DISSERTATION

PERINATAL FELINE IMMUNODEFICIENCY VIRUS INFECTION: A MODEL OF IN UTERO AND MILK-BORNE HIV-1 TRANSMISSION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATON PREPARED UNDER OUR SUPERVISION BY ROBIN WALSH ALLISON ENTITLED FELINE IMMUNODEFICIENCY MODEL OF HIV-1 VERTICAL TRANSMISSION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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A MODEL OF IN UTERO AND MILK-BORNE HIV-1 TRANSMISSION

Mother-to-child transmission of human immunodeficiency virus type-1 (HIV) occurs in utero, during delivery, and postnatally by breastfeeding. Milk-borne virus may be responsible for up to two-thirds of overall vertical transmission, and yet little is known about the role of cell-free and cell-associated virus in milk, and no studies have identified cellular sources of HIV in mammary gland. Most children of HIV-infected women are not detectably HIV-positive, although many have HIV-specific immune responses that suggest viral exposure. We studied mother-to-offspring transmission of feline immunodeficiency virus (FIV), the feline equivalent of HIV infection, and documented extensive viral shedding in milk, identified FIV-infected cells in mammary gland, and uncovered atypical 'covert' FIV vertical transmission. These results have relevance for the design of effective intervention strategies to reduce perinatal HIV transmission.

Kittens born to FIV-infected cats were assayed for virus in blood and/or tissues. DNA polymerase chain reaction (PCR) detected provirus in blood of all kittens on the day of birth, indicating in utero FIV infection. FIV antibody levels declined and became undetectable past 4 months of age, consistent with maternal antibody. No CD4 or CD8

T-cell abnormalities were observed, and kittens remained clinically healthy for over one year. Provirus was detected more easily in tissues than in blood, suggesting sequestration of virus with downregulated replication. This occult FIV vertical transmission may be a good model to investigate in utero viral exposure and potential protection from subsequent viral challenge.

Milk and blood samples were collected from FIV-infected cats. Viral RNA loads in early milk were as much as 4 log-fold higher than those in plasma, whereas proviral loads in milk cells were consistently lower than those in blood; thus virus appears to be concentrated in early milk. Proviral loads were equivalent in separated blood cells enriched for mononuclear cells versus polymorphonuclear cells, suggesting FIV infection of neutrophils. Additionally, treatment of cats during pregnancy with the antiretroviral drug PMPA was associated with decreased kitten birth weight and viability.

Cellular sources of FIV were sought in mammary gland biopsies from FIV-infected cats. In situ hybridization (ISH) revealed numerous FIV-infected cells in primarily intraepithelial locations. Combined immunohistochemistry and ISH identified the majority of these cells as CD3+ T-cells. Significantly more T-cells were observed in mammary glands of FIV-infected versus naïve cats. These results suggest a reservoir of FIV-infected cells in mammary gland that may promote viral dissemination in milk.

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and taking blood from 80-gram newborn kittens. Her expertise, dedication, and friendship will be sorely missed.

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DEDICATION

I dedicate this dissertation to my parents, Robert Walsh and Joyce O'Dowd, who not only believed I could do anything but convinced me that age was not an obstacle; and to my husband Wayne Jasper, whose love and humor continue to sustain and amaze me.

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CHAPTER ONE

INTRODUCTION TO LENTIVIRUS VERTICAL TRANSMISSION

Human immunodeficiency virus type 1 (HIV)

In the year 2001, 40 million people were living with HIV/AIDS; almost half were women while 2.7 million were children under the age of 15 [1]. Of the 3 million deaths attributed to HIV that year, 580,000 (19%) were children. Mother to child transmission was responsible for the vast majority of new infections in children, which occurred at the rate of 2,000 a day in 2001. The situation was particularly tragic in developing regions of the world such as sub-Saharan Africa, where 70% of new HIV infections worldwide occurred [2] and 55% of infected adults were women [1].

Vertical transmission of HIV can occur in utero, intrapartum, and postpartum via breastfeeding [3-5], however the relative contribution of each route of exposure remains a subject for debate. It can be assumed that infants testing positive for HIV by DNA polymerase chain reaction (PCR) or virus isolation (VI) on the day of birth were infected in utero, but babies testing negative at birth and positive at a later time are harder to categorize. The average period from infection to detectable virus in blood has been estimated to be 10 days, thus an initial negative test followed by a positive test after 10 days of age has been considered intrapartum infection [6]. However, it is difficult to

differentiate late in utero from intrapartum infection, and postnatal exposure to virus in breast milk confuses the picture [7]. The accepted view is that up to 70% of infections occur very late in utero or intrapartum [8-10]. Supporting evidence is provided from several sources: HIV has been detected in vaginal and cervical secretions at delivery, and in oropharyngeal aspirates from newborns [11-13]; caesarean section delivery has been associated with a lower risk of transmission [8, 14, 15], and antiretroviral treatment of mothers late in pregnancy and during labor significantly reduces transmission [16-19].

Breastfeeding is thought to increase risk of vertical transmission from 14 – 29%, and has been implicated in one-half to two-thirds of all new infections in infants [20-22]; maternal acquisition of HIV infection during lactation is associated with the highest risk [23-27]. While breastfeeding is not recommended for HIV positive women in developed countries, in underdeveloped nations malnutrition and infectious disease remain the primary causes of infant mortality; unsafe water supplies, lack of economic resources, and social stigma make bottle-feeding an unrealistic option for most [25, 26, 28-30]. Thus an understanding of milk-borne virus is crucial for making rational decisions about interventions designed for prevention of such transmission, such as early weaning or drug therapy.

Both cell-associated and cell-free virus have been detected in milk [31-35], however studies to evaluate their contribution to transmission rates have been contradictory. Very few studies have examined HIV RNA levels in milk; Lewis et al. reported RNA detection in 39% of samples, with more than half containing viral loads near the lower limit of detection [34]. Another study reported 63% prevalence of HIV RNA in milk, with mean viral loads of 1,000 copies/ml that did not change over a 3

months period; HIV RNA quantity was associated with an increased risk of vertical transmission [35]. Recently, Willumsen, et al. described intermittent shedding of virus in breastmilk, with frequent differences between samples from left and right breasts and viral loads up to 1.2 million copies/ml [36].

While some have found increased risk of transmission associated with detection of viral DNA in milk [37], others have not [38]. The cellular content of breast milk changes from early to late lactation; cell numbers are highest in colostrum and early milk, consisting primarily of macrophages and granulocytes. Cell numbers later decrease, and cell types change to include macrophages, lymphocytes, and epithelial cells [39]. If cell-associated virus is an important mode of transmission, it might be expected that infants would be most vulnerable during early lactation. In fact, while some studies have shown that most milk-borne transmission occurs early [30], others have not seen such an association [20, 40], and some implicate longer duration of breastfeeding with increased risk of transmission [25, 37].

There are likely many confounding factors influencing the outcomes of such observational studies – maternal factors such as CD4+ T-cell count, viral load, vitamin A deficiency, and presence of mastitis may be important [37, 41-43]. In addition, milk contains a variety of nonspecific anti-infective factors as well as HIV-specific antibody. A variety of protein and lipid substances found in milk may have protective effects, including lactoferrin and secretory leukocyte protease inhibitor [44-51]. HIV specific antibody can be detected in milk, but is not always associated with reduced risk of infection [52-56]; in fact there is in vitro evidence for antibody-mediated enhancement of

HIV infectivity [57-61] and an association between high maternal HIV antibody titer and increased perinatal transmission [62].

Host and virus genetic factors can influence vertical transmission rates as well. Examination of virus quasispecies transmitted from mother to infant has revealed apparent selection for some variants, which may depend upon the timing of transmission [63-66]. Discordance between mother and infant for MHC Class I alleles has been associated with reduced HIV transmission [67, 68]. Polymorphisms in chemokine receptors, which serve as coreceptors for HIV, or their ligands have been implicated in susceptibility to infection and disease progression among vertically exposed children [69-74].

Vertical transmission of other lentiviruses

It is clear from the complexities underlying observational studies of HIV vertical transmission that animal models are a necessity if we are to understand the host-virus interplay and design effective interventions. Mother-to-offspring transmission has been documented in other animal lentiviral infections. Several such infections of ruminants have been studied: bovine immunodeficiency virus (BIV) of cattle, caprine arthritis-encephalitis virus (CAEV) of goats and sheep, and ovine lentivirus (OvLV, maedi/visna virus) of sheep. Transmission through colostrum or milk has been documented for all three [75-78], and viral DNA has been identified in milk cells from BIV and CAEV infected animals [79-81]. In fact, mammary gland is a primary site of CAEV infection [76], and the major route of transmission is via ingestion of virus in milk [82]. While cells of the monocyte/macrophage lineage are the primary targets of CAEV and OvLV

[83, 84], and lymphocytes the primary target for BIV [85], many different cell types can be infected [86-88]. Interestingly, CAEV has been shown to replicate efficiently in mammary gland epithelial cells [89]. In utero transmission of BIV and OvLV has also been documented [90, 91]; estimates of the frequency of such transmission vary from 30 to 40% for BIV, to 11% for OvLV [90-92].

Equine infectious anemia virus (EIAV) is another lentivirus in which both in utero and milk-borne transmission has been documented; like CAEV and OvLV, monocytes and macrophages are the primary target of infection [93-95]. However, vector-borne transmission is by far the most important mode and therefore has received the most attention [96].

Simian immunodeficiency virus (SIV) of non-human primates is a well-studied model of HIV infection, with many similarities in terms of genome organization, cell tropisms, and receptor usage [97, 98]. However, there is a low prevalence of fetal infection despite the ability of SIV to infect placental cells in vitro [99, 100]; most vertical transmission documented has occurred postnatally via milk [101-103]. Therefore, studies of neonatal drug intervention to prevent in utero viral transmission using the SIV model have relied upon fetal inoculation for exposure to virus [104]. Additional drawbacks to the SIV model include the expense of primate research, limited animal availability, and zoonotic disease potential.

The feline immunodeficiency virus model

Feline immunodeficiency virus (FIV), the feline counterpart of HIV, causes a virtually identical disease syndrome which includes: (1) an acute phase characterized by a

burst of viral replication, a flu-like syndrome, and an early and progressive decline in CD4+ T-cells; (2) a prolonged midphase marked by virus downregulation and a continued gradual decline in CD4+ cell numbers; and (3) and a terminal phase marked by clinical immunodeficiency [105-109]. Importantly, transmission of FIV from mother to offspring has been documented to occur in utero and postnatally through milk.

Although early reports indicated vertical transmission to be rare, occurring only during acute maternal infection [110-112], later studies performed with different viral isolates revealed more frequent perinatal infections. O'Neil, et al. described vertical transmission rates of FIV-B-2542 and FIV-AB-2771 in queens delivering kittens that were later nursed by either their infected mothers or naïve surrogates; FIV DNA was detected in 34% of kitten blood samples on the day of birth, and 68% of samples at 6 months of age (FIV detected by viral isolation in 16% and 47%, respectively) [113]. Exposure to milk-borne virus was associated with a 13.5% increase in infection rate, and 56% of kittens non-viable at birth were FIV positive by viral isolation (VI) from tissues. Seven of nine colostrum samples tested were VI positive for FIV. Interestingly, not all infected kittens developed antibodies to FIV, and some were only transiently seropositive. The majority of FIV infected kittens (24 of 26) remained clinically healthy with no depletion of CD4+ T cells throughout the 6-month study.

In a later study of in utero FIV-B-2542 transmission, Rogers and Hoover found 40% of fetuses to be infected by 7 weeks gestation, and 60% by 9 weeks (term); DNA PCR detected ~20% more FIV-infected fetal tissues than did VI [114]. In addition, 40% of infected fetuses were FIV positive in tissues but not blood. A subsequent study examining term fetuses from queens infected with FIV-A-Petaluma (FIV-A) and FIV-C-

Pgmr (FIV-C) detected viral DNA in 67% and 92% of fetuses, respectively [115]. Thus, FIV has been detected more frequently in fetal or stillborn kitten tissue than in blood from newborn viable animals.

Another study of FIV vertical transmission examined 9 kittens that were delivered by caesarean and bottle-fed; therefore they received no colostral FIV antibody [116]. Two kittens (22%) developed rapid CD4+ T-cell depletion, were VI and PCR positive for FIV, and died by 9 weeks of age. However 7 kittens (78%) developed a 'regressive' infection; all were transiently seropositive and PCR positive in blood, while 3 were transiently VI positive. By 12 to 14 months of age all 7 kittens were seronegative, PCR, and VI negative; however 5 kittens had virus present in biopsied lymph node or bone marrow, detectable by DNA PCR or VI. These results suggested downregulation of FIV infection and/or viral sequestration to tissues.

Evidence for lentiviral containment

Taken together, these studies of FIV vertical transmission hint at a tantalizing prospect – that perinatal lentivirus infection can be sequestered and contained to below the level of detection, potentially even cleared, even without therapeutic intervention. In fact, there is some evidence that this may occur with HIV. Similar to the high prevalence of FIV documented in fetal tissues from infected queens, HIV has been detected in up to 90% of tissues from elective abortions by HIV positive women [117-119]. This observation has been difficult to explain, considering that only about 25% of children born to HIV-infected mothers have detectable HIV infections, even in the absence of retroviral therapy [7, 120, 121]. Sporadic reports of viral clearance in perinatally infected

children have recognized transient PCR or VI positivity without seroconversion [122-124]. Although some cases have come under fire and may have been laboratory error [125], others are better documented [123] and include children with transient organomegaly, which would support virus sequestration in tissues [122].

While transient HIV infection may be difficult to prove, recent investigations have focused on HIV-specific immune responses in apparently uninfected children of HIV-positive women. A wide range of such responses have now been demonstrated including: lymphocyte proliferation to HIV proteins [126], IL-2 production by CD4+ T-cells in response to HIV peptides [127-129], HIV stimulated β-chemokine production [129], HIV-specific cytotoxic T-lymphocyte (CTL) responses [130-134], and CD8+ T-cell noncytotoxic antiviral activity [135]. Most of these responses could be explained by intrauterine exposure to viral antigen, but the demonstration of CTL responses in cord blood suggests in utero exposure to live, replicating virus [120].

Similar types of immune responses have been identified in cohorts of people seemingly resistant to HIV infection. A variety of highly exposed, persistently seronegative (HEPS) adults have been investigated for correlates of protection, including sex partners of HIV-infected people [136, 137], commercial sex workers where HIV prevalence is high [138-141], and health care workers with occupational exposure [142, 143]; HIV-specific cellular immune responses have been demonstrated in all cases. Evidence from experimental SIV infection suggests that dose and route of exposure may be important; several studies have documented SIV-specific cellular immune responses in macaques inoculated mucosally with low doses of virus – these animals did not seroconvert, were intermittently PCR positive but VI negative, and were resistant to

subsequent mucosal challenge [144-147]. In another SIV study, transient treatment with a potent antiretroviral drug after intravenous virus inoculation resulted in viral containment and partial protection from subsequent challenge [148, 149]. The emerging picture is that host control of lentivirus infection may be possible, depending upon factors such as route of exposure, virus burden, degree of viral replication, and the host immune response.

Dissertation research

The initial goals of this research project were to use the FIV model of vertical transmission to investigate drug interventions aimed toward in utero and milk-borne virus exposure. However, preliminary studies using the antiretroviral drug PMPA during gestation revealed deleterious effects on kitten viability and birth weights. Additionally, observed vertical transmission rates of FIV-B-2542 were lower than previously reported, leading us to investigate the transmission potential of two different FIV isolates, FIV-A-Petaluma and FIV-C-Pgmr. Subsequently, we identified atypical 'covert' vertical transmission of FIV, a natural animal model for in utero HIV exposure and viral containment, which should shed light on the phenomenon of transient perinatal HIV infection and allow investigation of potential protection from future virus challenge.

During the initial studies, the observation of high viral loads in milk compared to plasma led to further characterization of cell-associated and cell-free virus in milk, and to exploration of potential cellular origins of virus in mammary gland tissues. Knowledge of specific virus-cell interactions in the mammary gland may impact the design of effective interventions to reduce milk-borne HIV transmission.

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CHAPTER TWO

COVERT VERTICAL TRANSMISSION OF FELINE IMMUNODEFICIENCY VIRUS

INTRODUCTION

Forty million people are living with human immunodeficiency virus (HIV) worldwide, and over half live in Sub-Saharan Africa, where HIV prevalence among pregnant women reaches 45% in some regions [1]. Mother-to-child transmission of HIV is responsible for the vast majority of infections in children, which caused the deaths of 580,000 in 2001[1]. Despite these stark numbers, it is intriguing that the majority of infants born to HIV-positive women (65 – 85%) remain apparently uninfected, even in the absence of therapeutic intervention [2, 3].

Vertical transmission of HIV may occur in utero, intrapartum, or postpartum during breastfeeding [4]. The accepted view is that most infections occur late in pregnancy or at delivery [4-8], while breastfeeding serves to increase the risk of transmission by 15 to 26% [9-11]. Nevertheless, there is evidence for frequent fetal infection as early as the second trimester; HIV has been detected by DNA polymerase chain reaction (PCR) in 50 to 90% of fetal tissues from elective abortions [12]. This is

surprising considering that only ~25% of children born to HIV-positive women have detectable HIV infections. There have been numerous recent reports of HIV-specific immune responses detected in HIV-negative children of HIV-positive women [13-18], suggesting at least exposure to viral antigens, and possibly limited viral replication. Evidence is mounting that abortive or transient HIV infections may occur in utero; understanding how viral containment or clearance might occur in these instances would contribute to the design of effective neonatal vaccination strategies.

Feline immunodeficiency virus (FIV) is the feline counterpart of HIV, causing an immune deficiency syndrome virtually identical to that caused by HIV [19-22]. Like HIV, FIV can be vertically transmitted in utero, through breast milk, and likely during delivery [23-27]. FIV has been detected in a large percentage of fetal tissues by DNA PCR [27, 28], and vertical transmission rates have recently been documented as ~22% (Chapter 3), similar to HIV. Similar to reports of transient HIV infections, regressive FIV infection has been reported in which kittens born to FIV-infected queens were transiently seropositive and PCR positive in blood; provirus remained detectable in lymph node biopsies while blood assays were negative {34}.

This work was initiated on the hypothesis that FIV would be transmitted frequently from mother to offspring, thus providing a model to investigate therapeutic interventions to interrupt such transmission. We report here unexpected occult FIV infection in kittens born to queens infected with FIV clades A and C, manifested by low-levels of provirus detected by PCR without CD4:CD8 abnormalities or detectable plasma antibody responses. Such covert FIV vertical transmission may represent a new model to investigate neonatal viral containment or clearance.

MATERIALS AND METHODS

Animals and sampling

All cats originated in a specific-pathogen-free (SPF) breeding colony maintained at Colorado State University and were housed in accordance with Animal Care and Use Committee regulations.

Three queens were inoculated intravenously with FIV-C-Pgmr (FIV-C; from an in vivo-passaged plasma pool source) and bred to FIV-negative males 4 to 7 months later. FIV infection was confirmed in the queens by DNA polymerase chain reaction (PCR) on blood as described below. All queens became pregnant and produced a total of 11 kittens. Blood was collected from queens prior to and at 2, 5, 8, 12, 16, 22, and 29 weeks post-inoculation (PI). Bone marrow aspirates were obtained from queens at 16 weeks PI. Blood was collected from queens and kittens within 24 hours of birth and biweekly for 8 to 10 weeks, then from kittens at 4, 6, 8, and 12 months of age. Milk was collected from queens within 24 hours of delivery and at 2, 4, and 6 weeks post-delivery. One kitten was euthanized for necropsy and terminal sample collection at 1 year of age.

Two queens infected with FIV-A-Petaluma for 12 to 15 months were bred to FIV-negative males. Both queens became pregnant, producing a total of 3 kittens that were non-viable at birth. Tissues were collected from these kittens at delivery. Queens were rebred 1 to 3 months later and produced a total of 5 kittens. Blood was collected from queens and kittens within 24 hours of birth and biweekly for 8 to 10 weeks. Three kittens were euthanized for necropsy and terminal sample collection at 11 weeks of age. Blood was collected from the two remaining kittens at 3, 4, 6, 12, and 14 months of age. Milk

was collected from queens within 24 hours of delivery and at 2, 4, and 6 weeks post-delivery. Naïve cats from the SPF breeding colony provided milk and blood samples for negative controls.

Sample processing

Blood was collected in EDTA under ketamine-acepromazine anesthesia (queens and kittens ≥ 6 weeks of age) or with manual restraint (kittens < 6 weeks of age) for complete blood cell count and CD4 and CD8 T-cell subset determinations. Whole blood was frozen in 200ul aliquots for DNA PCR. Plasma was separated from cells by centrifugation and stored at -70°C until use. Peripheral blood mononuclear cells (pbmc) were isolated by single-density ficoll-hypaque centrifugation (Histopaque-1077; Sigma, St. Louis, MO). In some experiments, pbmc were further sorted into CD8-enriched and CD8-depleted fractions using a FITC-conjugated anti-feline CD8 antibody (Southern Biotechnology Assoc., Birmingham, AL) and anti-FITC magnetic beads (Miltenyi Biotec, Auburn, CA).

Milk was collected by manual expression from queens under ketamine-acepromazine anesthesia. To facilitate milk release, queens received 2 U of oxytocin intramuscularly 10 minutes prior to collection. Milk cells and supernatant were separated by repeated centrifugation and removal of the fat layer. Cell-free milk supernatant was frozen for RT-PCR or used immediately for viral isolation as described below. Milk cells were washed in PBS and either pelleted and frozen for PCR or used immediately for viral isolation.

Bone marrow aspirates were collected from the proximal femur under anesthesia, and air-dried smears immediately prepared on glass slides. Slides were stained with Wright-Giemsa (EM Science Harleco, Gibbstown, NJ) for routine evaluation.

Terminal tissues collected from kittens included spleen, liver, thymus, brain, mesenteric lymph node, retropharyngeal lymph node, and bone marrow. Individual tissues were rinsed with sterile PBS and dissociated into cell suspensions through mesh sieves. Cells were pelleted and frozen for DNA PCR.

Flow Cytometry

Lymphocyte subset analysis was performed with a Coulter EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL) using feline CD4 and CD8 monoclonal antibodies as previously described [29, 30]. Absolute cell numbers were calculated from the total lymphocyte count.

Virus isolation

Naïve donor pbmc were obtained from SPF cats and stimulated with 10 μg/ml concanavalin A (Sigma, St. Louis, MO) for 3 days. One million naïve cells were cocultured in 24-well plates with sample pbmc, plasma, milk cells, or milk supernatant in RPMI medium supplemented with 20% fetal calf serum, 1% penicillin-streptomycin, 2% glutamine, 2 x 10^{-5} M 2-mercaptoethanol, and 100 U/ml interleukin-2 (Cetus/Roche, Emeryville, CA). Queen pbmc and milk cells were log diluted from 1 x 10^6 to 1 x 10^1; kitten pbmc cocultures were performed with 2 x 10^6 or 1 x 10^6 cells. Plasma and milk supernatant were added in serial 2-fold dilutions from 50 to 1.5 μl. In some experiments

CD8-enriched or CD8-depleted fractions from sample pbmc were cocultured with similar fractions from naïve pbmc. Culture supernatants were collected twice weekly for 4 weeks and assayed by FIV p26 capture enzyme-linked immunosorbent assay (ELISA) [31]. In some experiments cells were collected at the end of the culture period and frozen for DNA PCR. Negative controls consisted of naïve pbmc cultured alone, or with plasma or milk from naïve cats.

FIV antibody ELISA

Whole pelleted FIV, concentrated from persistently FIV-infected CrFK cell supernatants as previously described [38], was applied at 750 ng per well to 96-well microtiter plates in 0.01M borate buffer with 0.5% deoxycholic acid and blocked with 2% bovine serum albumin (BSA) in 0.05 M Tris/0.01 M EDTA/0.5 M NaCl (NTE). Duplicate plasma or milk samples were serially diluted 2X from 1:51 to 1:56,224 in ELISA diluent (NTE, 2% BSA, 5% fetal bovine serum, 0.5% Triton X-100) and incubated 30 minutes at room temperature. Bound antibody was detected using 100 µl per well of peroxidase-conjugated goat anti-cat IgG (Cappel, Organon Teknika Corp., Durham, NC) diluted 1:5000 in ELISA diluent with 5% mouse serum for 30 minutes at room temperature. Plates were developed with 3,3,5,5' tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories, Inc., Keene, NH) for 10 minutes and stopped with 2.5N H_2SO_4 before reading the optical density (OD), measured by absorbance at A_{450}. Titers were expressed as the inverse of the highest sample dilution that produced an OD > 0.1 and $\geq$ twice that of naïve SPF cat plasma.

In vitro FIV antibody stimulation

An in vitro method for polyclonal activation of B-cells to produce antibody to FIV was adapted from methods described by Jehuda-Cohen et al. [32]. Purified pbmc were cultured in triplicate at a concentration of 2×10^5 cells per 100 µl of medium alone (RPMI medium supplemented with 20% fetal calf serum, 1% penicillin-streptomycin, 2% glutamine, 2×10^{-5} M 2-mercaptoethanol) or medium containing 4 µg/ml pokeweed mitogen (ICN Biomedicals, Aurora, OH). Cultures were incubated for 4 days at 37°C in 5% CO_2 humidified atmosphere, centrifuged and supernatants collected and assayed for FIV antibody by ELISA described above. Cells from a naïve cat and a known FIV-positive cat were included as controls.

Provirus detection

Limiting-dilution DNA PCR using a nested set of primers located within the *gag* gene was used to estimate proviral burden. The sensitivity of this assay was determined using plasmid DNA containing the FIV-14 genome to be between one and ten copies of target sequence. DNA was extracted from either whole blood (200µl), pbmc, cocultured cells, or tissue cell pellets using a QIAamp Blood Kit (Qiagen Inc., Valencia, CA). Purified DNA was quantitated with a Beckman DU-70 spectrophotometer (Beckman-Coulter, Brea, CA). One microgram of sample DNA was amplified; DNA from positive samples was serially log-diluted and PCR-amplified to identify negative endpoints. Viral copy number per µg of DNA was estimated assuming at least one copy was detected at the highest positive dilution. For *gag* detection first round primers were gag 129 (5'-CGTAACTACAGGACGAGAACCTGG-3') and gag 802 (5'-CCAACTTTCCCAATG-

CTTCAAG-3'); second round primers gag 3 (5'-TTGACCCAAAAATGGTGTCCA-3') and gag 4 (5'-TTCTGCTTGTTGTTCTTGAGT-3') amplified a 293 base pair sequence nested within the first round product. PCR was performed in a Perkin-Elmer 9600 thermocycler (P-E Applied Biosystems, Foster City, CA). Cycling conditions for round one were: 95°C for 15 minutes to activate HotStarTaq™, 30 cycles of 94°C for 10 seconds, 57°C for 20 seconds, 72°C for 30 seconds, and a 7 minute 72°C extension. Round two used a 'touch-down' method as follows: 95°C for 15 minutes, 5 cycles with 94°C denaturing for 10 seconds, annealing for 10 seconds starting at 68°C with 4 consecutive 2°C decreases to 60°C, 72°C extension for 20 seconds; then 30 cycles of 94°C for 10 seconds, 60°C for 10 seconds, 72°C for 20 seconds, and a 7 minute 72°C extension. 25 µl reaction mixtures contained 3 mM (first round) or 2.5 mM (second round) $MgCl_2$, 1 X PCR buffer (Qiagen, Inc., Valencia, CA), 200 µM each dNTP (P-E Applied Biosystems), 0.1 µM each first round primer, 1 µM each second round primer (both Sigma-Genosys, The Woodlands, TX), and 2.5 U HotStarTaq™ polymerase (Qiagen, Inc., Valencia, CA).

Nested DNA PCR for *env* gene detection was also performed on some samples. First round primers that would amplify both FIV clades A and C were designed using MacVector® software (Genetics Computer Group, Inc., Madison, WI). First round primers were AC env 1 (5'-ACAGATCCATTACAAATCCCATTG-3') and AC env 2 (5'-GCCAACATAATATGGATAGCTGCT-3'); second round primers MB 1 (5'-ATA-CCAAAATGTGGATGGTG-3') and MB 2 (5'-CAAGACCAATTTCCAGCAAT-3') amplify a 550 base pair sequence nested within the first round product that includes the V3 region of the surface protein gp110 [33]. PCR was performed as described for *gag*

above, except that round one used a 55°C annealing temperature and primer concentrations were 1 μM in round one and 0.4 μM in round two. For both *gag* and *env* PCR, two microliters of the first round product were added to the second round reaction mixture. Products were visualized on 1.5% agarose gels containing Gelstar® (FMC Bioproducts, Rockland, ME). Specificity of the amplified products was determined by sequencing as described below. Negative controls included whole blood, pbmc, and cultured pbmc from naïve cats

DNA sequencing

Second round amplified *gag* and *env* products were purified by excision of the band of interest and gel extraction using QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA). Purified DNA was either directly sequenced (Macromolecular Resources Laboratory, Ft. Collins, CO) or first cloned into plasmid pCR®II-TOPO® using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Sequence data were analyzed using MacVector® software (Genetics Computer Group, Inc., Madison, WI).

Statistical analysis

Statistical analysis was performed with the Statview® software (Abacus Concepts, Inc., Berkeley, CA) by repeated measures ANOVA for CD4:CD8 ratios or multiple, one-tailed paired *t*-tests for neutrophil numbers. Significance was assumed at $p \leq 0.05$ for ANOVA and $p \leq 0.017$ for *t*-tests (0.05 divided by 3 comparisons).

RESULTS

FIV-C queens: transient neutropenia post-inoculation

All three cats inoculated intravenously with FIV-C became markedly neutropenic by 5 weeks post-inoculation (PI); neutropenia resolved by 5 to 6 months PI (Figure 2.1). Two of three queens (3843 and 3846) showed clinical signs of illness during this time period (fever, dehydration, and oral lesions) that required supportive care. Bone marrow aspirates taken at 16 weeks PI, when recovery from neutropenia was beginning, revealed normal myeloid:erythroid ratios with left shifts in the myeloid series and lymphoid hyperplasia (46 to 60% lymphocytes).

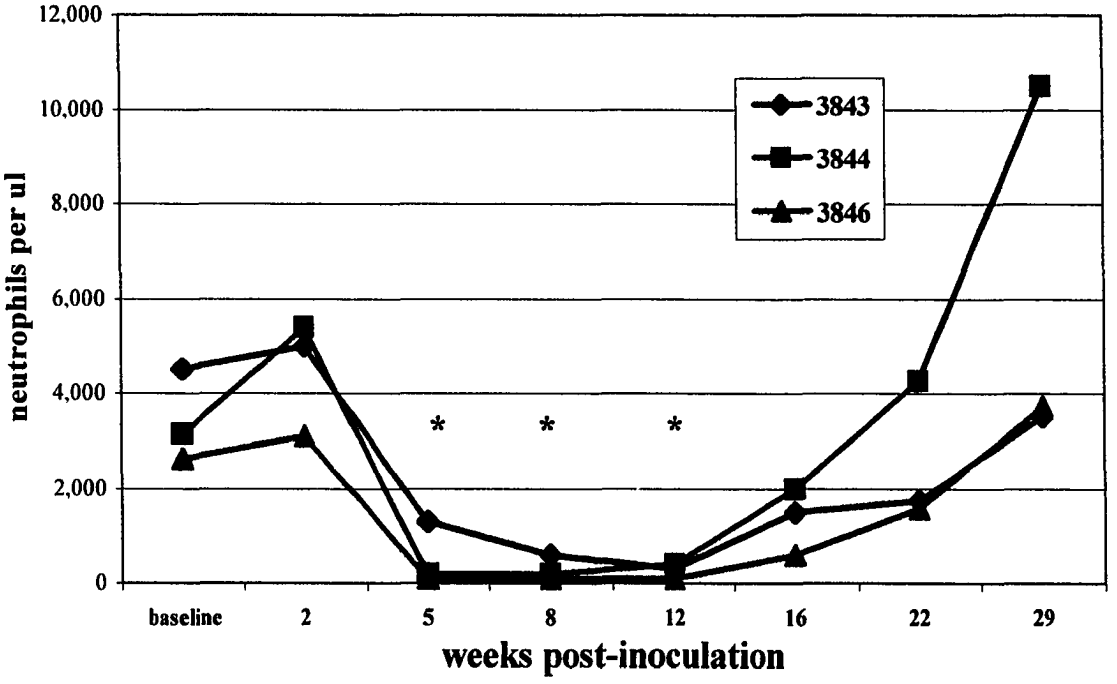


Figure 2.1: Neutrophil numbers in peripheral blood of 3 queens inoculated intravenously with FIV-C. Lower limit of our reference range is 2,000 cells/μl. * Significantly decreased from baseline, $p \leq 0.017$.

FIV-C queens: T-cell subset alterations

CD4+ lymphocyte numbers decreased while CD8+ lymphocyte numbers increased in all 3 queens post-inoculation with FIV-C, resulting in CD4:CD8 T-cell ratios of < 1 by 8 weeks post-inoculation (Figure 2.2). CD4:CD8 ratios remained low throughout the study period.

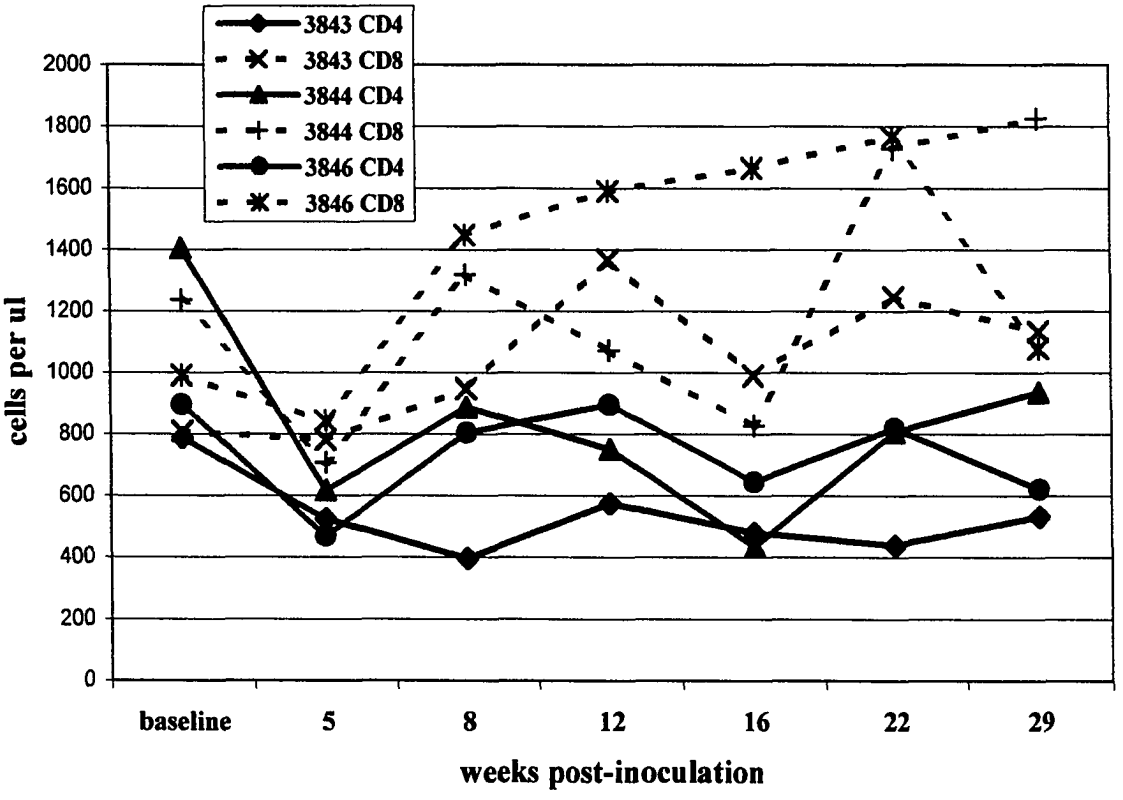


Figure 2.2: CD4+ (solid lines) and CD8+ (dashed lines) lymphocyte numbers in peripheral blood of 3 queens inoculated with FIV-C.

Queen proviral loads

Blood proviral loads for FIV-C infected queens were 1,000 to 10,000 copies/μg DNA, whereas blood from FIV-A infected queens contained ~ 2 log-fold less provirus (10 to 100 copies/μg DNA). Proviral levels in milk cells from all queens ranged from 1 to 100 copies/μg DNA, 2 to 3 log-fold lower than those detected in blood.

Queen FIV antibody responses

Plasma FIV antibody titers were quite variable among cats, but fairly stable during the 8 week period post-delivery. Titers were generally higher for FIV-C infected queens compared to FIV-A infected queens (Figure 2.3). As expected, antibody titers in milk were highest shortly after delivery, decreasing dramatically within 2 weeks (Figure 2.4).

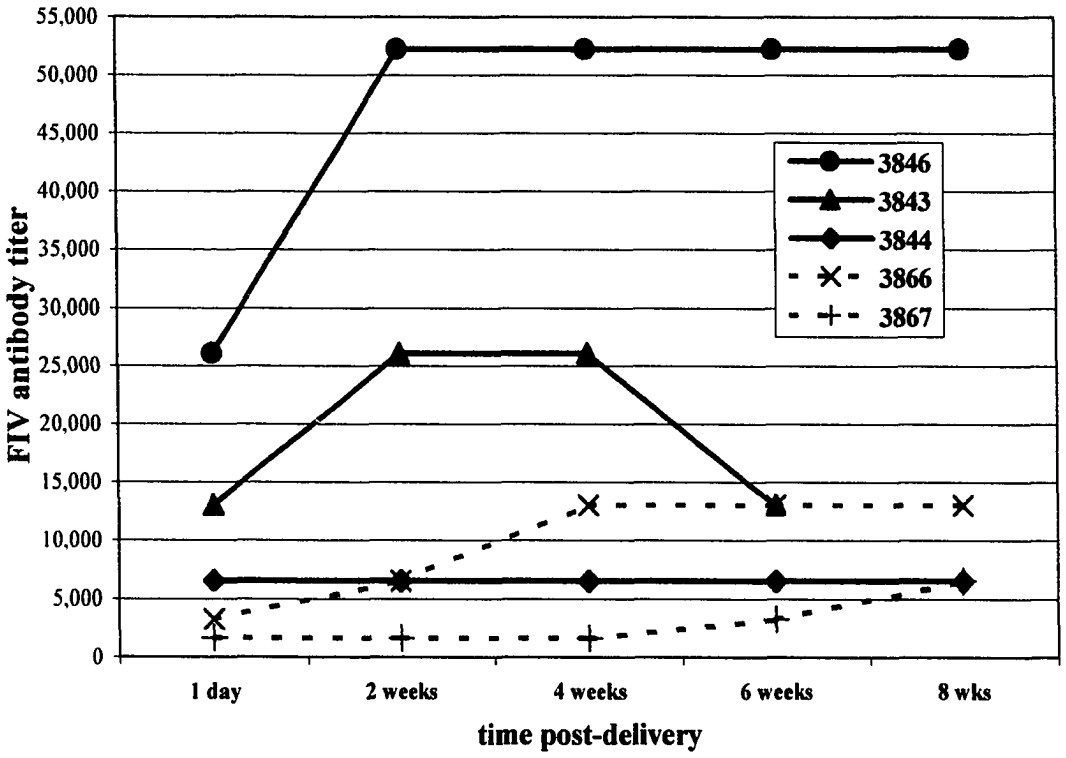


Figure 2.3: Plasma FIV antibody titers for 3 FIV-C infected queens (solid lines) and 2 FIV-A infected queens (dashed lines) following delivery of kittens. Titer expressed as the reciprocal of the highest plasma dilution positive by ELISA.

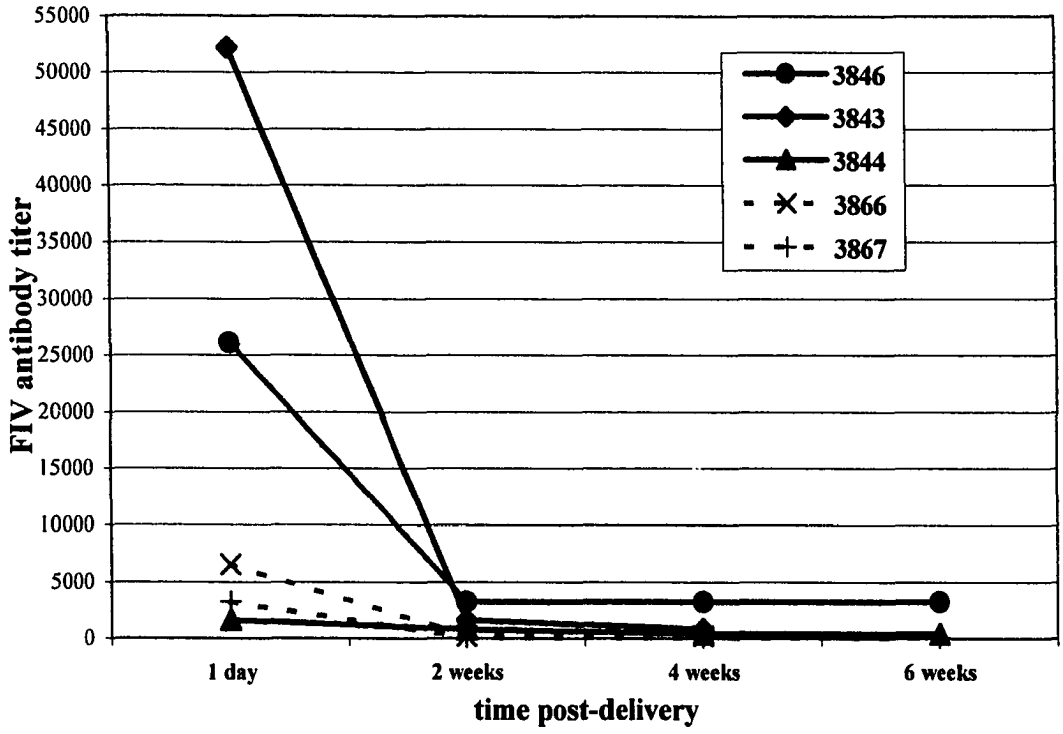


Figure 2.4: Milk FIV antibody titers for 3 FIV-C infected queens (solid lines) and 2 FIV-A infected queens (dashed lines) following delivery of kittens. Titers expressed as the reciprocal of highest milk dilution positive by ELISA.

Kitten FIV antibody responses

Fifteen of sixteen kittens born had detectable plasma FIV antibody at the time of initial sampling (within 48 hours of birth), reflecting maternal antibody passively transferred by colostrum. One kitten (3843 K1) was antibody negative on the first sample, but positive at two weeks of age. This kitten had been born during the night, with first blood samples obtained the following morning; it likely failed to nurse prior to sampling. Antibody levels in all kittens declined steadily with age, reaching undetectable levels by 10 to 16 weeks of age, consistent with loss of maternal antibody (Figure 2.5).

Figure 2.5: Plasma FIV antibody titers for kittens born to FIV-C queens (solid lines) and FIV-A queens (dashed lines). Titers expressed as reciprocal of highest plasma dilution positive by ELISA. *Initial titers for 3 kittens that are not shown were 52,224.

In-vitro FIV antibody stimulation

Isolated pbmc from 4 kittens collected at 14 months of age were investigated for their ability to produce antibody to FIV after in vitro stimulation. No FIV antibody was detected in any supernatant after 4 days in culture, with or without pokeweed mitogen (PWM) stimulation. Pbmc isolated from known FIV-positive cats were positive for FIV antibody in this assay, however there was no difference in OD between samples with and without PWM stimulation (Figure 2.6).

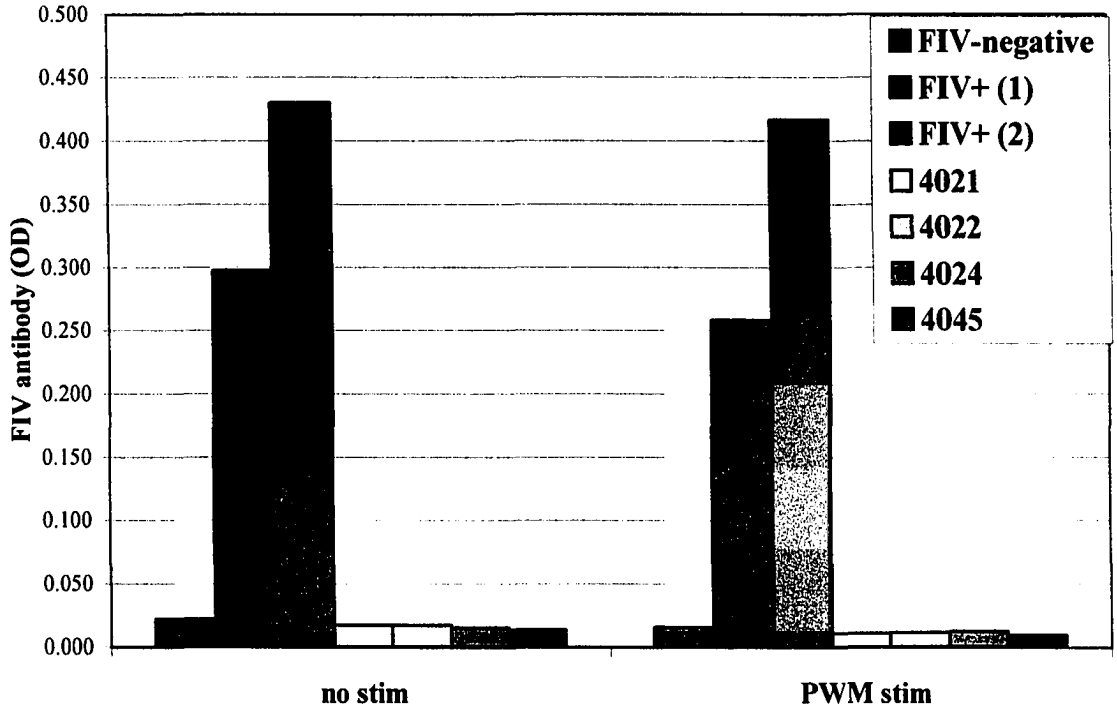


Figure 2.6: FIV antibody production in vitro for isolated pbmc from kittens born to FIV-A (4021, 4022) and FIV-C (4024, 4045) queens, with or without pokeweed mitogen (PWM) stimulation. Samples were collected at 14 months of age. Results represent the mean OD of triplicate wells assayed by FIV antibody ELISA. Antibody was detected from pbmc of two known FIV positive cats (FIV+ 1 and 2) in both stimulated and unstimulated cultures.

Kitten lymphocyte subsets

There were no significant differences in mean CD4:CD8 ratios for FIV-C and FIV-A infected kittens compared to uninfected controls (Figure 2.7). CD4:CD8 ratios for all kittens declined with age but remained ≥ 1, except for a single kitten. Kitten 4022, born to an FIV-A infected queen, had a ratio of 0.8 at the last sampling time (14 months of age); however absolute CD4+ lymphocytes numbers were not abnormally low (1000 cells/μl).

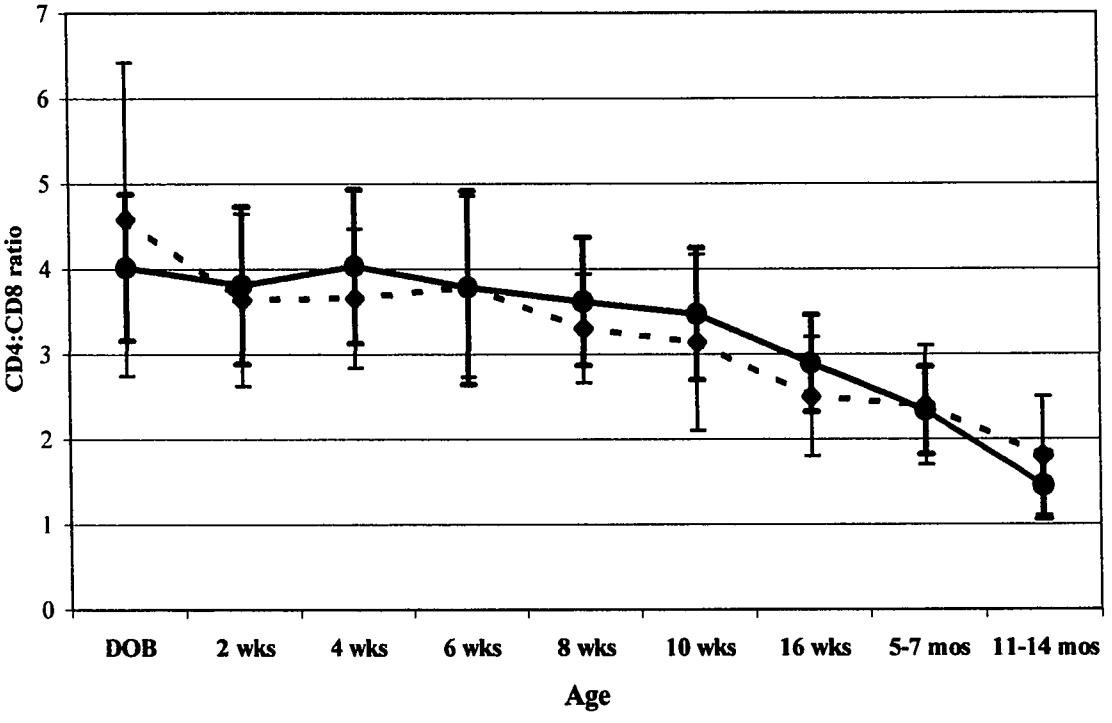


Figure 2.7: CD4:CD8 lymphocyte ratios for FIV-C and FIV-A infected kittens (solid line) compared to uninfected controls (dashed line). Mean, error bars indicate standard deviation. Repeated measures ANOVA demonstrated a significant effect of time ($p = 0.005$) but no significant differences between groups ($p = 0.89$).

Detection of provirus in kitten blood

Provirus was detected by DNA-PCR in the earliest blood samples (24 to 48 hrs after birth) from all 16 kittens. Initial PCR was performed with primers that amplify a conserved region of the *gag* gene. Subsequent blood samples from all kittens were positive for gag at least once, however only with the highest DNA input (1μg); multiple PCR reactions were often performed because of low-intensity signal that was difficult to interpret (Figures 2.8 and 2.9). DNA sequencing confirmed the presence of FIV *gag*

sequences when performed on suspicious amplified product from 10 of 11 kittens born to FIV-C queens and 2 of 5 kittens born to FIV-A queens, either from blood or cells collected from cocultures (Tables 2.1 and 2.2).

Additional PCR was performed to amplify a region of the *env* gene. *Env* sequences were obtained in blood from 9 of 11 kittens born to FIV-C queens and 2 of 5 kittens born to FIV-A queens; most of these were confirmed by sequencing (Table 2.1). Despite repeated attempts in over 125 separate PCR reactions, *env* could not be amplified from blood for any kitten over 6 weeks of age.

Viral isolation

Cocultures performed with isolated pbmc from all kittens were only rarely positive for presence of FIV p26 by ELISA (3 of 41 cocultures) (Table 2.2), and optical densities (OD's) were typically quite low compared to those from positive cocultures from queen pbmc done in parallel (data not shown). Similarly, cocultures performed with kitten plasma were positive for p26 in 3 of 13 experiments (Table 2.2), while 2 of 4 cocultures performed with queen plasma were positive (data not shown). Interestingly, DNA-PCR for provirus performed on cells retrieved from either pbmc or plasma cocultures was frequently positive for *gag* and/or *env* (Table 2.2).

Cocultures performed with cell-free milk supernatant were p26 ELISA-positive in 1 of 5 experiments, however cells retrieved from the cocultures were positive for provirus by DNA-PCR in all cases. Two cocultures were performed with isolated milk cells; 1 of 2 was positive by ELISA but cells from both were positive by DNA-PCR.

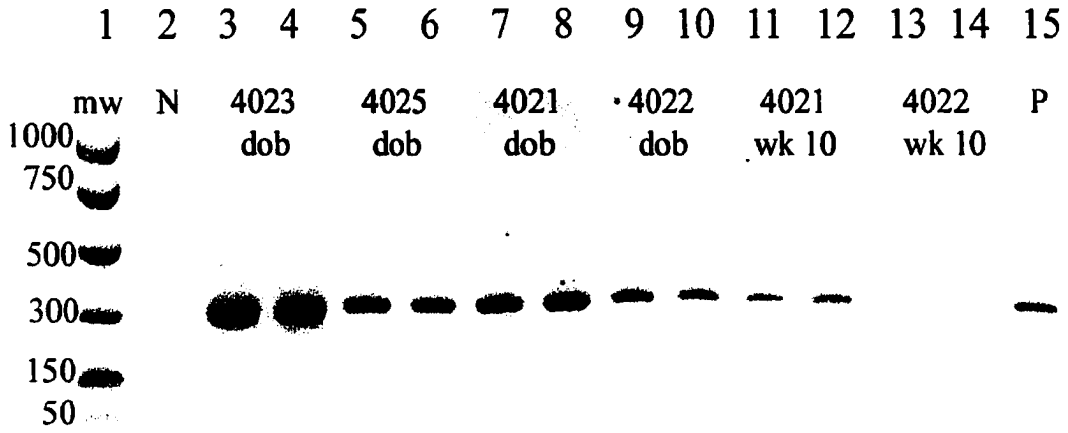


Figure 2.8: Nested DNA-PCR for FIV *gag* in blood of FIV-C (4023, 4025) and FIV-A (4021,4022) kittens. Amplified product of about 300 base pairs from duplicate PCR reactions are shown for samples from day of birth (dob) and 10 weeks of age (wk 10). Note decrease in signal intensity for kitten 4021 from day of birth to 10 weeks (lanes 7 & 8 compared to lanes 11 & 12), and loss of signal in kitten 4022 by 10 weeks of age (lanes 9 & 10 compared to lanes 13 & 14). Lane 1 = molecular weight markers (base pairs). Lane 2 = negative control (blood DNA from naïve cat). Lane 15 = positive control (100 copies FIV plasmid).

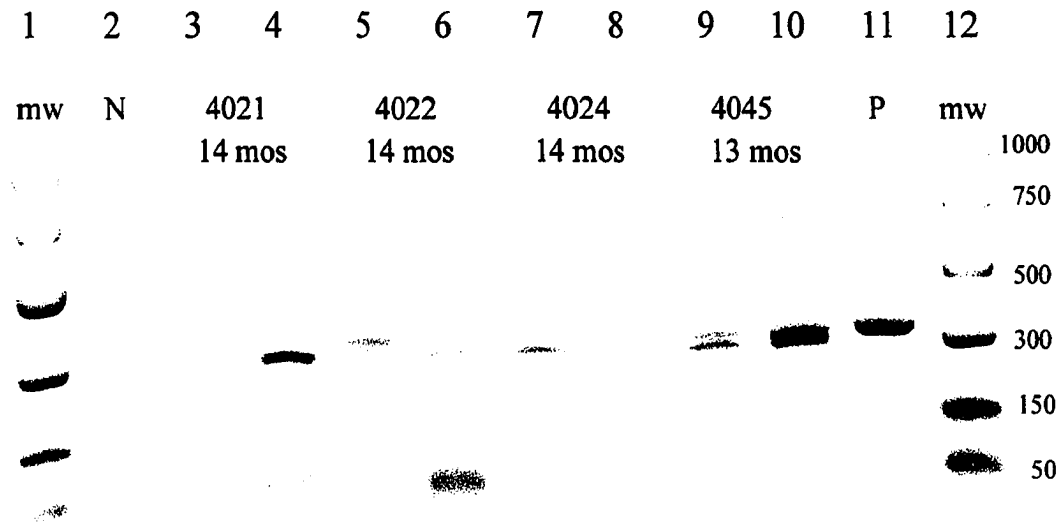


Figure 2.9: Nested DNA-PCR for FIV *gag* in blood of FIV-A (4021, 4022) and FIV-C (4024, 4045) kittens at 13 to 14 months of age. Amplified products from duplicate PCR reactions are shown, demonstrating variability in band intensity between duplicate samples (compare lane 3 to lane 4, lane 7 to lane 8, lane 9 to lane 10). DNA sequencing confirmed *gag* sequence for product in lanes 4 and 10. Lanes 1 & 12 = molecular weight markers (base pairs). Lane 2 = negative control (blood DNA from naïve cat). Lane 11 = positive control (100 copies FIV plasmid).

Table 2.1: Summary of nested DNA-PCR for FIV provirus in blood of kittens born to FIV-C and FIV-A infected queens.

	Age										
	1-2 days	2 wks	4 wks	6 wks	8 wks	10 wks	16 wks	5-6 mos	7-8 mos	1 yr	14 mos
FIV-C											
4023	+	-	-	-	+		+	+	-		
4024	+	-	-	+	+		+	+	-	(+)	(+)
4025	+		-	+	(+)		+	+	-		
4026	+		-	+	(+)		+	+			(+)
4027	+	(+)	-	-	(+)		+	+	-	(+)	
4043	+	+	-	(+)		+	(+)	+			+
4044	+	+	-	-		+	+	+			
4045	+	+	-	(+)		+	+	+		-	+
4046	+	+	-	+	-	+	+		+	+	
4047	+	+	-	-	-	+	-		-	-	
4048	+	+	+	-	-	+	-		-	-	
FIV-A											
4021	+	-	-	+	(+)	+		+		-	+
4022	+	(+)	-	+	(+)	+		+		+	(+)
3867K1	+	+	+			+					
3867K2	+	-	+			+					
3867K3	+	+	(+)			+					

+ = positive for both *gag* and *env*
+ = positive for *gag;* (+) = pale *gag* band
- = negative for *gag* or *env*
Shaded cells indicate amplified FIV sequences were confirmed by DNA sequencing.

Table 2.2: Summary of viral isolation results for kittens born to FIV-C and FIV-A queens. Viral isolation was performed by coculture of kitten pbmc (c) or plasma (p) with pbmc from FIV-negative cats; supernatants were tested for FIV p26 by ELISA.

	Age									
	1-2 days	2 wks	4 wks	6 wks	8 wks	10 wks	16 wks	5-6mos	7-8 mos	1yr
FIV-C										
4023			- c		- c					
4024			- c		- c			- c		
4025			- c		- c			- c		
4026			- c		- c			- c		
4027			- c		- c			- c		
4043							- c*, p*			
4044							- c*, p*			
4045							- c*, p*			
4046		- c, p*	- c	- c	- c				+ c	- c#
4047		- c, p*	- c	- c	- c				- c	- c#
4048		- c, p*	- c	+ c					+ c	- c#
FIV-A										
4021			- c		- p			- c, p*		
4022			- c		- p			- c, p*		
3867K1					- c	+ p				
3867K2					- c	+ p				
3867K3						+ p				

- = negative by ELISA, + = positive by ELISA, for cocultures of pbmc (c) or plasma (p). Outlined cells indicate samples that were positive for provirus by DNA-PCR performed on cells retrieved from the cocultures; * indicates which cocultures were positive. Black outlines = positive for *gag*, blue outlines = positive for both *gag* and *env*. Shaded cells indicate amplified FIV sequence was confirmed by DNA sequencing.
Cocultures performed with sorted cells (CD8-enriched and CD8-depleted).

Detection of provirus in kitten tissues

Gag and/or *env* sequences were detected by DNA-PCR in at least 2 tissues from all FIV-A kittens examined (3 stillborn kittens and 3 kittens euthanized at 11 weeks of age). Positive bands were only observed with the highest DNA input (1 μg), and band

intensity was low. Similarly, provirus was detected in several tissues from one FIV-C kitten (cat number 4027) euthanized at 1 year of age. Multiple PCR reactions were required to demonstrate convincing positive bands. DNA sequencing confirmed the presence of FIV *gag* sequences in three tissues (Table 2.3).

Table 2.3: Summary of nested DNA-PCR for FIV provirus in tissues of kittens born to FIV-A and FIV-C infected queens.

		Tissue					
	Age*	**MLN**	**Thymus**	**Spleen**	**BM**	**RPLN**	**Brain**
FIV-A							
3866K1	**dob#**				+		+
3866K2	**dob#**		+	+	(+)		+
3867K1	**dob#**	+	+	-			-
3867K1	**11 wks**	+	+		+	-	
3867K2	**11 wks**	+	+		+	+	
3867K3	**11 wks**	+	+		+	-	
FIV-C							
4027	**1 yr**	+	(+)	(+)	(+)	+	

* Age at time of necropsy and tissue collection.
# Kittens were non-viable at birth.
+ = positive for *gag*, - = negative for *gag*, (+) = pale *gag* band
+ = positive for *gag* and *env*, (+) = pale bands for *gag* and *env*
Shaded cells indicate amplified FIV sequence confirmed by DNA sequencing.
MLN = mesenteric lymph node, BM = bone marrow, RPLN = retropharyngeal lymph node.

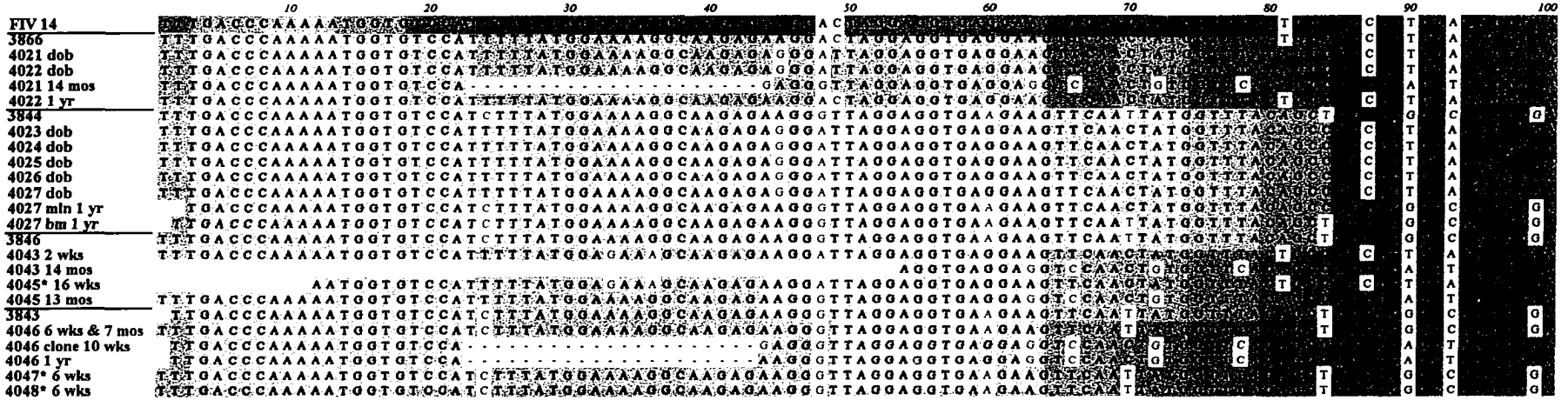
DNA sequencing

DNA sequencing was performed on *gag* and *env* amplified product from all three FIV-C infected queens (3843, 3844, 3846) and one FIV-A infected queen (3866) in order to compare to sequences obtained from their kittens (Tables 2.4 and 2.6). Sequencing was also performed on *gag* and *env* amplified product from the plasmid (containing the

FIV-14 genome) used as a positive control for DNA-PCR, in order to compare to kitten sequences and rule-out potential low-level FIV plasmid contamination. Negative control DNA from FIV negative cats was amplified in tandem with sample DNA; 45 separate control DNA samples were assayed. Occasionally very pale bands were observed in the 300 base pair range. These were sequenced on 3 occasions, and the sequences obtained had no homology to FIV. On two occasions bright positive bands were observed in the negative control lanes. These were sequenced and found to be identical to the FIV-14 sequence, confirming plasmid contamination of the naïve DNA. No *env* sequences obtained from kittens or queens were identical to the FIV-14 sequence. *Gag* sequences from the FIV-A queen and kittens were identical to the FIV-14 sequence (a clone of FIV-A-Petaluma), which was expected for this conserved region of *gag*.

All *env* and *gag* sequences obtained from all three FIV-C infected queens were identical to each other, while *env* and *gag* sequences from FIV-A infected queen 3866 were most similar to the FIV-14 plasmid sequences. In general, *gag* sequences from kittens contained silent base substitutions that did not result in amino acid changes (Table 2.5). However, a similar spectrum of mutations including a 21-base pair deletion was observed in 3 samples from 2 kittens representing clades A and C. *Gag* amplicons examined from these 2 kittens at different time points did not contain these deletions. As expected there was more variation among the *env* sequences, with more of these differences corresponding to amino acid changes (Tables 2.6 and 2.7).

Table 2.4: DNA sequences for 293 base pair region of FIV *gag* amplified from: plasmid DNA (FIV 14), FIV-A queen (3866), FIV-A kittens (4021, 4022), FIV-C queens (3844, 3846, 3843), and FIV-C kittens (4023, 4024, 4025, 4026, 4027, 4043, 4045, 4046, 4047, 4048). Horizontal lines separate individual queens and their kittens. Most products were amplified from blood; * indicates product amplified from cells retrieved from cocultures, mln = mesenteric lymph node, bm = bone marrow. Ages of kittens when samples were obtained are indicated; dob = day of birth. 'Clone' indicates the DNA was cloned prior to sequencing. Note the highlighted 21-bp deletion present in 3 samples from 2 different kittens.



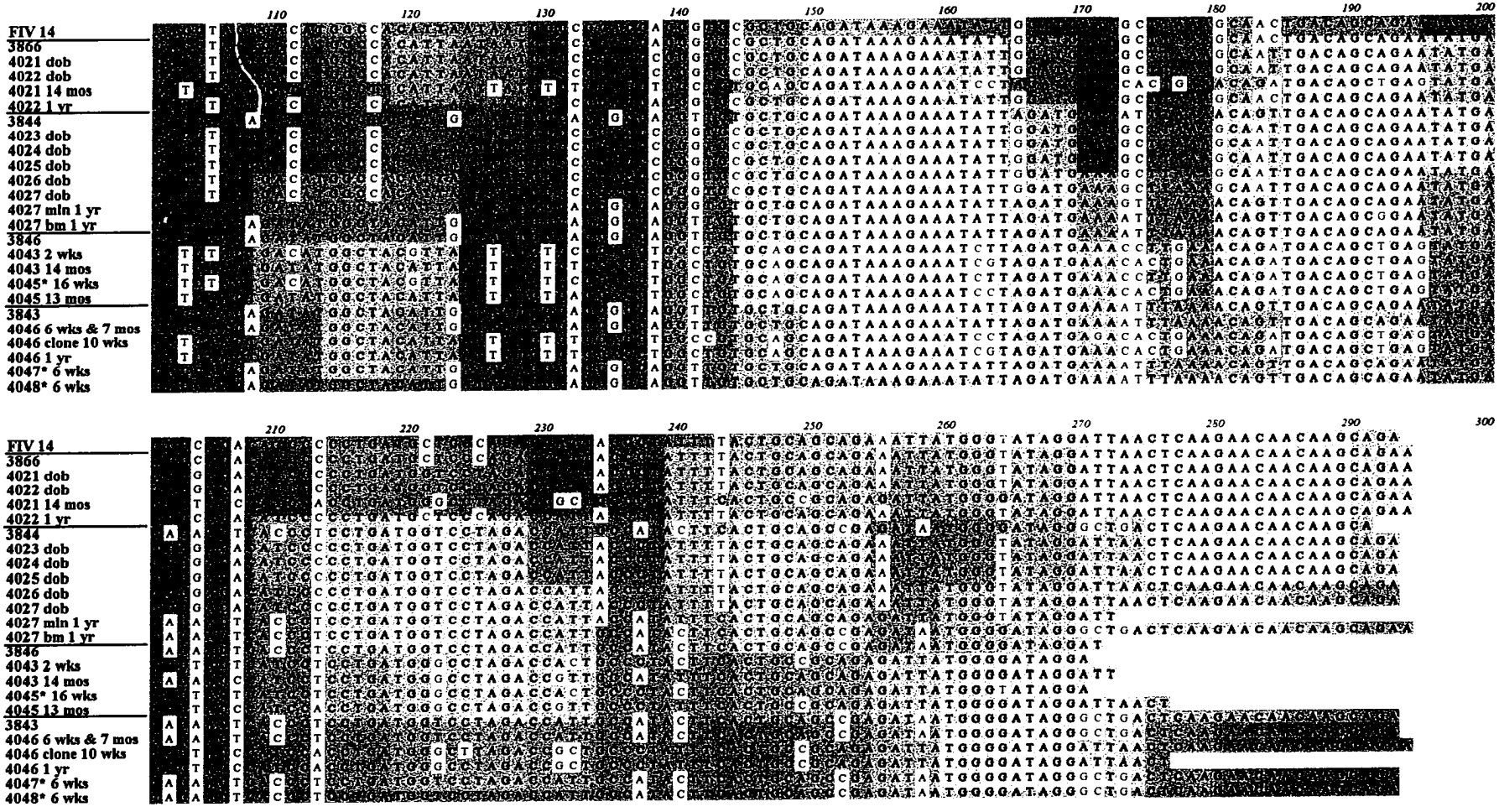


Table 2.5: Amino acid sequences translated from the 293 base pair region of FIV *gag* amplified from: plasmid DNA (FIV 14), FIV-A queen (3866), FIV-A kittens (4021, 4022), FIV-C queens (3844, 3846, 3843), and FIV-C kittens (4023, 4024, 4025, 4026, 4027, 4043, 4045, 4046, 4047, 4048). Horizontal lines separate individual queens and their kittens. Note the highlighted amino acid deletions corresponding to highlighted base-pair deletions in the DNA sequence.

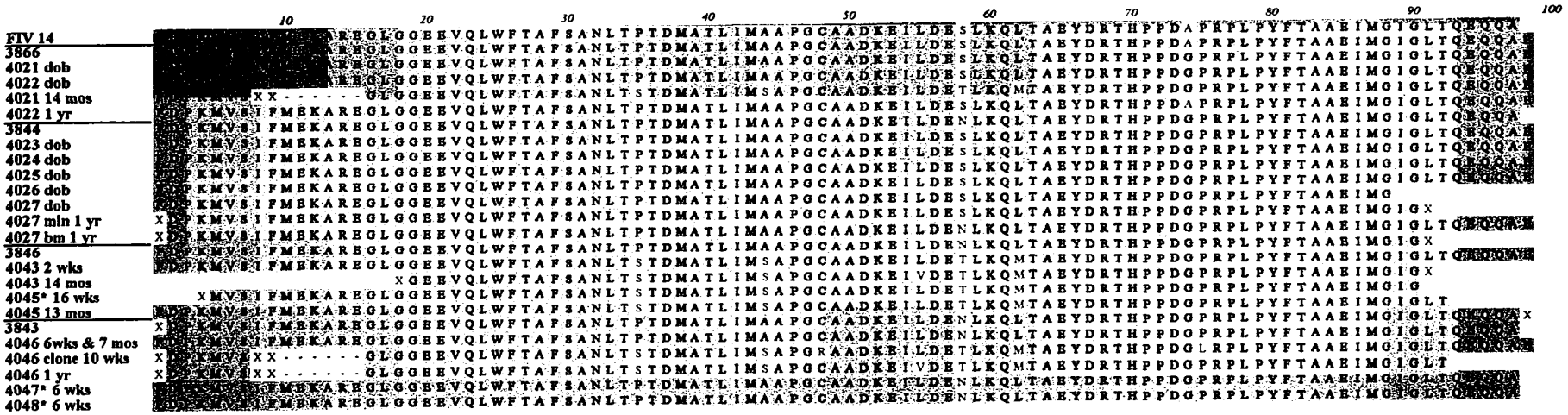
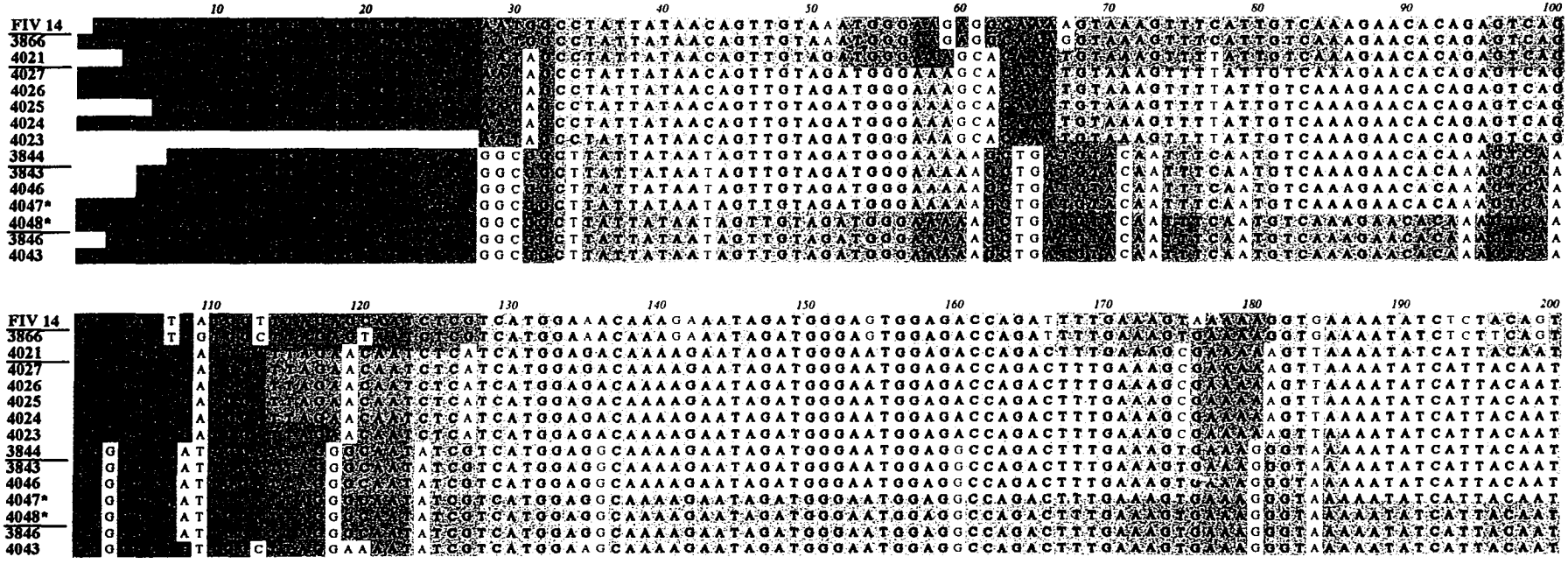


Table 2.6: DNA sequences for a 500 base pair region of FIV *env* amplified from plasmid DNA (FIV-14), FIV-A queen (3866), FIV-A kitten (4021), FIV-C queens (3844, 3843, 3846), FIV-C kittens (4027, 4026, 4025, 4024, 4023, 4046, 4047, 4048, 4043). Horizontal lines separate individual queens and their kittens. Most products were amplified from blood; * indicates product amplified from cells retrieved from cocultures. Note the highlighted 3-bp deletion present in 7 samples.



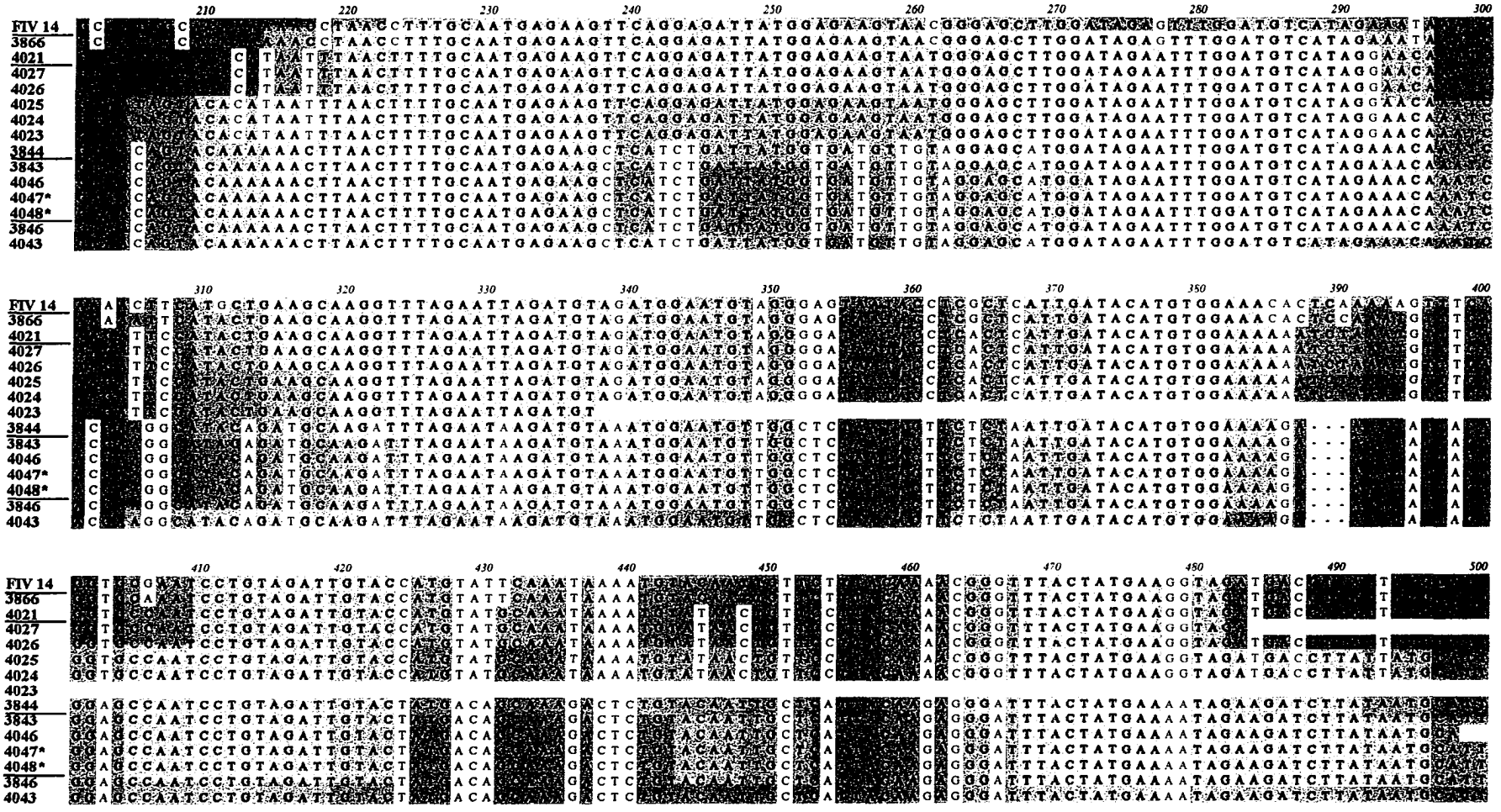
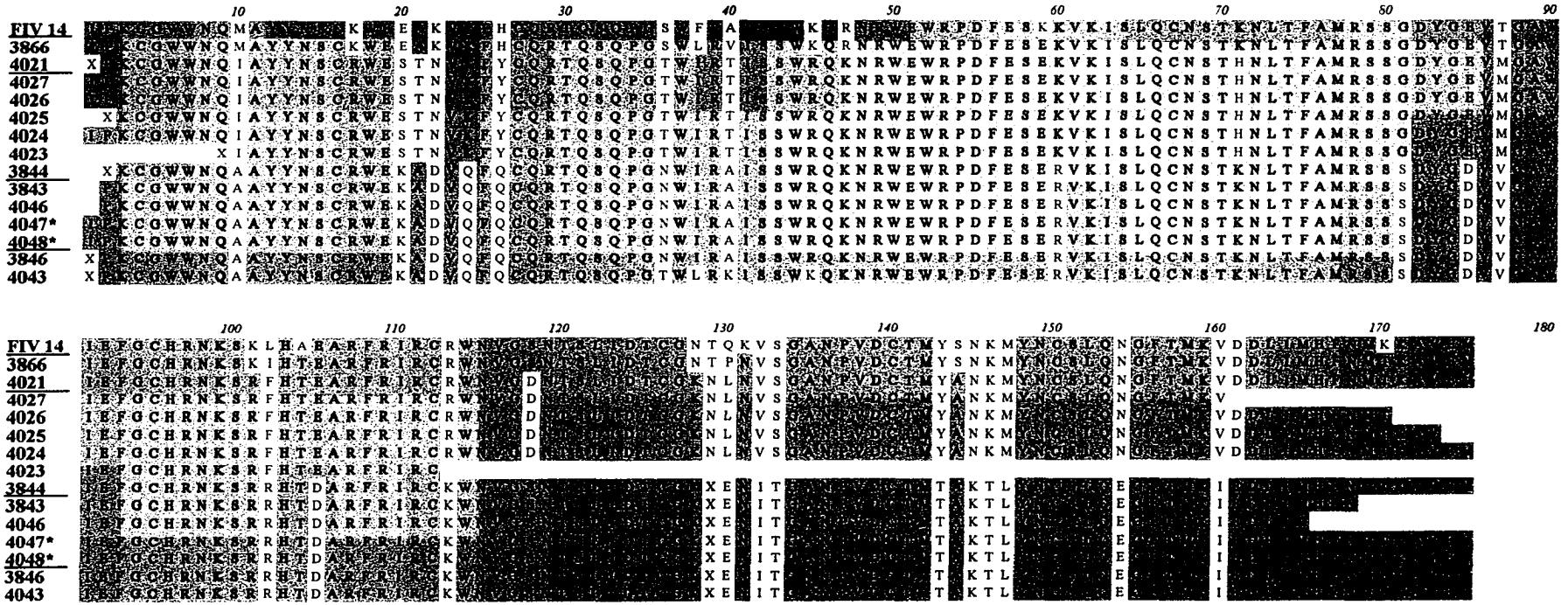


Table 2.7: Amino acid sequences translated from the 500 base pair region of FIV *env* amplified from plasmid DNA (FIV-14), FIV-A queen (3866), FIV-A kitten (4021), FIV-C queens (3844, 3843, 3846), FIV-C kittens (4027, 4026, 4025, 4024, 4023, 4046, 4047, 4048, 4043). Horizontal lines separate individual queens and their kittens. Highlighted in yellow is the amino acid deletion corresponding to the highlighted 3 base-pair deletions shown in the DNA sequence.



DISCUSSION

We describe covert vertical transmission of two different FIV isolates representing FIV-A-Petaluma and FIV-C-Pgmr. DNA PCR detected FIV provirus in blood samples from all viable kittens within a day or two of birth, and in tissues from all kittens non-viable at birth. However, provirus became increasingly more difficult to amplify from blood as the kittens aged, often requiring as many as six separate reactions to obtain one distinct positive band. We believe this reflects the presence of very few proviral copies, as similar indistinct and pale positive bands were occasionally observed in limiting-dilution PCR reactions from FIV-A queens, which had relatively low proviral loads. In general it was easier to amplify provirus from kitten tissues than from blood, suggesting that most cells harboring provirus are sequestered and not in the circulating pool. In particular, for one FIV-C kitten (4027) that was euthanized at one year of age, FIV *gag* was amplified from multiple tissues and *env* from two tissues while concurrent blood PCR resulted in a weak *gag* band only. These results are consistent with previous studies in our laboratory demonstrating detection of provirus more often in fetal tissues than in fetal blood [27]. DNA PCR for *gag* was positive more often than for *env*; *env* was amplified only in tissues or early time points in blood. This apparent difference in sensitivity has been reported previously in regressive FIV infection [34], and may reflect the relative sensitivities of the two assays, although limiting-dilution PCR performed on blood DNA from queens suggested equal limits of detection.

Although most experimentally FIV-infected cats seroconvert [22, 35, 36], detection of antibody in vertically infected kittens has been variable in previous studies [37].

Maternal antibody to FIV is transferred via colostrum [38], and expected to disappear gradually with age. Kitten FIV antibody titers in the current study declined steadily and remained undetectable past 4 months of age, consistent with maternal antibody. However, regressive FIV infection has been documented in which kittens of FIV-positive queens were delivered by caesarean and bottle-fed, thus acquiring no maternal antibody [34]. These kittens had transient antibody responses after 5 weeks of age, were initially VI positive in blood, and remained PCR positive in blood and lymph node for a period of time after antibody was no longer detectable, suggesting an active infection that was either completely cleared or sequestered at low levels. Thus, in the current study the absence of detectable FIV antibody after 4 months of age does not rule out a previous active infection.

Viral isolation only rarely confirmed presence of infectious virus in kitten blood samples; however small sample volumes precluded coculture assays from day of birth samples, when provirus was detected most easily by DNA-PCR. Previous studies in our laboratory compared VI and DNA PCR results in fetal tissues and found good correlation, which would suggest that most virus detected by PCR in newborns is infectious [27]. DNA PCR performed on cells retrieved from cocultures revealed presence of provirus even in cultures that were negative by p26 ELISA. Interestingly, this occurred not only in pbmc cocultures but also in cultures of kitten plasma with naïve pbmc. This suggests that virus was present and able to infect cells in culture, but did not replicate efficiently enough to be detected by our assay. These findings are similar to observations by Dandekar et al. in which naïve cats housed with FIV-positive cats became PCR positive but antibody and VI negative [39]. In that study, transient viral RNA was detected by in situ hybridization in cultured cells, but FIV antigen could not be

detected in culture supernatants. CD8+ lymphocytes have been shown to suppress viral replication for HIV, SIV, and FIV [40-43]. However, CD8-depleted pbmc cocultures from 3 kittens in this study were negative for FIV antigen production, similar to cultures in 3 cats reported by Dandekar et al [39].

DNA PCR evidence indicates that all kittens in this study were infected with FIV in utero, consistent with previous fetal infection studies [27, 28]. However, all kittens remain clinically healthy at > 1 year of age (except for those born dead or euthanized for tissue collection). Based on results from tissues examined thus far, we expect that virus is sequestered in tissues at low levels, and suggest that viral replication is markedly downregulated, such that no antibody response can be detected. Such low-level, sequestered viral infection, without a detectable antibody response, has been well-documented in SIV [44, 45], and suspected in reports of occult HIV infection in high-risk adults [46].

Cellular immune responses were not investigated here, but would seem the plausible mechanism for viral containment. A variety of cell-mediated immune responses have been detected in apparently uninfected children born to HIV-positive mothers, including lymphocyte proliferation in response to recombinant HIV proteins [47], IL-2 production in pbmc or cord blood after stimulation with HIV peptides [15, 48], and HIV-specific cytotoxic lymphocytes (CTL) in pbmc or cord blood [17, 18, 48-51]. Because CTL responses are thought to require intracellular replication, and in light of sporadic reports of viral clearance or transient infections in infants [52-55], it is possible that at least some degree of active infection may be occurring in these infants in utero. The pertinent question is whether or not these immune responses can confer protection

against future virus exposure; there is some evidence that this might be the case. In HIV, cohorts of highly exposed, persistently seronegative people (HEPS) have been studied that have similar cellular immune responses and remain HIV negative despite ongoing exposure [56-60]. In SIV, several studies of low-dose mucosal inoculation have resulted in cellular immune responses without seroconversion; animals remain healthy, VI negative, and PCR positive in tissues, and have been resistant to subsequent mucosal challenge [44, 45, 61-63]. Interestingly, previous studies in which kittens born to naïve queens were foster-nursed by FIV-infected queens resulted in 6 of 10 kittens rapidly becoming virus-positive; 3 kittens became symptomatic and 2 of those died by 14 weeks of age [37, 38]. Kittens in the current study nursed their mothers, yet remained healthy; this might suggest a protective effect of in utero FIV exposure. Indeed, one study of infants born to HIV positive women found those with anti-HIV cellular immune responses at birth were less likely to acquire HIV by breastfeeding during the first year [15].

Taken together, these studies suggest that host control of lentiviral infection is possible if the initial exposure occurs under specific circumstances, including low virus burden and limited viral replication. The covert FIV vertical transmission described here may provide an animal model in which to study the natural generation of such low-level infections and investigate potential protection from future viral challenge, information which would contribute to the design of effective neonatal vaccination strategies.

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CHAPTER THREE

MILK-BORNE FELINE IMMUNODEFICIENCY VIRUS

INTRODUCTION

An estimated 17.6 million women are infected with HIV worldwide; the vast majority live in developing countries where breastfeeding is still recommended to decrease infant mortality from other infectious diseases [1-4]. Although vertical transmission of HIV from mother to child can occur in utero, intrapartum, or postpartum through breast milk, breastfeeding has been implicated in one to two-thirds of overall HIV vertical transmission [5-8].

While HIV DNA and RNA have been demonstrated in milk, their relative importance in viral transmission remains unclear. The presence of HIV provirus in milk was associated with increased risk in one study [9] while no correlation was found in another [10]. Similarly, duration of breastfeeding has been correlated with increased transmission in some studies [9, 11] but not in others [10]. The contribution of cell-free virus is uncertain; Pillay et al. found a correlation between cell-free virus loads and vertical transmission, and similar viral loads throughout lactation [12], while Lewis et al. found the prevalence of cell-free virus to be higher in immature milk than in colostrum [13].

Simple, cost-effective interventions to prevent HIV vertical transmission are still lacking for developing countries. While abbreviated antiretroviral regimens have been effective in non-breastfed infants [14], a successful strategy to protect children from continued exposure to milk-borne virus has not yet been developed. The reverse transcriptase inhibitor 9-(2-phosphonylmethoxypropyl)adenine (PMPA) has been effective controlling simian immunodeficiency virus (SIV) in macaques infected parenterally [15, 16] and in newborn macaques infected orally, even with limited treatment [17-19]; it may hold promise for prevention of milk-borne infection.

Feline immunodeficiency virus (FIV) induces an immune deficiency syndrome similar to that caused by HIV [20-23]. As with HIV, vertical transmission of FIV occurs in utero, postnatally via milk, and potentially during delivery [24-28]. Thus, the FIV model can be used to investigate the role of milk-borne virus in vertical transmission, and potential therapeutic strategies to prevent such transmission. We hypothesized that frequent vertical transmission of FIV would be prevented by PMPA treatment of queens and kittens, and that virus would be concentrated in milk early in lactation. Here we report that viral RNA appears to be selectively concentrated in the milk of FIV-infected queens, despite low levels of provirus in milk cells. In addition, when FIV-infected queens were treated with PMPA during gestation we observed deleterious effects on kitten viability and birth weight.

MATERIALS AND METHODS

Animals and sampling

All cats originated in a specific-pathogen-free (SPF) breeding colony maintained at Colorado State University and were housed in accordance with Animal Care and Use Committee regulations.

Seven queens previously infected with FIV-B-2542 for at least 36 months were bred to FIV-negative males; 4 queens became pregnant and produced a total of 6 litters. Queens and their kittens received either 9-(2-phosphonylmethoxypropyl)adenine (PMPA, generously provided by Dr. Norbert Bischofberger, Gilead Sciences) or sham treatment (saline) by subcutaneous injection, at a dose of 30 mg/kg/day for queens and 20 mg/kg/day for kittens, starting the second trimester of pregnancy and continuing through weaning at 6 weeks postpartum. Two queens contributed litters to both the PMPA and sham-treatment groups. Blood was collected from queens at 4 and 6 weeks gestation, and from queens and kittens within 24 hours of birth and biweekly thereafter. Milk was collected from 3 queens within 24 hours of delivery. Kittens were euthanized for necropsy and terminal sample collection at 10 weeks of age.

Ten queens were inoculated intravenously with FIV-B-2542 (from an in vivo-passaged plasma pool source) and bred to FIV-negative males 2 – 6 months later. FIV infection was confirmed in the queens by DNA polymerase chain reaction (PCR) on blood as described below. Seven queens became pregnant and produced a total of 7 litters. Two queens and their kittens received PMPA treatment as described above.

Blood was collected from queens prior to and at 2, 5, 8, 12, and 16 - 18 weeks post-inoculation (PI). Blood was collected from queens at 4 and 6 weeks gestation, and from queens and kittens within 24 hours of birth and biweekly thereafter. Milk was collected from queens within 24 hours of delivery and at 2, 4, and 6 weeks post-delivery. Kittens were euthanized for necropsy and terminal sample collection at 10 weeks of age, except for four kittens born to queen 3748 that were transferred to another study. Naïve cats from the SPF breeding colony provided milk and blood samples for negative controls.

Sample processing

Blood was collected under ketamine-acepromazine anesthesia (queens and kittens ≥ 6 weeks of age) or with manual restraint (kittens < 6 weeks of age) in EDTA for complete blood cell count and CD4 and CD8 T-cell subset determinations. Whole blood was frozen in 200ul aliquots for DNA PCR. Plasma was separated from cells by centrifugation and stored at -70°C until use. In some cases platelet-rich plasma was prepared by low-speed centrifugation; platelets were then pelleted and frozen for DNA PCR. Peripheral blood mononuclear cells (pbmc) were isolated by single-density ficoll-hypaque centrifugation (Histopaque-1077; Sigma, St. Louis, MO). In some experiments pbmc and polymorphonuclear cells (pmnc) were separated by double-density centrifugation (Histopaque-1119 and Histopaque-1077; Sigma, St. Louis, MO) as previously described by Toth, et al. [29]. Separated cells were spotted onto glass slides and stained with Wright-Giemsa (EM Science Harleco, Gibbstown, NJ) for differential cell counts.

Milk was collected by manual expression from queens under ketamine-acepromazine anesthesia. To facilitate milk release, queens received 2 U of oxytocin intramuscularly 10 minutes prior to collection. Milk cells and supernatant were separated by repeated centrifugation and removal of the fat layer. Cell-free milk supernatant was frozen for later use or used immediately for viral isolation as described below. Milk cells were washed in PBS and either pelleted and frozen for later use or used immediately for viral isolation.

Terminal tissues collected from kittens included spleen, liver, thymus, brain, mesenteric lymph node, and bone marrow. Individual tissues were rinsed with sterile PBS and dissociated into cell suspensions through mesh sieves. Cells were pelleted and frozen for DNA PCR.

Flow Cytometry

Lymphocyte subset analysis was performed with a Coulter EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL) using feline CD4 and CD8 monoclonal antibodies as previously described [30, 31]. Absolute cell numbers were calculated from the total lymphocyte count.

Virus isolation

Naïve donor pbmc were obtained from SPF cats and stimulated with 10 μL/ml concanavalin A (Sigma, St. Louis, MO) for 3 days. One million naïve cells were cocultured in 24-well plates with serial two-fold dilutions of cell-free milk supernatant (100 μl to 1.5 μl) or serial log-fold dilutions of milk cells (1×10^5 to 1×10^1 or 2×10^6 to

2 x 10^3) in RPMI medium supplemented with 20% fetal calf serum, 1% penicillin-streptomycin, 2% glutamine, 2 x 10^{-5} M 2-mercaptoethanol, and 100 U/ml interleukin-2 (Cetus/Roche, Emeryville, CA). Culture supernatants were collected twice weekly for 4 weeks and assayed by FIV p26 capture enzyme-linked immunosorbent assay (ELISA) [32].

Provirus detection

Limiting-dilution DNA PCR using a nested set of primers located within the *gag* gene was used to estimate proviral burden. The sensitivity of this assay was determined using plasmid DNA containing the FIV-14 genome to be between one and ten copies of target sequence. DNA was extracted from either whole blood (200µl), pbmc, pmnc, platelets, or tissue cell pellets using a QIAamp Blood Kit (Qiagen Inc., Valencia, CA). Purified DNA was quantitated with a Beckman DU-70 spectrophotometer (Beckman-Coulter, Brea, CA). One microgram of sample DNA was amplified; DNA from positive samples was serially log-diluted and PCR-amplified to identify negative endpoints. Proviral copy number per µg of DNA or infected cells per million was estimated assuming at least one copy or positive cell was detected at the highest positive dilution, and that 1 µg of DNA represents 2 x 10^5 cells. The first round primers were gag 129 (5'-CGTAACTACAGGACGAGAACCTGG-3') and gag 802 (5' CCAACTTTCCCAATG-CTTCAAG-3'). The second round primers gag 3 (5'TTGACCCAAAAATGGTGTCCA-3') and gag 4 (5'-TTCTGCTTGTTGTTCTTGAGT-3') amplified a 293 base pair sequence nested within the first round product. Hot-start PCR was performed in a Perkin-Elmer 480 thermocycler (P-E Applied Biosystems, Foster City, CA) with 35

cycles of 94°C, 56°C (round 1) or 63°C (round 2), and 72°C for 1 minute each. Reaction mixtures contained 2.5 mM $MgCl_2$, 1.25 X Gene Amp 10X PCR buffer II, 200 μM each dNTP (all P-E Applied Biosystems), 0.1 μM each first round primer, 1 μM each second round primer (both Gibco BRL, Rockville, MD), and 2.5 U Taq polymerase (Sigma, St. Louis, MO). Two microliters of the first round product were added to the second round reaction mixture. Products were visualized on 1.5% agarose gels stained with ethidium bromide or containing Gelstar® (FMC Bioproducts, Rockland, ME). Negative controls included water and DNA extracted from naïve cat samples.

Viral RNA quantitation

Plasma and cell-free milk supernatant was tested for viral RNA with a quantitative-competitive reverse transcriptase PCR (QC-RT PCR) assay based on the method described by Diehl, et al. [33] RNA was extracted from plasma (200 μl) or milk (25 to 100 μl) using a QIAamp Viral RNA Kit (Qiagen Inc; Valencia, CA). Serial dilutions of a 21-base deleted competitor RNA were added to the sample RNA before amplification. Aliquots of competitor RNA were kept frozen at -70°C, thawed and serially diluted in TE buffer pH 7.0 (Ambion, Inc; Austin TX) just before use. Primers used in the single round reaction were gag 3 and gag 4 (described above). Reverse transcription (cDNA synthesis) followed by DNA PCR was performed in a single tube using the EZ rTth RNA PCR kit and a Perkin-Elmer 9600 thermocycler (P-E Applied Biosystems, Foster City, CA). 50 μl reaction mixtures contained 1X EZ buffer, 250 μM each dNTP, 2.5 mM Mn(OAc)2, 5 U rTth polymerase (all P-E Applied Biosystems), and 0.2 μM each primer (Gibco BRL, Rockville, MD). Reverse transcription was performed

at 65°C for 20 minutes, followed by a one minute denaturation at 94°C and 40 cycles of 94°C for 10 seconds, 62°C for 20 seconds, and 72°C for 30 seconds, with a final extension of 4 minutes at 72°C. Products were visualized on 3% Metaphor gels containing Gelstar® (FMC Bioproducts, Rockland, ME). The 21-base pair difference allowed identification of competitor and wild-type products; the band intensity equivalence point was used to calculate plasma or milk viral RNA load per milliliter of sample. Negative controls included water, plasma from naïve cats, and sample plasma amplified with the reverse transcriptase step omitted to control for DNA contamination.

Statistical analysis

Kitten viability and birth weight data were analyzed using a chi-square test and 2-tailed *t*-test for unpaired data, respectively. Statistical analysis was performed with the Statview® software (Abacus Concepts, Inc., Berkeley, CA); significance was assumed at $p \leq 0.05$.

RESULTS

Kitten viability

Eleven of sixteen kittens (69%) born to 4 queens in the PMPA-treatment group were stillborn. Seven of twenty-seven kittens (26%) born to 9 queens that did not receive PMPA were stillborn (Figure 3.1). PMPA treatment of queens at 30 mg/kg/day was associated with significantly decreased kitten viability at birth (Chi square $p = 0.006$).

Kitten birth weights

The mean birth weight of kittens born to PMPA-treated queens was 77 grams, significantly lower ($p < 0.001$) than birth weights of kittens born to untreated queens (104 grams).

Vertical transmission rate

Six of 27 kittens in the placebo group were determined to be FIV-positive by DNA-PCR in blood or tissues, for an overall vertical transmission rate of 22%. Three of these kittens were non-viable at birth. A positive *gag* band was only detectable with a large DNA input (≥ 1 μg) in tissues from two kittens. Similarly, three FIV-positive kittens viable at birth were positive by DNA-PCR in blood only at the highest DNA input, and were negative in both thymus and bone marrow. Only one of the 6 FIV-positive kittens had readily detectable provirus present in multiple tissues (Table 3.1).

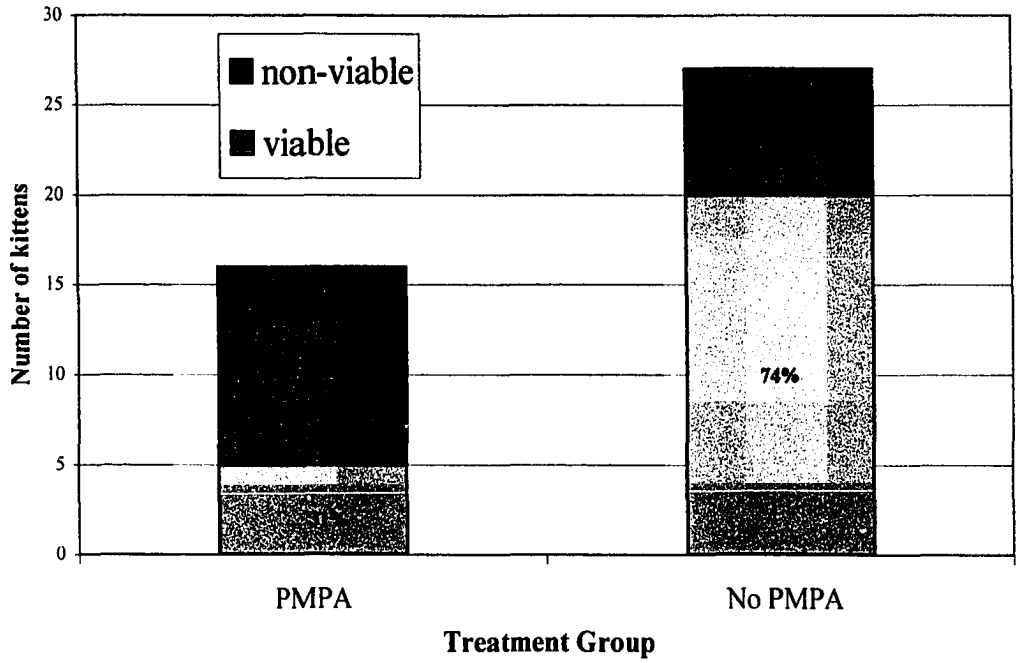

Figure 3.1: Numbers of kittens born to FIV-B-2542 infected queens within each treatment group that were viable (■) or non-viable (■) at birth. PMPA-treatment was associated with significantly decreased kitten viability (Chi square p = 0.006).

Table 3.1: Summary of proviral loads in blood and tissues from 6 kittens (3 viable and 3 non-viable at birth) vertically infected with FIV-B-2542. Values expressed as minimum viral copies present per μg of DNA determined by limiting-dilution DNA-PCR.

Kitten number	Blood	Bone marrow	Thymus	Mesenteric lymph node
Viable				
3468-1	1	Neg	Neg	nd
3468-2	1	Neg	Neg	nd
3468-3	1	Neg	Neg	nd
Non-viable				
3711-1	nd	1	1	nd
3711-2	nd	1	1	nd
AH04-1	nd	1,000	100	10,000

Neg = no provirus detected, nd = not done

Provirus in blood and milk cells

Proviral loads in blood and milk cells stayed relatively stable in individual cats throughout the study period, generally varying by no more than a log between time points. Eleven queens had limiting-dilution nested DNA-PCR performed on both blood and milk cells collected on the day of delivery. Ten of eleven queens (91%) had higher levels of provirus detected in blood cells than in milk cells (Figure 3.2). The exception, queen 3711, gave birth to two non-viable kittens, thus subsequent milk samples were not available for testing. The discrepancy in proviral loads was noted throughout lactation in all five queens for which milk samples were available at multiple time points (Table 3.2).

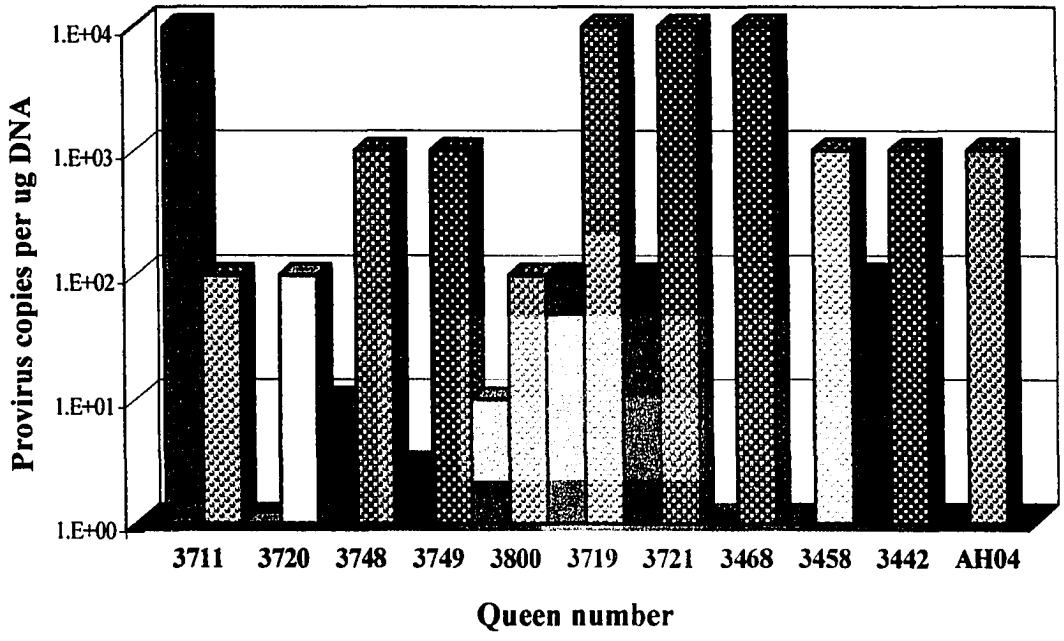

Figure 3.2: Proviral loads in cells from milk (solid bars) or blood (hatched bars) for eleven FIV-B-2542 infected queens on the day of delivery. Values expressed as minimum viral copy number per μg of DNA as determined by limiting-dilution nested DNA-PCR. Note that 10 of 11 queens have higher levels of provirus detectable in blood cells than milk cells.

Table 3.2: Comparison of proviral loads in cells from blood and milk during lactation from five FIV-B-2542 infected queens. Values expressed as minimum viral copy number present per μg of DNA as determined by limiting-dilution nested DNA-PCR.

	Day of delivery		Week 2		Week 4		Week 6	
Queen	**blood**	**milk**	**blood**	**milk**	**blood**	**milk**	**blood**	**milk**
3720	100	0	100	3	100	10	100	10
3748	1,000	10	100	10	100	2	1,000	2
3749	1,000	3	100	1000	1,000	10	100	100
3719	10,000	100	10,000	100	1,000	10	1,000	10
3721	10,000	100	1,000	10	1,000	10	100	20

Viral RNA in plasma and milk

Plasma viral loads varied widely among queens; queens that had been infected for over 3 years had viral loads of 1,000 to 10,000 copies/ml, while those infected only a few months ranged from 10,000 to 5 million copies/ml (sample QC-RT-PCR result shown in Figure 3.3). Ten queens had QC-RT-PCR performed on plasma and cell-free milk supernatant collected on the day of delivery. In contrast to the results for proviral load, nine of 10 queens had higher levels of viral RNA detected in milk than in plasma (Figure 3.4). The exception, queen 3720, had increasing amounts of viral RNA in milk as lactation progressed; by week 4 the milk viral load was greater than the plasma viral load.

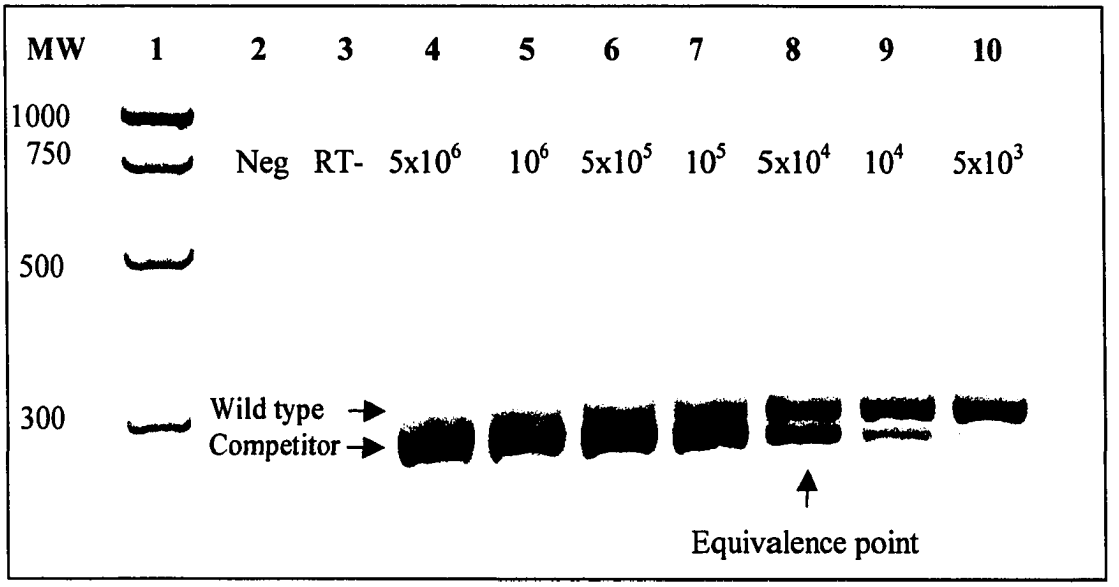

Figure 3.3: QC-RT-PCR gel for queen AH04 milk supernatant from day of delivery. Lane key: 1 = molecular wt markers (base pairs), 2= water control, 3 = RT negative control, 4 through 10 = competitor RNA dilutions ($5x10^6$ copies to $5x10^3$ copies) co-amplified with RNA extracted from 25 µl of milk supernatant. Equivalence point of $5x10^4 = 2x10^6$ viral RNA copies per milliliter of milk.

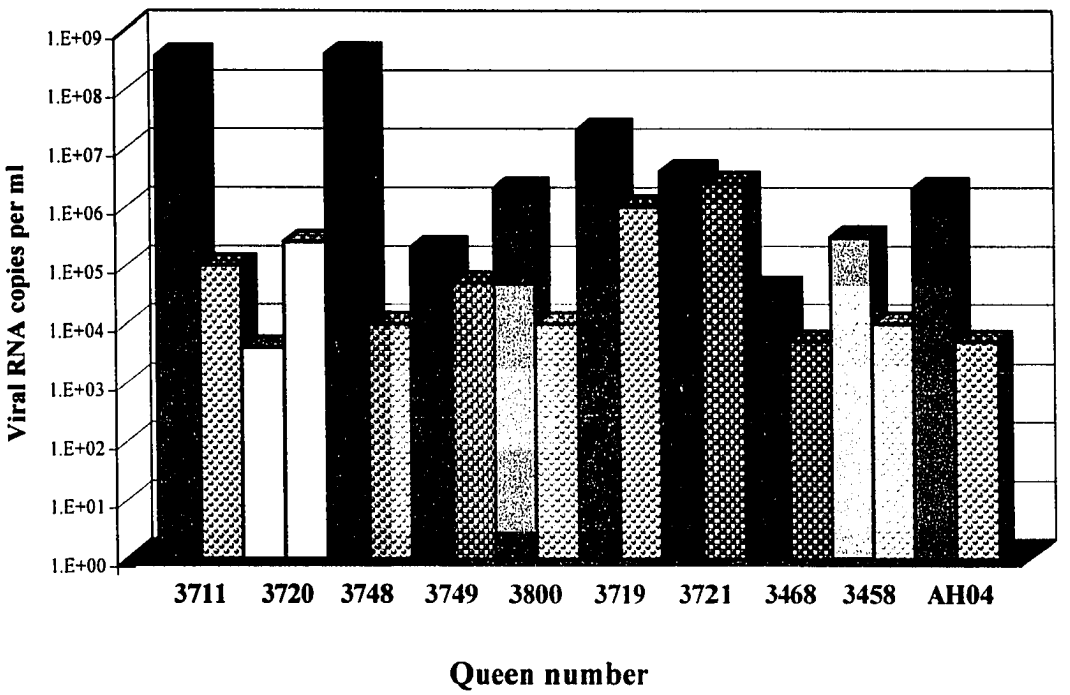

Figure 3.4: Viral RNA loads in milk (solid bars) or plasma (hatched bars) for ten FIV-B-2542 infected queens on the day of delivery. Values expressed as viral copy number per milliliter of sample as determined by QC-RT-PCR. Note that 9 of 10 queens have higher levels of viral RNA detectable in milk than plasma.

Viral isolation

Virus isolation (VI) by coculture confirmed presence of infectious virus in cell-free milk supernatant from the day of delivery in two of three queens tested, while two samples from week 4 of lactation were negative (Table 3.3). Coculture supernatants became positive for FIV p26 by ELISA after 3 weeks in culture, and only at the lower milk input volumes (≤ 6 µl). Cells retrieved from these cocultures were positive for provirus by DNA-PCR. In contrast, milk cell cocultures from the day of delivery in two queens were both negative for infectious virus.

Provirus comparisons in blood components

Because minimal volumes of blood could be obtained from newborn kittens (about 600 µl from a 100 gram kitten), PCR was often performed on DNA extracted from whole blood rather than isolated pbmc. To assure confidence in the results, DNA was extracted in parallel from whole blood and isolated peripheral blood mononuclear cells (pbmc) from FIV-B-2542 infected queens and subjected to limiting-dilution DNA-PCR. In 24 separate comparisons, the proviral load per µg of DNA was the same for pbmc and whole blood in 9 cats; in 5 cats the proviral load was higher in pbmc than in whole blood, and in 10 cats the proviral load was higher in whole blood than in pbmc. The proviral load detected varied by no more than one log between samples from any individual.

In order to examine the possibility that components of whole blood other than pbmc might be responsible for the observed proviral loads, double-density centrifugation was used to separate pbmc and polymorphonuclear cells (pmnc) from the same blood sample. In addition, platelet pellets were prepared from two samples. PCR was

performed on DNA extracted from platelets and separated pbmc and pmnc. Whereas platelets contributed little to the detectable proviral load, the pmnc-enriched cell populations contained viral DNA equivalents equal to or greater than that in the pbmc-enriched cell populations (Figure 3.5).

Table 3.3: Virus isolation in milk supernatant or milk cells for three FIV-B-2542 infected queens on the day of delivery and week 4 of lactation. (-) = VI negative as assessed by p26 antigen ELISA, (+) = VI positive, nd = not done.

	Milk supernatant		Milk cells
Queen	**Delivery**	**Week 4**	**Delivery**
3748	+	-	- ($1x10^5$)*
3749	-	-	- ($2x10^6$)
3800	+	nd	nd

* = maximum number of milk cells in a log-dilution series

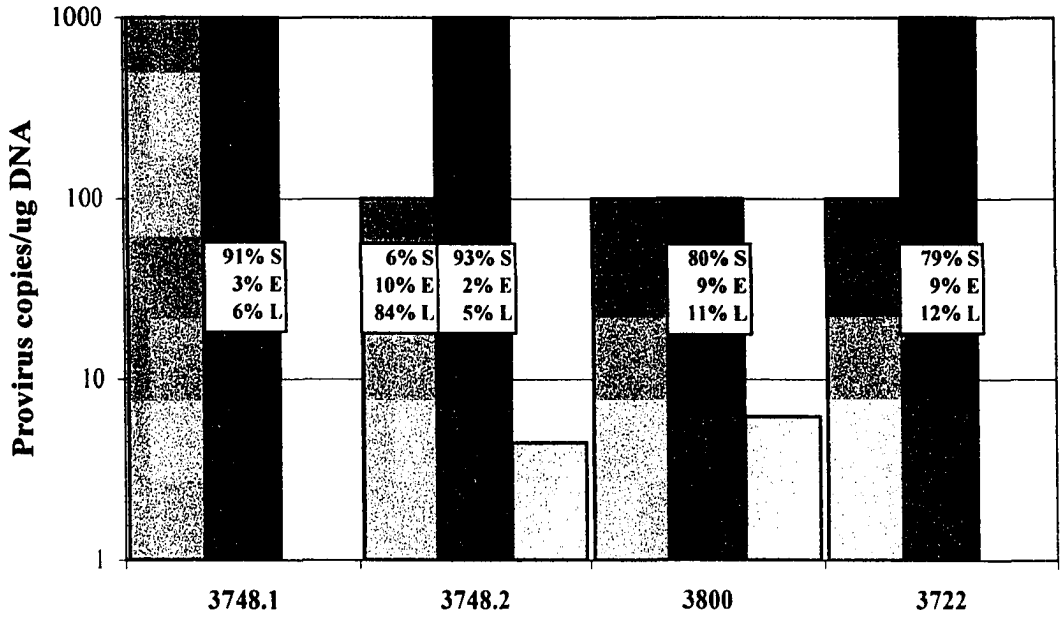

Figure 3.5: Proviral loads determined by limiting-dilution DNA-PCR in separated pbmc (▩), pmnc (■), and platelets (▩) from 3 FIV-B-2542 infected queens. Two different experiments for queen 3748 are shown. Percentages indicate cell distribution determined by differential counts on stained films; S = segmented neutrophils, E = eosinophils, L = lymphocytes.

DISCUSSION

Although previous studies with FIV-B-2542 performed in our laboratory had reported transmission from mothers to approximately 50% of offspring [34, 35], in the present studies using current generation virus pools we observed a vertical transmission rate of 22%, which is similar to that reported for HIV [36]. This lower rate, however, made chemotherapeutic intervention studies problematic; assessment of the efficacy of PMPA to reduce or prevent vertical FIV transmission would require substantially greater numbers of animals. Reasons for the difference in vertical transmission rates are not clear, but may reflect genotypic changes in the virus pool used to inoculate the queens.

In our initial studies with PMPA, however, we identified adverse fetal effects of PMPA when administered to pregnant queens at 30 mg/kg day, the dose used for macaques in SIV studies [37-39], and well-tolerated by juvenile cats in a previous study in our laboratory (unpublished data). We observed significantly higher kitten mortality and lower birth weights in kittens born to queens that received PMPA. Similarly, a recent study of PMPA administration to pregnant macaques identified decreased infant body weights, growth restriction and bone-related toxicity, and alterations in serum phosphorus and alkaline phosphatase [40]. Future studies of PMPA efficacy in vertical transmission should take into account the toxicities identified here; a shortened treatment time and/or lower dose might have fewer negative effects and still be effective.

We documented queen proviral loads in DNA extracted from both whole blood and isolated pbmc by limiting-dilution DNA PCR and found them to be strikingly similar, and often higher in whole blood. Only a fraction of the DNA extracted from a

whole blood sample would be expected to originate from mononuclear cells; blood cell differential counts varied among samples, but neutrophils almost always outnumbered lymphocytes, sometimes by as much as 3 or 4 to 1. Indeed, we found just as much provirus present in isolated polymorphonuclear cells as in mononuclear cells. FIV infection of bone marrow stromal cells, megakaryocytes, and unidentified marrow mononuclear cells has been demonstrated [41-44], as has HIV infection of some hematopoietic progenitor cells [45]. Although HIV DNA has been amplified from purified human neutrophils and eosinophils [46-50], we are unaware of previous reports of FIV infection of neutrophils. We also report here low levels of viral DNA present in platelets, which is not surprising considering the evidence of megakaryocytic infection [43, 44].

We identified relatively low levels of viral DNA present in milk cells collected throughout lactation. Although we did not quantify cell numbers in milk samples, cell numbers appeared to be highest in early milk, based on the total μg of DNA that could be extracted from cell pellets. This is consistent with what has been reported for human milk; cell numbers are reported to decrease from 10^7 per ml in colostrum to 10^5 per ml in late milk [51]. In humans, macrophages and granulocytes predominate in colostrum and early milk, while late milk contains mostly macrophages, almost no granulocytes, ~20% T cells, and ~10% epithelial cells [51]. Assuming this distribution to be similar in cats, we might conclude that provirus levels were lower in milk cells than in blood because we were detecting provirus present mainly in macrophages. While FIV-infected macrophages can be readily demonstrated in situ in various tissues [43, 52-54], their relative proviral burden compared to lymphocytes is unknown. We were unable to

demonstrate infectious virus in two milk cell samples that were cocultured for viral isolation, however cell numbers were limited. One of two milk cell cocultures performed in another study (Chapter Two) was positive by VI, and two previous reports have documented infectious virus in feline milk cells [34, 35].

In striking contrast to the low levels of viral DNA present in milk were the high levels of viral RNA detected in cell-free milk supernatant. Viral loads in early milk were as much as 4 log-fold higher than viral loads in plasma for any individual queen. This contrasts with reports of HIV RNA in milk, which suggest a prevalence of 40 to 60% and viral loads lower than that of plasma [12, 13]. However, very few studies have addressed this specific aspect of HIV vertical transmission. A recent comparison of HIV viral loads between left and right breasts early in lactation revealed viral loads ranging from 600 to 1.2 million copies/ml, with virus present unilaterally on at least two occasions in 20% of women [55]. Presence of mastitis has been associated with increased milk viral loads and risk of HIV vertical transmission [56], however we saw no clinical evidence of mastitis in these queens when samples were collected.

The marked disparity between viral loads in plasma and milk observed here suggest that virus is actively concentrated in milk early in lactation. Several potential mechanisms exist to explain this observation. Subclinical inflammation comprised primarily of lymphocytes, many of which could be productively infected, might result in such concentration. Mammary epithelial cells, which are adept at transcellular transport of immunoglobulins into milk [57], might be responsible for transporting virus into milk as well. There is some evidence to suggest that mammary epithelial cells are capable of productive infection with HIV, and that viral replication can be upregulated by lactogenic

hormones [51, 58, 59]. FIV should be an excellent model in which to explore these possibilities by direct examination of mammary gland tissues from infected queens.

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CHAPTER FOUR

FELINE IMMUNODEFICIENCY VIRUS IN MAMMARY GLAND

INTRODUCTION

Of the 40 million people living with HIV-AIDS in 2001, almost 3 million were children less than 15 years of age; another 580,000 children died of their disease that year [1]. In 2001, HIV was responsible for 70% of deaths among children under 5 years of age in Zimbabwe alone [1]. Vertical transmission of HIV from mother to child continues to be the main source of new infections in children; one-half to two-thirds of this transmission worldwide can be attributed to breastfeeding [2-6].

Feline immunodeficiency virus (FIV) is the feline counterpart of HIV, causing a virtually identical disease syndrome [7-10]. Like HIV, FIV can be transmitted via mucosal exposure, blood transfer, and vertically from mother to offspring [9, 11-13]. Vertical transmission of FIV occurs in utero, postnatally via milk, and potentially during delivery [14-18]. Breastfeeding has been reported to increase the risk of HIV transmission by 14% to 26%, depending on the stage of maternal infection [19-21]; similarly the risk of FIV vertical transmission has been shown to increase by 13.5% when kittens nurse their infected mothers [22].

Previous studies in our laboratory (Chapter 3) have revealed concentration of viral RNA in milk early in lactation, with relatively low levels of provirus present in milk cells. Although HIV has been documented in both cell-free and cell-associated milk fractions [23-27], little is known about potential cellular sources of virus in the mammary gland. In vitro studies with cultured human mammary gland epithelial cells have demonstrated their susceptibility to productive HIV infection, raising the possibility of a cellular reservoir for virus that becomes activated during pregnancy [28, 29]. Similarly, milk epithelial cells from goats infected with caprine arthritis encephalitis virus (CAEV) produced high viral titers in culture [30]. However, there have been no reports identifying HIV-infected cells of mammary gland *in situ*. Here, using the FIV model, we report the first identification of FIV-infected cells in the lactating mammary gland. In addition, we observed differences in lymphocyte distribution in the mammary glands of FIV-infected versus naïve cats that may impact viral dissemination in milk.

MATERIALS AND METHODS

Animals and sampling

All cats originated in a specific-pathogen-free (SPF) breeding colony maintained at Colorado State University and were housed in accordance with Animal Care and Use Committee regulations.

Three queens were inoculated intravenously with FIV-B-2542 and three with FIV-C-Pgmr (from an in vivo-passaged plasma pool source) and bred to FIV-negative males 2 – 7 months later. FIV infection was confirmed by DNA polymerase chain

reaction (PCR) on blood as described below. All queens became pregnant and produced a total of 27 kittens.

Two queens infected with FIV-A-Petaluma (FIV-A) for 1 to 1.5 years were bred to FIV-negative males. Both queens became pregnant, producing a total of 5 kittens.

Mammary gland biopsies were obtained from 3 FIV-B queens prior to breeding and within 48 hours after delivery. Biopsies were obtained from three FIV-C queens 48 to 96 hours after delivery and 10 to 14 days after weaning kittens. One FIV-A queen was biopsied 6 days after delivery and one week after weaning; the other within 24 hours after delivery. Three naïve queens from the SPF breeding colony provided mammary gland biopsies for negative controls; two were obtained one day after delivery and one just before delivery during a terminal caesarean section procedure as part of an unrelated study.

Lymph nodes were collected at necropsy from two of the FIV-B queens and from several acutely infected cats that were part of another study. These samples were used as reference points for in situ hybridization assays and to evaluate cell phenotype markers for immunohistochemistry.

Sample processing

Mammary gland biopsies consisting of one or two tissue samples measuring 1 x 2 cm were obtained from a cranial gland under ketamine-acepromazine anesthesia. Tissues were fixed for 12 to 18 hours in 10% buffered formalin followed by processing into paraffin blocks using a 'short cycle' technique that minimized further exposure to formalin and liquid paraffin (Colorado State University Histology Laboratory, Ft.

Collins, CO). Lymph nodes obtained at necropsy were identically processed. Routine 5 μm paraffin sections were placed on positively charged slides with minimal heat treatment.

Cell phenotype immunohistochemistry (single label)

Tissue sections were deparaffinized with heat and xylene, rehydrated through a series of graded alcohols to dH_2O and rinsed in Tris-buffered saline (TBS). Staining was performed with a Ventana™ ES automated slide stainer that utilizes a universal anti-mouse/rabbit biotinylated secondary antibody, an alkaline phosphatase-streptavidin conjugate, a substrate chromagen (fast red A), and a hematoxylin counterstain (Ventana Medical Systems, Tucson, AZ). Primary antibodies for cell phenotype used were: polyclonal rabbit anti-CD3 for T lymphocytes, monoclonal anti-BLA.36 for B lymphocytes, monoclonal anti-Fascin (p55) for dendritic cells, monoclonal Mac 387 for macrophages (all Dako Corp., Carpinteria, CA), monoclonal anti-CD45R (B220) for B lymphocytes (Cedarlane Laboratories Ltd., Ontario, Canada), and monoclonal anti-pankeratin for epithelial cells (Ventana Medical Systems, Tucson, AZ). Negative controls consisted of polyclonal or isotype-matched monoclonal irrelevant primary antibodies applied to both mammary gland and lymph node sections and processed in parallel with experimental sections.

Antigen retrieval methods were required for optimal staining of tissues. Pre-treatment with protease (Ventana Medical Systems, Tucson, AZ) was performed prior to application of Mac 387 and pankeratin primary antibodies. Autoclaving at 250°C for 8

minutes in Dako® Target Retrieval Solution (Dako Corp., Carpinteria, CA) was utilized prior to application of all other primary antibodies.

In situ hybridization

Tissue sections were deparaffinized and rehydrated sequentially with xylene, 100% ethanol, 80% ethanol, 50% ethanol, and RNase-free water. The slides were then incubated with 5 mM levamisole for 20 minutes (this step was omitted when fluorescent detection was used), washed with 1X SSC buffer (Sigma, St. Louis, MO), incubated in 0.2 N HCl for 20 minutes, and washed again with SSC buffer. Endogenous peroxidase was blocked with 5% H_2O_2 in methanol for 30 minutes. The sections were digested with 10 - 25 μg/mL proteinase K (PK) in buffer containing 10mM Tris (pH 7.4) and 2 mM $CaCl_2$ for 5 minutes at 37°C. The PK concentration was optimized empirically for each section. Digestion was stopped with 0.1 M glycine in PBS. The slides were then incubated in 0.1 M triethanolamine-0.25% acetic anhydride solution for 10 minutes, washed in 2X SSC, incubated in 0.1 M Tris (pH 7.4)-0.1M glycine solution for 15 minutes, and washed in 2X SSC. Prehybridization was done at 50°C for 15 minutes with hybridization solution containing 50% deionized formamide, 1X SSC, 1X Denhardt's solution, 5 mM $NaPO_4$ (pH 6.8), 0.1% sodium dodecyl sulfate, 250 μg/ml of salmon sperm DNA, 5% dextran sulfate, and 250 μg/ml tRNA (all Sigma, St. Louis, MO). Hybridization solution containing 50 ng digoxigenin-labeled whole FIV antisense or sense probe per slide was applied and slides were coverslipped. For detection of viral RNA, sections with antisense probe were heated to 65°C for 10 minutes, chilled for 5 minutes on ice, and hybridized overnight in a humid chamber at 50°C. For detection of

viral DNA, sections with sense probe were heated to 95°C for 10 minutes to denature DNA before chilling and hybridizing overnight. Following hybridization, the coverslips were removed and the slides washed with 4X SSC-50% formamide for 1 hour at 53°C and then in 2X SSC for 2 minutes. An RNase mixture (1 unit/ml RNase T1 and 20 μg/mL RNase A (both Sigma, St. Louis, MO) for 30 minutes at 37°C) was used to digest excess probe and wash steps were repeated. Slides were blocked for 1 hr in a buffer containing 5% horse serum, 2.5% sheep serum, 2.5% goat serum 0.3% Tween 20, 1% Boehringer blocking agent (Boehringer Mannheim, Indianapolis, IN), 100 mM Tris (pH 7.4), and 150 mM NaCl. Slides were blocked for an additional 30 minutes in TNB blocking buffer (TSA™ Systems, NEN Life Science Products, Boston, MA).

For chromogen signal detection, a sheep anti-digoxigenin peroxidase-conjugated antibody (Roche Molecular Biochemicals, Indianapolis, IN) diluted 1:100 in TNB buffer was applied for 30 minutes at room temperature. The sections were washed in TNT buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, and 0.05% Tween 20) three times for 5 minutes each. Signal was amplified with tyramide using the TSA™ Plus DNP System (NEN Life Science Products, Boston MA) according to manufacturer's directions. Briefly, the tyramide-DNP reagent, diluted 1:50 in the diluent provided in the NEN kit, was applied to the slides for 15 min. After three washes with TNT buffer, the alkaline phosphatase-conjugated anti-DNP antibody provided in the kit, diluted 1:50 to 1:100 in TNB buffer, was applied for 30 minutes. After three more washes with TNT buffer, the sections were incubated with 5-bromo-4-chloro-3-indolyl phosphate(BCIP)-nitroblue tetrazolium (NBT) substrate (Amresco, Solon, OH) for 10 to 20 minutes in the dark. Sections were examined microscopically frequently during the development process and

washed at the first indication of non-specific background signal. The sections were washed in TNT buffer, counterstained with nuclear fast red (Vector Laboratories, Burlingame, CA), dehydrated in graded ethanols, cleared in xylene, and mounted with Cytoseal XYL (Stephens Scientific, Kalamazoo, MI). Negative controls included tissue sections without probe added, sections from FIV-negative cats with antisense probe added, and sections from FIV-positive cats with sense probe added and no DNA-denaturing step.

For fluorescent detection, the anti-digoxigenin peroxidase-conjugated antibody was applied at a dilution of 1:1000 for 30 minutes, slides were washed three times in TNT buffer, the tyramide-DNP reagent diluted 1:500 was applied for 10 minutes and slides again washed three times. A peroxidase-conjugated anti-DNP antibody diluted 1:500 in TNB buffer was applied for 30 minutes. After three more TNT washes, FITC-conjugated tyramide reagent diluted 1:50 in the provided diluent was applied for 10 minutes. Slides were finally washed three times in TNT buffer, once in dH_20, and mounted immediately with Vectashield® mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Combined in situ hybridization and immunohistochemistry

In order to identify the phenotype of cells in mammary gland that were productively infected with FIV, in situ hybridization (ISH) for FIV RNA was performed as described above using tyramide amplification and fluorescent detection. After microscopic examination for signal quality, coverslips were removed and slides were washed in TBS for 20 minutes to remove the mounting medium. Individual slides were

then stained with one of the phenotype markers as described for single-label immunohistochemistry (IHC) above, except that no further antigen retrieval methods were employed. Finally, the slides were washed in TBS and coverslipped with Vectashield® mounting medium with DAPI (Vector).

Microscopy and image analysis

Digital images of stained sections were captured using a CoolSnap™ camera and software system (Roper Scientific, Munich, Germany) and an Olympus BX60 fluorescent microscope. Quantitation of cells positive for FIV by ISH was performed manually by observation of at least 5 low-power fields (10X objective, final magnification 100X, field of view 2.2 mm) for each tissue section. Average number of positive cells per low-power field were recorded as: negative if no positive cells identified, 1+ if 1 to 10 positive cells were present, 2+ if 10 to 25 positive cells were present, 3+ if 25 to 50 positive cells were present, and 4+ if > 50 positive cells were present. Quantitation of cells positive for Mac 387 by IHC was performed in the same way. CD3 positive cells identified by IHC were counted in 20 high-power fields (40X objective, final magnification 400X, field of view 0.55 mm) for each section and recorded in an Excel® spreadsheet (Microsoft Corp., Redmond, WA). Statistical analysis was performed with the Statview® software (Abacus Concepts, Inc., Berkeley, CA) by ANOVA with multiple comparisons made by the method of Bonferonni and Dunn; significance was assumed at $p \leq 0.05$.

For sections that underwent both ISH and IHC, multiple digital images of the same field were captured using brightfield illumination and 3 different fluorescent channels (blue for DAPI nuclear staining, green for FITC-labeled FIV positive cells, red

for cell phenotype markers stained with Fast red A). These images were imported into the Adobe Photoshop® software (Adobe Systems, Inc., Mountain View, CA) and merged to demonstrate signal colocalization.

RESULTS

Mammary gland biopsy quality

Biopsies from three FIV-B queens obtained before breeding contained little glandular tissue, consisting primarily of connective tissue and fat. These were not deemed sufficient quality for analysis. Biopsies obtained shortly after delivery consisted predominately of glandular tissue; those obtained 10 to 14 days after weaning contained glandular tissue that was involuting and partially replaced with connective tissue.

Cell phenotype immunohistochemistry (single label)

The CD3 antibody (Dako) worked well in both lymph node and mammary gland and revealed many T-cells present in mammary gland tissues. In biopsies taken within the first few days after delivery, most T-cells were located between epithelial cells lining alveoli, with fewer cells noted in the interstitium (Figure 4.1). This pattern changed in biopsies taken post-weaning; T-cells appeared in large clusters mainly in interstitial areas of the gland (Figure 4.2). There were significantly more T-cells present in FIV-infected cats from each clade than in naïve cats in biopsies from near date of delivery (Figures 4.3 and 4.4). Subjectively, moderate to large clusters of T-cells, ranging in size from 15 cells to > 50 cells, were noted only in tissues from FIV-infected cats.

Macrophages identified by the Mac 387 antibody (Dako) were present in low to moderate numbers (1+ to 2+, or <10 to 10 – 25 cells per low-power field) in all mammary glands examined. They had primarily an interstitial distribution, with a few cells identified within alveolar lumina (Figure 4.5). The pankeratin antibody (Ventana) brightly stained all mammary gland epithelial cells lining alveoli and ducts (Figure 4.6).

Phenotype markers that seemed to work well in lymph node, staining the expected population of cells, did not always work well in mammary gland. For example, p55 (fascin) brightly stained a population of cells in the lymph node consistent with interdigitating dendritic cells along with lighter staining of follicular dendritic cells. However, when used on mammary gland tissues this antibody was inconsistent; staining endothelial cells brightly on some slides but not others, along with staining of smooth muscle cells and small histiocytes in addition to rare cells with an appearance consistent with dendritic cells. Similarly, CD45R (B220) brightly stained B-cell areas of lymph node but no staining was observed in any mammary gland section. Another B-cell marker, BLA.36, which is reported to label feline B-cells, some macrophage subsets, interdigitating dendritic cells, and granulocytes [31], appeared to stain a wide variety of cell types in mammary gland and so was not specific for B-cells.

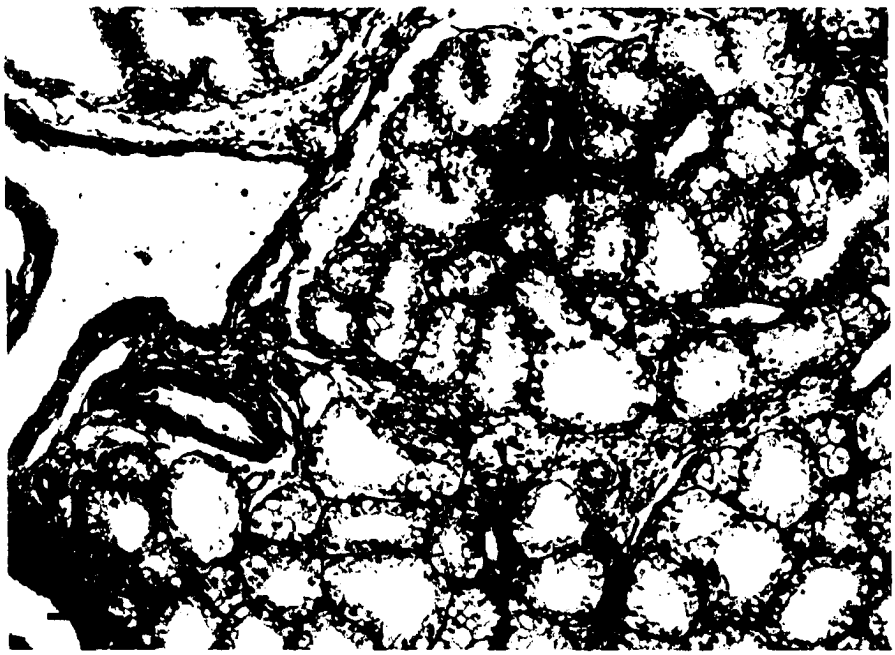

Figure 4.1: Immunohistochemistry, mammary gland, FIV-B queen, 3 days post-delivery. Numerous CD3+ T-cells (red) are present between epithelial cells lining alveoli, with fewer cells located in the interstitium. One moderate cellular cluster is shown here. Brightfield overlaid with fluorescent image, Fast red A, hematoxylin counterstain. Bar = 50 μm.

Figure 4.2: Immunohistochemistry, mammary gland, FIV-C queen, 2 weeks post-weaning. CD3+ T-cells (red) are present in clusters primarily in interstitial locations. Brightfield overlaid with fluorescent image, Fast red A, hematoxylin counterstain. Bar = 50 μm.

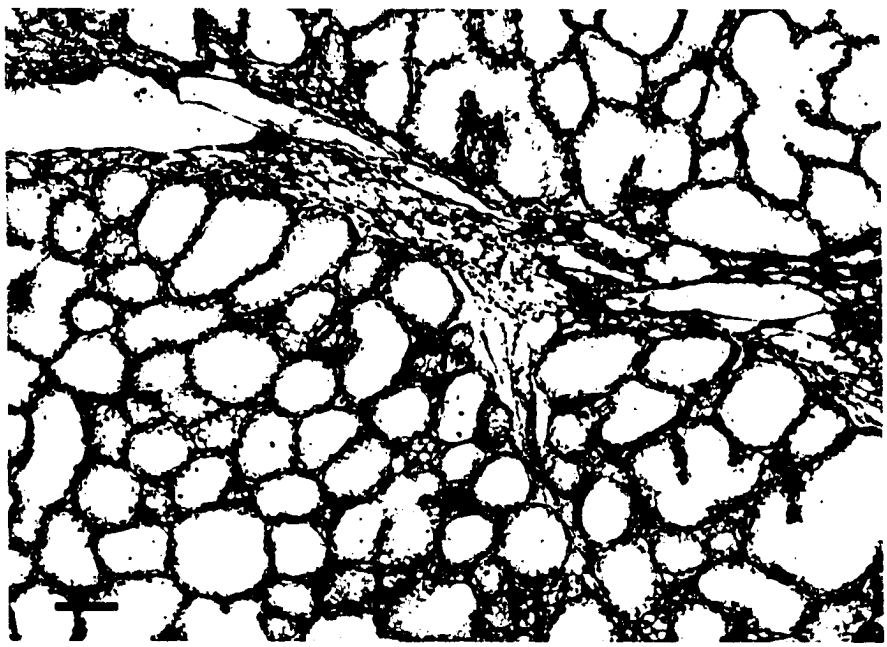

Figure 4.3: Immunohistochemistry, mammary gland, naïve queen, 2 days post-delivery. CD3+ T-cells (red) are present individually between epithelial cells lining alveoli. Brightfield overlaid with fluorescent image, Fast red A, hematoxylin counterstain. Bar = 50 μm.

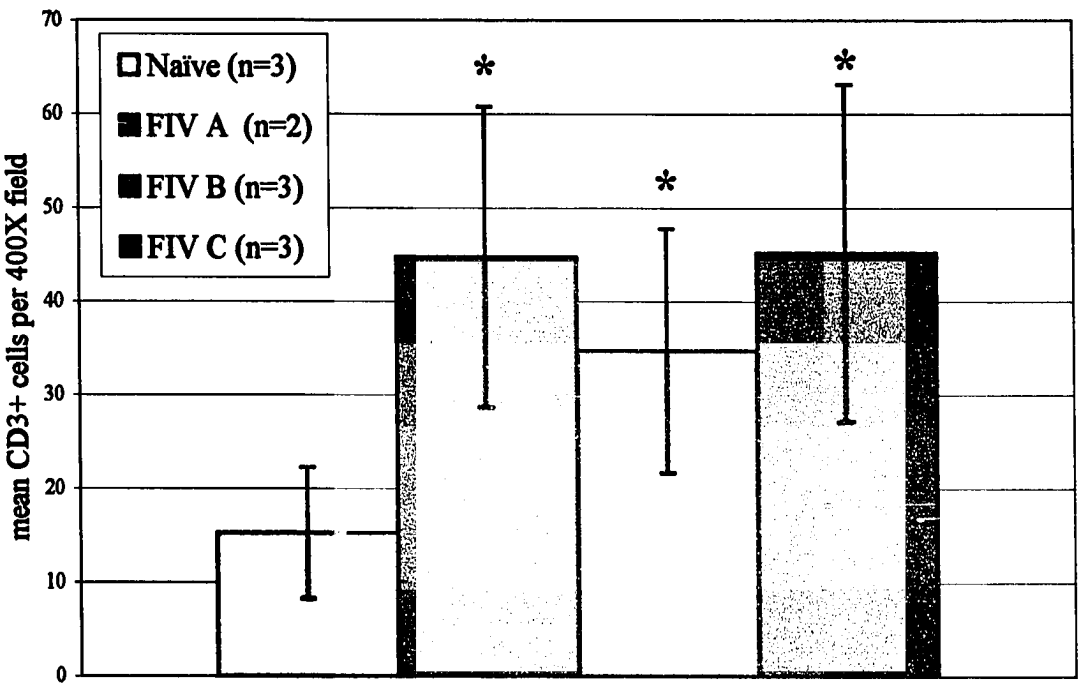

Figure 4.4: CD3+ lymphocytes in mammary gland biopsy sections from naïve (□), FIV-A (■), FIV-B (■), and FIV-C (■) infected queens obtained near the time of delivery. Cells were counted in twenty 400X fields; mean cell counts are reported. Error bars indicate standard deviation. * Significantly different from naïve, $p < 0.0001$.

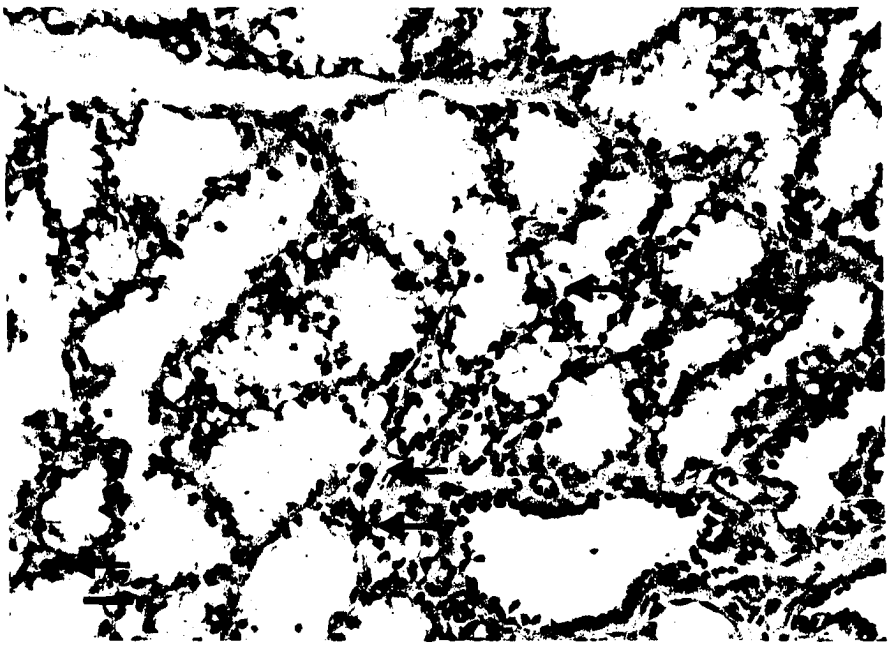

Figure 4.5: Immunohistochemistry, mammary gland, FIV-B queen, 3 days post-delivery. Four Mac 387+ macrophages (red, arrows) are shown. Fast red A, hematoxylin counterstain. Bar = 20 μm.

Figure 4.6: Immunohistochemistry, mammary gland, FIV-B queen, 3 days post-delivery. Pankeratin antibody labels all alveolar and ductal epithelial cells (red). Fast red A, hematoxylin counterstain. Bar = 50 μm.

In situ hybridization

Numerous cells (3+ to 4+, or 25 to >50 per low power field) productively infected with FIV were demonstrated by RNA in situ hybridization (ISH) in lymph nodes from acutely-infected animals used as positive controls (Figure 4.7), while low numbers were identified in lymph nodes of the chronically-infected queens used in this study (Figure 4.8). Interestingly, mammary gland tissues from these queens contained moderate to large numbers of cells positive for FIV RNA by ISH (Figures 4.8, 4.9, Table 4.1). Most positive cells were lining alveolar lumina in a distribution pattern similar to that of CD3+ T-cells identified with immunohistochemistry.

When DNA in situ hybridization was performed to detect cells harboring FIV provirus, the number of positive cells identified increased for both lymph node and mammary gland, although the magnitude of the increase was greater in lymph node than in mammary gland (Figures 4.11 – 4.13; Table 4.1). No positive cells were identified in negative control slides for either RNA or DNA ISH (Figure 4.14). Pale, non-specific, stippled staining was observed in some control slides; this usually occurred in association with milk proteins inside alveoli, or in connective tissue.

Figure 4.7: RNA in situ hybridization, lymph node, acute FIV infection. Numerous cells positive for FIV RNA (purple) are present. FIV – BCIP/NBT, nuclear fast red counterstain. Bar = 50 μm.

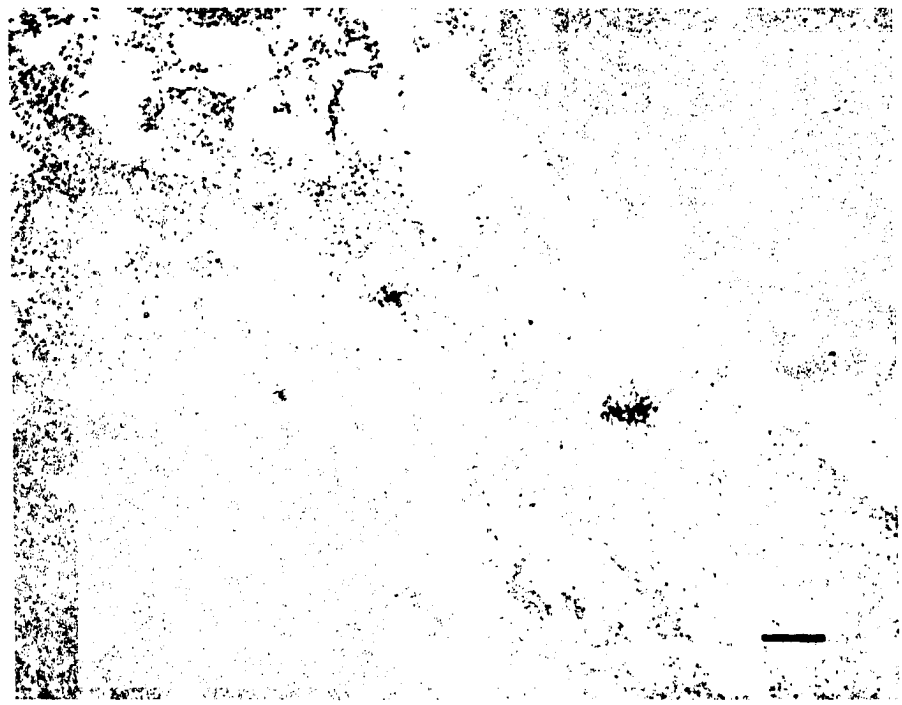

Figure 4.8: RNA in situ hybridization, lymph node, FIV-B queen (3800). Relatively few cells are positive for FIV RNA (purple). FIV – NBT/BCIP, nuclear fast red counterstain. Bar = 50 μm.

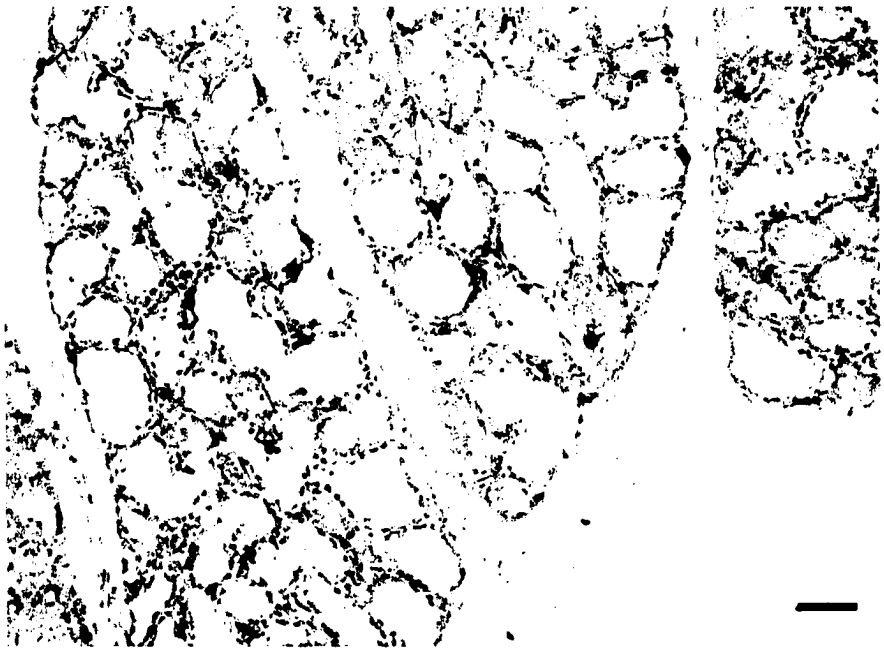

Figure 4.9: RNA in situ hybridization, mammary gland, FIV-B queen (3800). Numerous cells positive for FIV RNA are present lining alveoli. Compare large numbers of positive cells shown here to positive cells in lymph node of this queen shown in figure 4.8. FIV – BCIP/NBT, nuclear fast red counterstain. Bar = 50 μm.

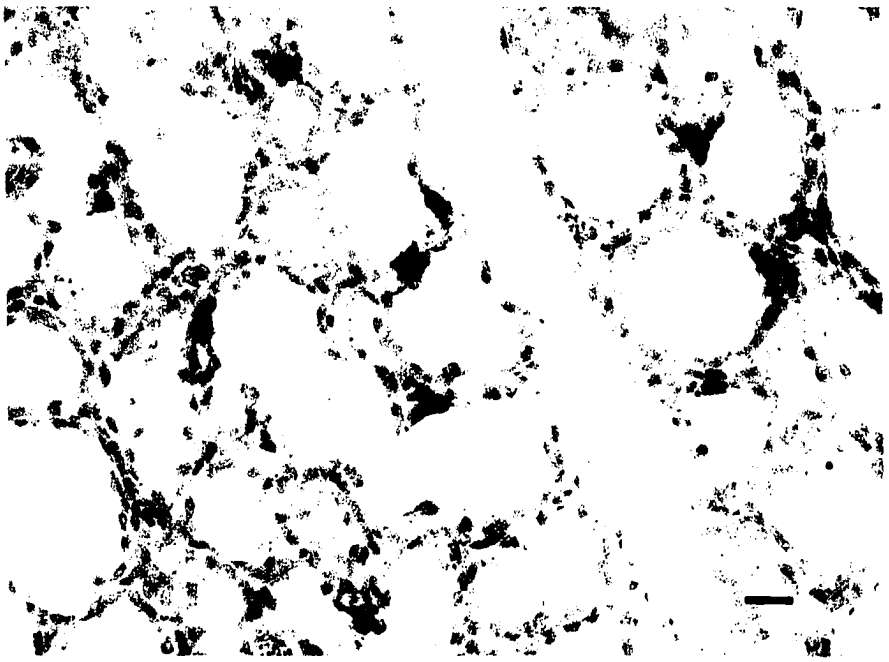

Figure 4.10: RNA in situ hybridization, mammary gland, FIV-B queen (3800). Higher magnification demonstrating position of FIV positive cells (purple) surrounding alveolar lumina. FIV – BCIP/NBT, nuclear fast red counterstain. Bar = 20 μm.

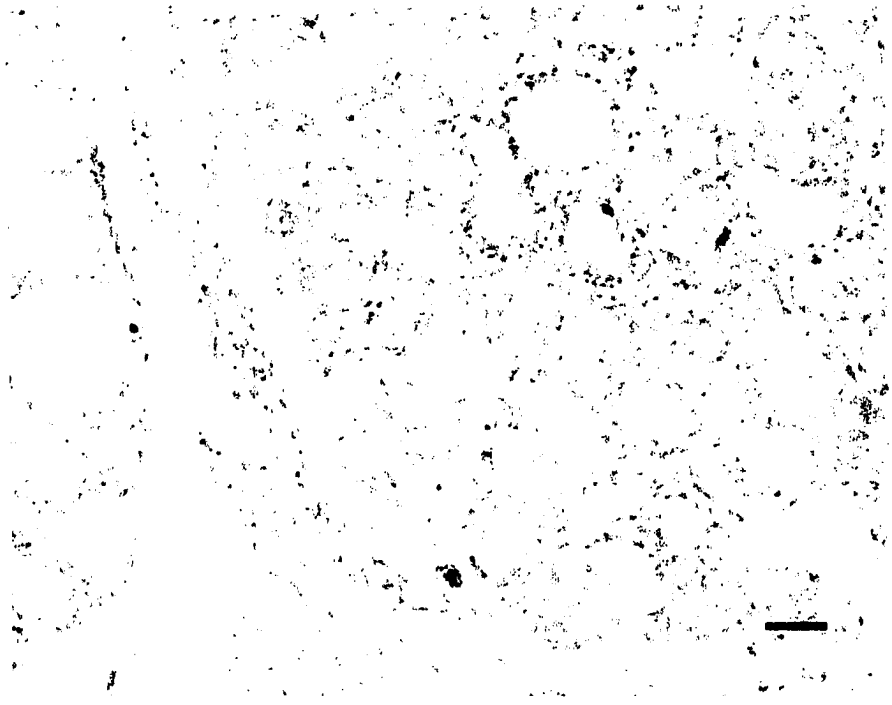

Figure 4.11: RNA in situ hybridization, mammary gland, FIV-B queen (3748). 2+ FIV positive cells (purple) were identified. FIV – BCIP/NBT, nuclear fast red counterstain. Bar = 50 μm.

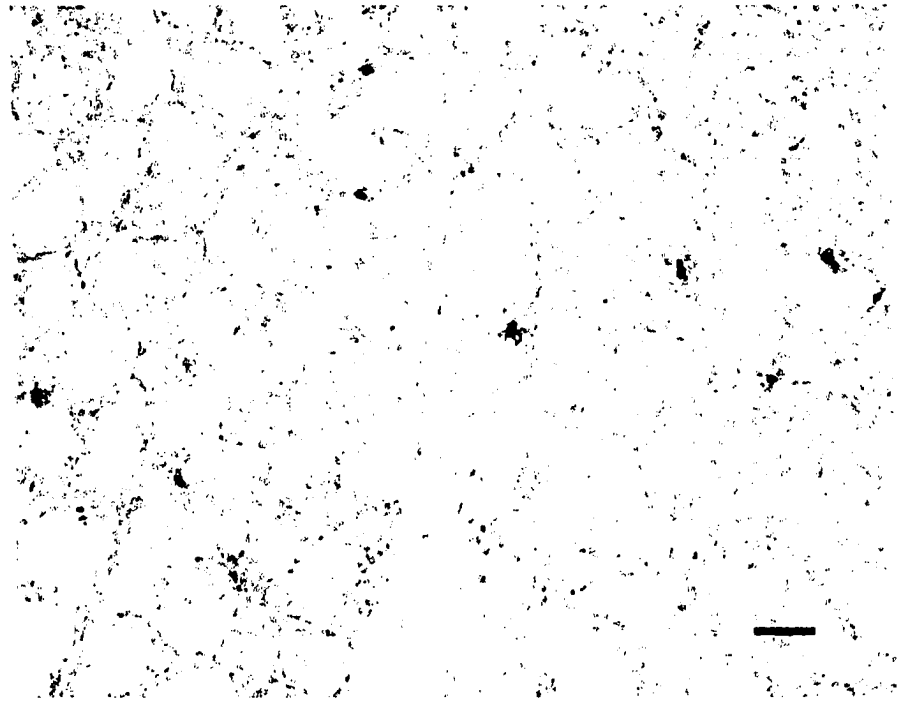

Figure 4.12: DNA in situ hybridization, mammary gland, FIV-B queen (3748). About twice as many cells (3+) were positive for FIV provirus (purple) as for FIV RNA in this queen (Figure 4.11). FIV – BCIP/NBT, nuclear fast red counterstain. Bar = 50 μm.

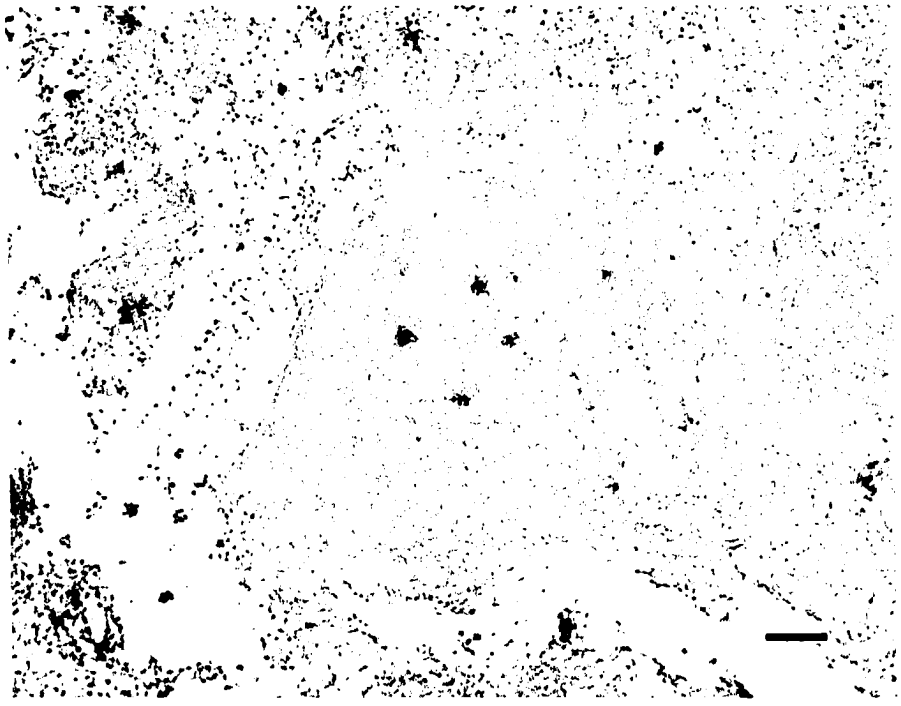

Figure 4.13: DNA in situ hybridization, lymph node, FIV-B queen (3800). Compare 3+ cells positive for provirus here (purple) to 1+ positive for FIV RNA in Figure 4.8. FIV – BCIP/NBT, nuclear fast red counterstain. Bar = 50 μm.

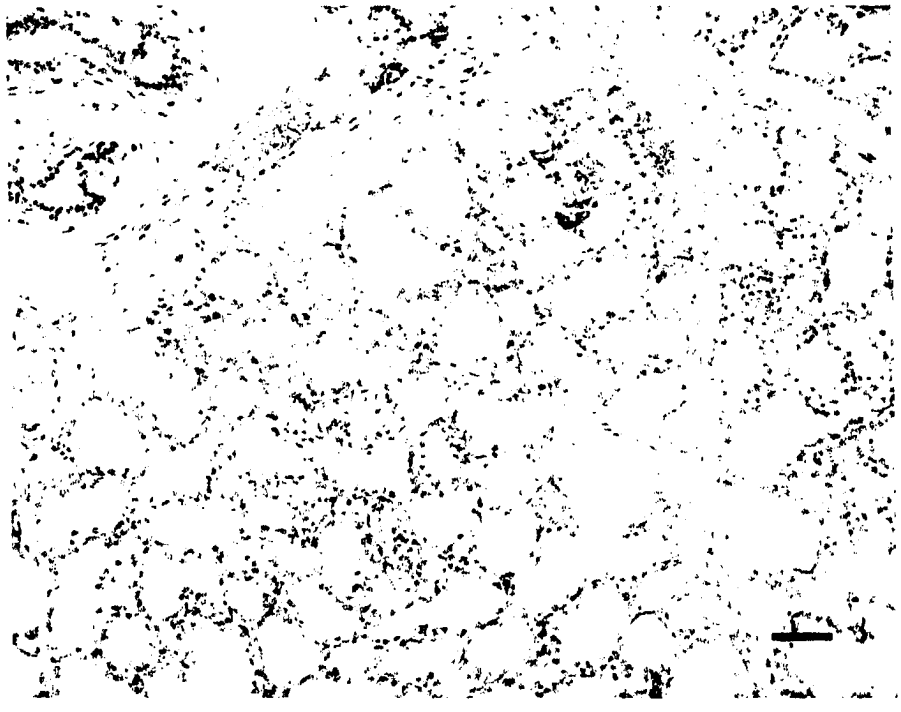

Figure 4.14: RNA in situ hybridization, mammary gland, naïve queen. There are no FIV-positive cells present, and background stain is negligible. FIV – BCIP/NBT, nuclear fast red counterstain. Bar = 50 μm.

Table 4.1: Relative numbers of cells containing FIV RNA or FIV provirus in lymph node (LN) and mammary gland (MG) of 3 FIV-B chronically infected queens compared to lymph node from an acutely infected cat (3257).

Cat	Tissue	FIV RNA*	FIV provirus¶
3257 (acute)	LN	3 - 4+	4+
3748	MG	2+	3+
3749	MG	2+	nd
	LN	1+	nd
3800	MG	3+	3+
	LN	1+	3+

*positive by RNA in situ hybridization; productively infected cells
¶positive by DNA in situ hybridization; cells harboring provirus
Results reported as the number of positive cells per low power field (100X):
1+ = < 10, 2+ = 10 – 25, 3+ = 25 – 50, 4+ = > 50.

Phenotype of FIV-infected cells in mammary gland

In situ hybridization for viral RNA using fluorescent detection was followed by immunohistochemistry for cell phenotype and slides were examined for colocalization of virus with phenotype markers. In the three FIV-B queen mammary gland sections examined to date with this technique, the majority of cells productively infected with FIV were CD3+ T-cells (Figures 4.15, 4.17). Occasional cells positive for Mac 387 (macrophages) were also found to contain FIV RNA (Figures 4.16, 4.18). On slides

stained for CD3, there were moderate numbers of cells that were positive for FIV RNA but negative for CD3.

The ISH signal for FIV RNA was significantly quenched when followed with IHC; this phenomenon was most striking in sections stained with pankeratin. Convincing colocalization of FIV RNA with cells positive for pankeratin (epithelial cells) could not be demonstrated (Figure 4.19, 4.20), considering the distribution of CD3+ T-cells (between epithelial cells lining alveoli) shown with IHC.

Figure 4.15: Combined ISH-IHC, mammary gland. Numerous CD3+ cells (red) are shown. Four CD3+ cells contain FIV RNA (green-yellow); two cells contain FIV RNA (green) but do not stain for CD3 (arrowheads). FIV – FITC, CD3 – Fast red A, nuclei – DAPI (blue). Bar = 20 μm.

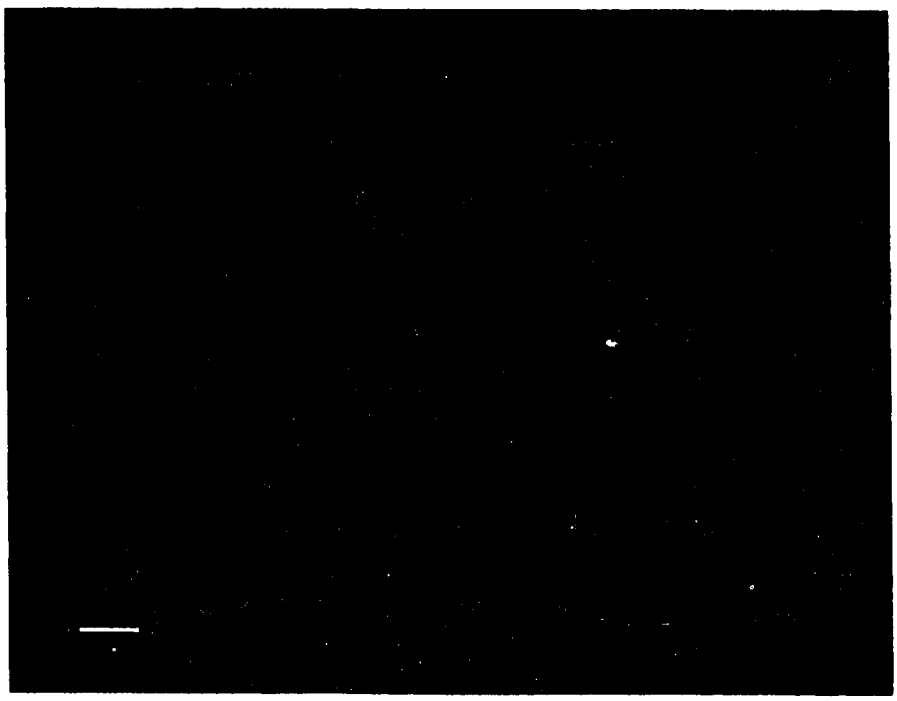

Figure 4.16: Combined ISH-IHC, mammary gland. Several Mac 387 positive cells (red) are seen here; one contains FIV-RNA (green-yellow). FIV RNA – FITC, Mac 387 – Fast red A, nuclei – DAPI (blue). Bar = 10 μm.

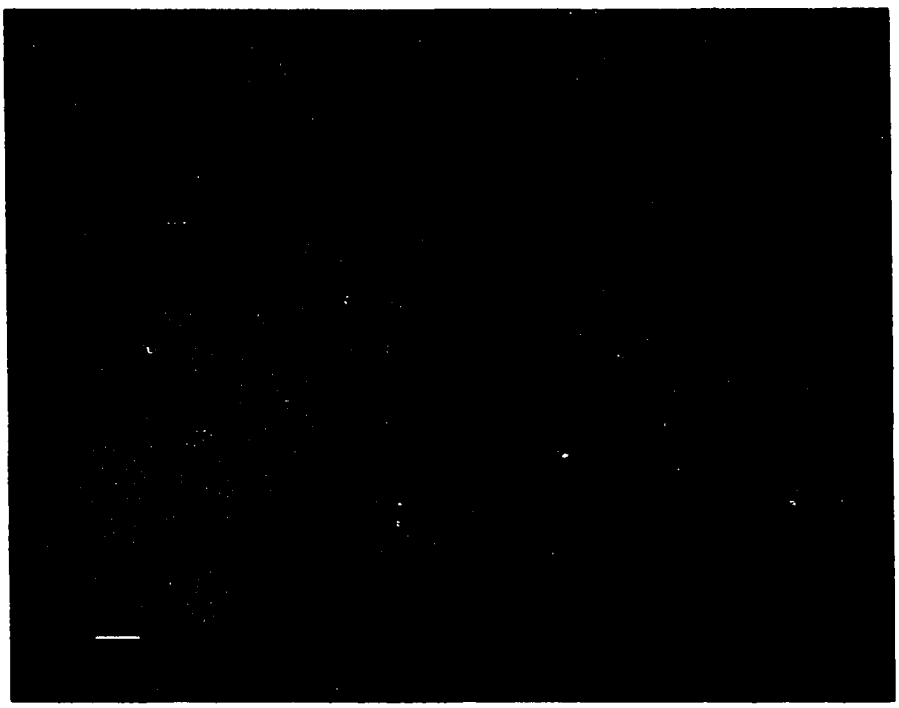

Figure 4.17: Combined ISH/IHC, mammary gland. FIV RNA (green-yellow) in a CD3+ T-cell (red). FIV RNA – FITC, CD3 – Fast red A, nuclei – DAPI (blue). Bar = 10 μm.

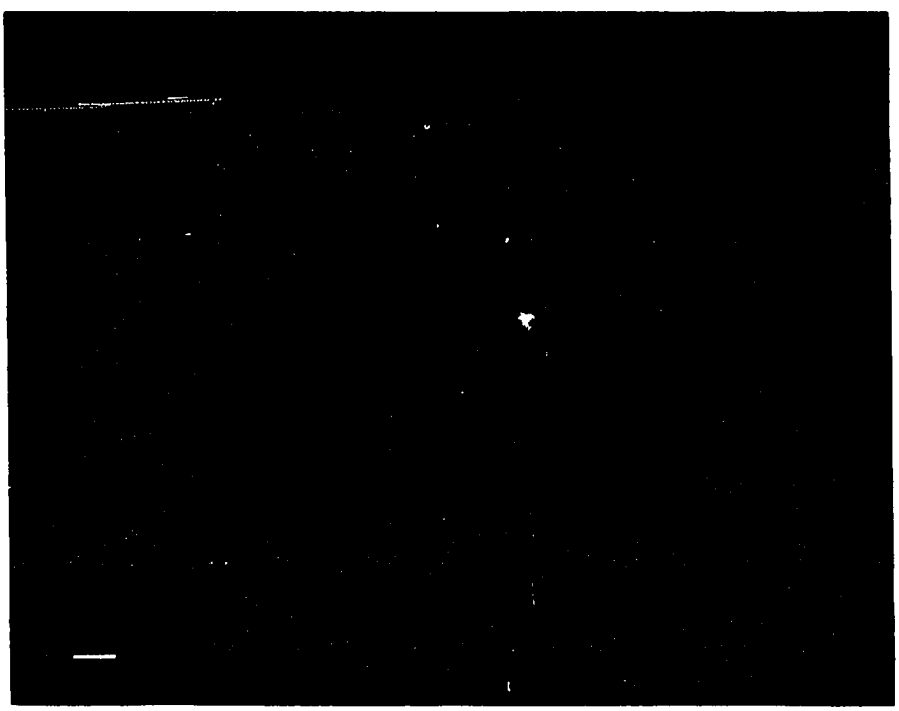

Figure 4.18: Combined ISH/IHC, mammary gland. FIV RNA (green-yellow) in a Mac 387 positive cell (red). FIV RNA – FITC, Mac 387 – Fast red A, nuclei – DAPI (blue). Bar = 10 μm.

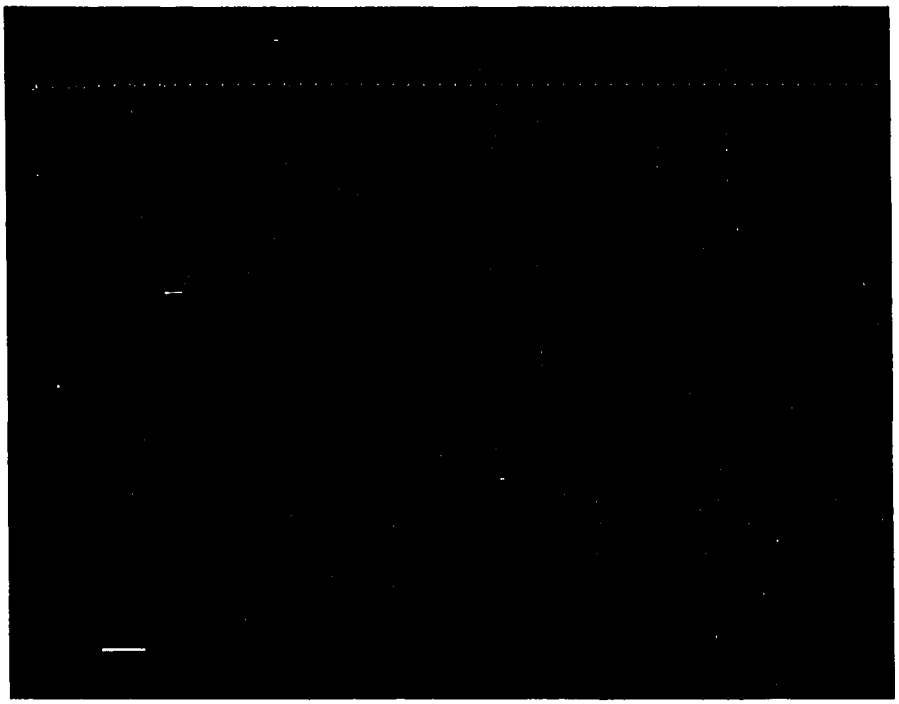

Figure 4.19: In situ hybridization, mammary gland. FIV RNA (green) is shown in a cell lining a small duct. FIV RNA – FITC, nuclei – DAPI (blue). Bar = 10 μm.

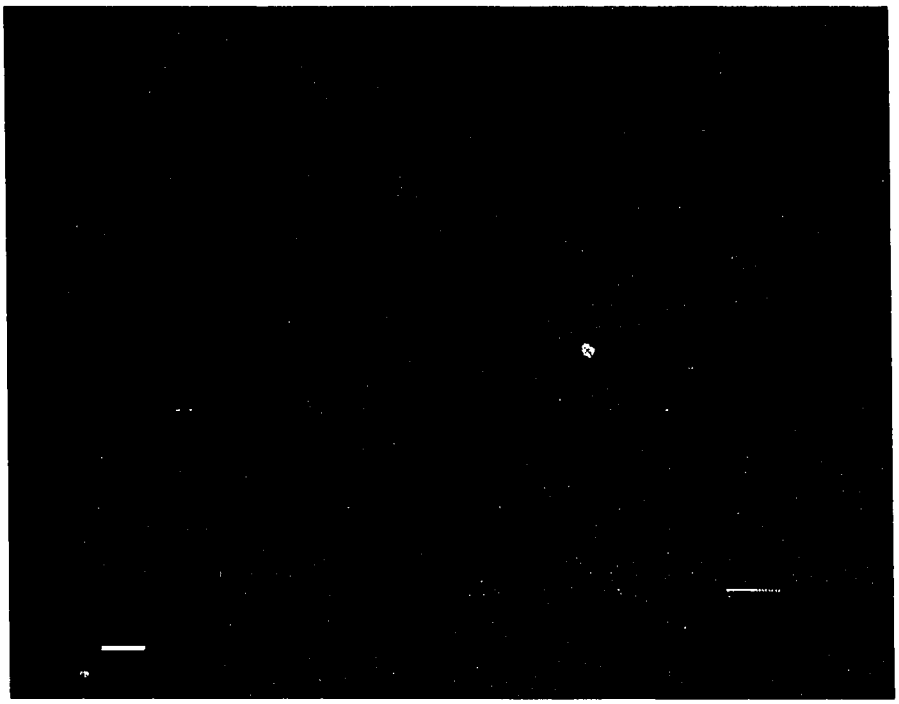

Figure 4.20: Combined ISH/IHC, mammary gland. Same section as in Figure 4.19, following IHC for pankeratin (red). Note significant quenching of FIV RNA signal (green) compared to ISH alone. FIV RNA – FITC, pankeratin – Fast red A, nuclei – DAPI (blue). Bar = 10 μm.

DISCUSSION

We sought to identify FIV infected cells in mammary gland that might account for the previously described concentration of viral RNA in early milk from FIV infected queens (Chapter 3). We found that mammary gland tissues from chronically infected queens contain more infected cells than can be detected in lymph nodes. Thus, mammary gland may act as a reservoir for virus production that ultimately results in virus concentration in milk.

While previous studies in our laboratory and by others have detected numerous productively infected cells in various tissues of acutely infected cats, as infection becomes chronic fewer of these cells can be identified [32-38]. In the current study, DNA in situ hybridization performed on queen lymph nodes detected provirus in three times as many cells as were positive for viral RNA, consistent with the downregulation of most viral replication. In mammary gland, however, the numbers of cells detected by DNA and RNA ISH were more similar, suggesting that a larger proportion of infected cells were supporting active viral replication.

Immunohistochemistry revealed surprisingly large numbers of CD3+ lymphocytes present in mammary glands from both naïve and FIV infected queens. However, this is consistent with information available from studies in other species. The presence of a mucosal lymphoid system in mammary gland analogous to that of the intestinal tract has been described [39, 40]. Intraepithelial lymphocytes residing in mammary gland alveoli have been described in bovine, caprine, ovine, and porcine studies and reported to be primarily CD8+ T-cells, with CD4+ T-cells more often

residing in subepithelial locations [41-47]. These cells generally express an activation or memory phenotype, as do the majority of lymphocytes found in milk [43, 48-52]. Studies in mice have shown that T-cells home to the developing mammary gland in response to increased expression of the mucosal addressin MAdCAM-1 during pregnancy, reaching peak numbers just before delivery [53]. Thus, a normal mechanism exists to recruit T-cells to mammary gland during pregnancy, which is consistent with our observations. We found significantly more T-cells in FIV-infected versus naïve cats, and large cellular clusters were only observed in the FIV-positive cats. FIV infection results in widespread lymphocyte activation [54-56], thus increased T-cell numbers in mammary glands of FIV-infected cats might simply reflect a larger population of target cells for normal recruitment. However, presence of lymphoid aggregates is consistent with reports of hyperplastic lymphoid lesions of a variety of tissues in FIV, SIV, and HIV infections [57-60].

Combined ISH/IHC identified the majority of cells that co-labeled for FIV RNA and a cell phenotype marker as CD3+ T-cells, with rare FIV positive macrophages identified by Mac 387. However, there were other FIV positive cells that did not label for either marker. These cells could represent either a macrophage subset that did not stain for Mac 387, dendritic cells, B cells, or epithelial cells. Macrophages would seem the likely candidates, as Mac 387 is known to identify only a subset of macrophages, and infected macrophages are reportedly identified more frequently in tissues from cats with chronic FIV infection [61, 62]. Several other macrophage markers need to be investigated in mammary gland tissues; preliminary experiments with monoclonal anti-CD68 (Dako) suggest it may stain a larger proportion of mammary gland macrophages

(data not shown). The dendritic cell marker used in these studies, p55 (fascin), was unreliable in mammary gland. Similarly, the B cell antibody CD45R (B220), which worked well in lymph node sections, did not stain any cells in mammary gland, suggesting that a different subset of B cells (likely plasma cells) needs to be investigated.

Human and caprine mammary epithelial cells grown in culture support productive infection of HIV and CAEV, respectively, suggesting they may be a source of virus production in vivo [29, 30]. We found that the pankeratin antibody brightly stained all mammary epithelial cells, but it was not possible to definitively co-localize FIV RNA within them; future studies will need to employ confocal microscopy to make that determination.

An advantage of the Ventana® Fast red A stain was the ability to view the stained cells with either brightfield or fluorescent microscopy. Fluorescence was useful for rapidly scanning sections for low numbers of positive cells, which were difficult to see with brightfield alone. By overlaying a fluorescent image over the brightfield image, the visibility of individual positive cells was enhanced. Background staining with phenotype markers was slightly increased following ISH, likely due to the harsh tissue digestion steps required; background was easily identified by viewing the section with brightfield first, thus eliminating false fluorescent signal colocalization in combined ISH/IHC experiments.

In summary, we report here evidence for FIV-related alterations in the mucosal lymphoid system of feline mammary gland, as well as the presence of substantial numbers of productively infected cells, the majority of which are CD3+ lymphocytes. These results suggest a mammary gland reservoir of virus available for dissemination in

milk, which has relevance for the design of effective strategies to prevent milk-borne transmission of HIV.

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CONCLUSION AND FUTURE DIRECTIONS

The research described in this dissertation has yielded novel information about FIV in milk and mammary gland, and unexpected results regarding mother-to-offspring FIV transmission, raising many new questions worthy of investigation. We have identified covert vertical transmission of FIV from mother to kitten as documented by the following observations: (a) FIV proviral sequences readily detectable by PCR in newborn kittens, (b) increasing difficulty in demonstrating FIV proviral sequences with age, (c) presence of only maternal vs. kitten-origin antibody to FIV, and (d) absence of CD4+ or CD8+ T-cell abnormalities in the kittens. The cats under study remained clinically healthy at 14 months of age, however it is unknown whether or not these animals will seroconvert later in life, or whether virus will remain sequestered in tissues or be completely cleared. This animal model will allow investigation of these potential outcomes, which cannot easily be explored in HIV. Perhaps the most intriguing question is whether or not this natural low-level lentivirus infection can result in resistance to subsequent challenge, and if so what are the immune correlates of such protection? Future studies to address these questions in cats identified with covert FIV infection will include examination of lymphocyte proliferation responses, FIV-specific CD4+ or CD8+ lymphocytes identified by cytokine production, CD8+ T-cell noncytolytic virus suppression, and mucosal FIV challenge.

We also found that FIV is prevalent in early postpartum milk, both as cell-free virus and as provirus in milk cells. Milk provirus levels were lower but cell-free virus levels were higher than in blood, suggesting that infectious virus was being concentrated in mother's milk. These findings raised the question of how offspring of FIV/HIV-infected mothers escape or resist infection and disease, despite exposure to high concentrations of virus in milk. In previous foster nursing experiments, FIV-naïve kittens that nursed FIV-infected queens developed rapidly progressive disease, suggesting that neither antibody nor other antiviral substances in milk are responsible for the protection observed in kittens born to FIV-infected queens. Additional foster nursing studies, with larger numbers of animals to include investigation into cellular and innate immune responses, could provide insight into mechanisms of protection against vertical transmission of AIDS-like viruses

We demonstrated increased numbers of CD3+ T-cells in the mammary glands of FIV-infected queens; many of these cells contained replicating virus. Further phenotypic characterization of this cell population by immunohistochemistry and flow cytometry would help us understand how virus and antiviral immunity is selectively transferred perinatally. Additional work to characterize FIV-infected cells that are not CD3+ should focus on identifying additional phenotype markers for macrophages, plasma cells, and dendritic cells that are reliable in mammary gland and suitable for combined in situ hybridization and immunohistochemistry. Confocal microscopy could determine whether or not mammary gland epithelial cells are infected in vivo, as some in vitro studies of HIV might suggest. Study of cell surface adhesion molecules, which may direct selective

lymphocyte trafficking to the mammary gland in infected animals, might also provide unique insight into milk-borne lentivirus transmission.

Although PMPA treatment of FIV-infected queens during gestation was associated with detrimental fetal effects, less frequent administration of this drug should be evaluated, such that its effects on viral load in milk and virus-infected cells in mammary gland could be evaluated. Limited treatment might result in a marked reduction of virus-infected cells in mammary gland, and perhaps have a significant effect on viral dissemination in milk. Thus, PMPA treatment could help provide a correlation between milk-borne virus and the cellular source of such virus in mammary gland.

No facile animal model to investigate mother-to-offspring transmission of AIDS-like viruses exists. The studies of FIV vertical transmission comprising this dissertation contribute to the understanding of lentivirus vertical transmission, and set the stage for future investigations of transient infection and the role of mammary gland in milk-borne virus transmission. Knowledge obtained from these studies will influence the design of effective intervention strategies to prevent mother-to-child HIV transmission.