another potential outcome of in utero FIV exposure is "regressive" infection, characterized by tissue-sequestered latent provirus in the absence of detectable virus or antibody in blood. Our laboratory has previously documented cases of vertical transmission of FIV resulting in inapparent infections post-natally. Kittens testing positive for FIV in the neonatal period later became seronegative and were negative for viral RNA and DNA in blood. Ongoing presence of virus was only identified through PCR analysis on tissues [47] . Vertical FIV transmission may be more commonly reported now that we know that very sensitive assays must be applied to a complete spectrum of tissues before discounting infection. An alternative explanation for our finding of vertical transmission of FIV-A-Pet is that the sample sent to us may have been an unusually virulent variant. Widely divergent virulence patterns in different stocks of FIV-A-Pet have been observed by others (E. Sparger, personal communication). More work will be required to clarify the true incidence of vertical FIV transmission both in the laboratory and in the field.

In conclusion, our findings in the present and prior studies demonstrate vertical transmission of FIV isolates representing the three major clades. Significant variability is encountered in the prevalence, degree, and distribution of fetal infection among FIV isolates. Fetal tissue sequestration of FIV is common. Thus, blood assays alone are unreliable indicators of infection in the perinatal period. These findings raise concerns about conclusions drawn from the blood-based protocols used to define the timing of perinatal HIV-1 transmission. We have shown that high placental provirus burden may indicate increased risk of fetal infection, but that virus localization to placenta is neither necessary nor sufficient for fetal infection. Information gained from further study of the

mechanisms of maternal-fetal FIV transmission might aid in the identification of new strategies for intervention of mother-to-child HIV-1 transmission.

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