JOURNAL OF VIROLOGY, May 1994, p. 3380–3385
0022-538X/94/$04.00+0

Vol. 68, No. 5

Feline Immunodeficiency Virus Can Be Experimentally Transmitted via Milk during Acute Maternal Infection

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Received 8 November 1993/Accepted 31 January 1994

Postnatal transmission of feline immunodeficiency virus (FIV) in neonates nursed by acutely infected mothers and infection resulting from oral inoculation of kittens with FIV were evaluated. Ten of 16 kittens nursed by four queens with FIV infection established immediately postpartum developed FIV infection. Five of 11 neonates orally administered cell-free FIV culture supernatant developed FIV infection. Kittens that developed FIV infection had greater proportions of $CD4^+$ and Pan-T^+ lymphocytes at birth than negative kittens. Infectious virus was recovered from the milk of acutely infected mothers. We conclude that FIV may be experimentally transmitted via milk from queens with acute infections and that oral administration of FIV to neonatal kittens results in infection.

Vertical virus transmission, defined as transmission either in utero, during the intrapartum period, or in the early postnatal period via milk, is well documented for several lentiviruses of the retrovirus family. Human immunodeficiency virus (HIV) is transmitted by both in utero and lactogenic routes (9, 23, 24, 26, 45, 50) and probably during the intrapartum period (13, 15, 22). HIV has been isolated in cell-free and cellular fractions of human breast milk (5, 41), further supporting a role for milk in the vertical transmission of HIV. The importance of the different routes of transmission and the risk factors that govern vertical transmission are debated. It is conceivable that different routes will be more important than others under different circumstances. Some of these issues could be addressed by the development of an animal model of vertical lentivirus transmission.

As in the case of HIV, there is evidence for in utero and postnatal transmission of simian immunodeficiency virus from infected female monkeys (28). Maedi/visna virus in sheep and caprine arthritis-encephalitis virus in goats are both efficiently transmitted via colostrum and/or milk (2, 12, 21). Equine infectious anemia virus has also been reported to be transmitted by both in utero and milk routes (17, 20, 40), though these modes of infection are far less important than inoculation of the virus into the bloodstream. The use of these animal models in studies of vertical transmission of lentiviruses is hindered by long gestation periods, few offspring per pregnancy, and zoonotic risk in the case of simian immunodeficiency virus.

Cats are susceptible to infection with a number of retroviruses, including feline leukemia virus, feline syncytium-forming virus, and feline immunodeficiency virus (FIV). Among these feline viruses, vertical transmission is considered important for feline leukemia virus and feline syncytium-forming virus, both viruses being transmitted in utero (25, 32). Early studies of the pathogenesis of FIV found no evidence of vertical FIV transmission (49), and other studies examining vertical transmission of FIV (44) have found no evidence of such in chronically infected cats. However, there have been recent reports of FIV infections consistent with vertically acquired infections in neonatal and juvenile kittens (6, 46). We have also observed a case of FIV infection consistent with vertical transmission in a kitten (unpublished observations). The kitten was delivered and nursed by a mother inoculated with FIV 4 days prior to parturition. The timing of maternal infection in relation to parturition in our cat, in conjunction with the timing of infection in the cats reported by Wasmoen et al. (46), raises the possibility that transmission occurred via milk. In all these cases, however, other routes of vertical transmission could not be eliminated. We therefore addressed the hypothesis that FIV could be transmitted via milk during acute maternal infection. We addressed this route of transmission in acutely infected specific-pathogen-free cats because this represented a stage of FIV infection not addressed in earlier studies of vertical FIV transmission.

Neonates are susceptible to FIV infection after oral inoculation. We first explored the possibility that neonates are susceptible to oral infection with FIV. Eleven kittens 1 to 3 days of age were orally administered 2×10^6 50% tissue culture infective doses ($TCID_{50}$) of cell-free FIV obtained from a culture supernatant of FCD4E cells, a feline lymphocyte cell line established in our laboratory that is highly susceptible to FIV infection (14). The kittens were evaluated for FIV infection every 2 to 3 weeks after inoculation for 7 months. Of these 11 kittens, 5 developed FIV infection. Infection with FIV was confirmed by PCR amplification and Southern analysis (Fig. 1; data for two positive kittens are not shown) of a 780-bp segment of the FIV *gag* gene (14, 46) from lysates (19) of peripheral blood mononuclear cells (PBMC) and by detection of FIV-specific antibodies by a commercial enzyme-linked immunosorbent assay (ELISA) (Idexx, Portland, Maine) and by Western blotting (immunoblotting) (31) (data not shown).

There was some variability in outcome of exposure to orally administered FIV within and among three litters (Table 1); two of two kittens in one litter developed infection, while one of four and two of five kittens in the other two litters developed FIV infection. All of the kittens that developed FIV infections were provirus positive by 5 weeks postinoculation (p.i.), with the earliest positives apparent at 3 weeks p.i. Antibody responses were generally detected by 8 to 9 weeks p.i. by ELISA,

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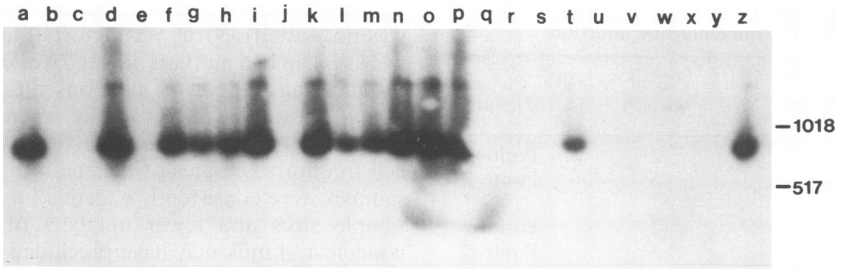

FIG. 1. Southern blot of FIV *gag* gene segments amplified from PBMC from 5- to 10-week-old kittens by PCR. One hundred microliters of whole blood was lysed, and 10 μl of the lysate was added to a PCR mixture (14, 46). Primers flank a 780-bp segment of the *gag* gene that has 95% homology with two distinct cloned isolates (34) and were generated from a published FIV sequence (39). Products were resolved on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labelled probe for autoradiography. Lanes contain products from kittens as follows: a to n, kittens that were nursed by their acutely infected mothers (mother DE3, a to e; mother LH2, f to i; mother KX3, j to n); o to w, kittens orally inoculated with FIV that were nursed by their FIV-negative mothers (mother CO4, o to s; mother LC3, t to w). Lane x is empty, lane y is the negative template control, and lane z is the product amplified from a lysate of FIV-Black-infected CRFK cells. Numbers on the right are relative sizes (in base pairs) of amplified products. Results for four kittens (two orally inoculated FIV-positive kittens nursed by negative mother VL3 and two FIV-negative kittens nursed by infected mother DC3) are not presented.

and FIV specificity and persistence were later confirmed by demonstration of antibodies to the p24^{gag} protein by Western analysis at 4 months of age. Generalized lymph node enlargement, a feature consistently observed by us and others following parenteral inoculation of FIV (49), was observed in two of the five positive kittens. None of the FIV-positive kittens developed clinical disease during the 7-month study period, though one of the positive kittens developed an abscess at the site of injection of an identification chip. Evaluation of the mothers that nursed the orally inoculated kittens by PCR and by ELISA for FIV antibody 7 months postpartum found no evidence that orally infected kittens transmitted the virus to their FIV-negative mothers, nor did any of the originally negative kittens ever develop FIV infections.

Mothers inoculated with FIV immediately postpartum transmit FIV to their offspring. The fact that FIV infections could be established in kittens orally inoculated with FIV supported the possibility that FIV could be transmitted in the postnatal period via milk. To further assess this route of transmission, four mothers were intravenously inoculated with 5×10^5 $TCID_{50}$ of a supernatant of FIV-infected FCD4E cells in the immediate postpartum period (within 24 h of delivery of the last kitten) to definitively exclude in utero or intrapartum transmission from mother to kitten. All four intravenously inoculated female cats developed FIV infections as determined by the presence of provirus in PBMC at 2 weeks p.i. and by the development of FIV-specific antibodies (data not shown).

TABLE 1. Results of oral inoculation of neonatal kittens with FIV[a]

Mother	No. of FIV-positive kittens/total[b]
CO4	2/5
LC2	1/4
VL3	2/2
Total	5/11 (45%)

[a] Eleven kittens delivered by FIV-negative mothers were orally inoculated with 2×10^6 $TCID_{50}$ of cell-free FIV-$NCSU_1$ at 24 to 72 h of age. The kittens were allowed to nurse their FIV-negative mothers and were evaluated for FIV infection every 2 to 3 weeks.

[b] Number of kittens from each litter that developed FIV infection. FIV infection was confirmed by detection of FIV *gag* gene segments by PCR and detection of FIV antibodies by ELISA and Western analysis. Negative kittens remained FIV negative through the 7-month study period.

A total of 16 kittens were delivered of the four females described above. Kittens were evaluated for FIV infection every 2 to 3 weeks beginning at birth. Of these 16 kittens, 10 developed FIV infections as evidenced by the presence of FIV provirus in PBMC (Fig. 1; data for two negative kittens are not shown) and the detection of antibodies to the FIV p24^{gag} protein by Western blotting and ELISA (data not shown). Maternal anti-FIV antibody was not the source of kitten antibody because antibodies persisted after weaning, and none of the negative kittens ever had a detectable antibody response.

One kitten, though consistently positive for FIV by PCR and Southern analysis and by ELISA, did not have a positive Western blot. To confirm the presence of infection in this kitten, Percoll-enriched PBMC (1×10^6 cells) were cocultured with FCD4E cells (2.5×10^6 cells). The supernatant from the coculture of this kitten's PBMC was positive for p24 antigen by a commercial ELISA (Idexx), confirming the presence of infection in this kitten. In no instance was a kitten that was negative by ELISA found to be positive by Western blotting.

There was some variation in mother-to-infant transmission observed among the four litters (Table 2), with some queens showing a high rate of transmission (cat LH2, four of four kittens positive for FIV; cat KX3, four of five kittens positive for FIV) and others having lower rates or no transmission (cat DE3, two of five kittens positive; cat DC3, neither of two kittens positive). None of the kittens had FIV *gag* sequences detectable by PCR at 2 weeks of age, six were positive at 4 weeks of age, and an additional four became positive for provirus by 8 weeks of age. FIV antibodies were detected by ELISA generally 4 weeks after detection of FIV *gag* sequences; specificity of FIV antibody was confirmed by detection of antibodies to p24^{gag} by Western blotting at 4 months of age (data not shown). All positive kittens still had antibody responses detectable by ELISA at 7 months of age. There was no statistically (chi-square) significant difference between the transmission rates of milk-exposed kittens and orally inoculated kittens ($P = 0.38$). As was the case for the orally inoculated kittens, not all of the positive kittens developed lymph node enlargement: eight of the positive kittens had lymph node enlargement, while the other two positive kittens did not. Like the kittens given FIV orally, none of the six remaining nursed kittens had developed evidence of FIV infection, as determined by absence of FIV provirus in their

TABLE 2. Postnatal transmission of FIV and recovery of virus from milk from mothers with acute FIV infection[a]

Mother	No. of FIV-positive kittens/no. of kittens in litter[b]	No. of wk p.i.[c]	Culture result[d]
DE3	2/5	2	Positive
		3	Positive
DC3	0/2	2	Positive
		3	Positive[e]
LH2	4/4	2	Negative
		4	Positive[f]
KX3	4/5	NA[g]	NA[g]
Total	10/16 (62%)		

[a] Four adult specific-pathogen-free female cats were inoculated with 2×10^5 $TCID_{50}$ of cell-free FIV-$NCSU_1$ intravenously within 24 h of delivery of the last kitten of a litter. Infection of the mothers was confirmed by PCR detection of FIV *gag* gene segments and by detection of FIV antibodies by ELISA and Western analysis.

[b] Number of kittens from each litter that developed FIV infection in the postnatal period. FIV infection was confirmed as for the mothers. Negative kittens remained FIV negative through the 7-month study period.

[c] Milk samples were obtained at the indicated intervals p.i. A total of six milk samples were obtained from the three mothers (two samples per mother) indicated. Milk was added to FCD4E cells and the cells were cultured for 21 days.

[d] Cultures were monitored for FIV infection by observing syncytium formation, by detecting p24 antigen in the culture supernatant, and by demonstration of FIV *gag* gene segments by PCR in digests of cultured cells.

[e] The milk sample was passed through a 0.45-μm-pore-size filter, and the filtrate was used to inoculate FCD4E cells.

[f] Positive as determined by the presence of syncytia and detection of *gag* sequences by PCR.

[g] NA, samples could not be obtained for evaluation.

PBMC and an absence of FIV antibody when the study ended at 7 months postparturition.

Mothers inoculated with FIV have infectious virus in their milk. To assess the possibility that milk was the source of infectious virus for the kittens, milk samples (50 to 100 μl) from the mothers were obtained and used to inoculate FCD4E cells. Difficulties in obtaining milk samples of a sufficient volume for culture on a consistent basis precluded a thorough evaluation of the temporal appearance of virus in the milk, and milk could not be obtained from one of the four nursing mothers. Two milk samples from each of the other three queens were evaluated for the presence of FIV. Of the six samples tested, five were positive for FIV (Table 2) as determined by the presence of syncytia and detection of FIV provirus in all five cocultured samples and by the presence of p24 antigen in the culture supernatants in four of five (the fifth was not tested). Virus was detected in the milk as early as 2 weeks p.i. in two mothers and at 4 weeks p.i. in the third. In one instance, an aliquot of milk was filtered (0.45-μm-pore-size filter) and was positive for FIV when cocultured, suggesting that, at least in this cat, infectious virus was present in a cell-free form. Lysates of pellets from all six centrifuged milk samples were consistently negative for FIV by PCR, but small sample sizes and fewer numbers of mononuclear cells in noncolostral milk may have precluded detection of FIV DNA in milk cells. In conjunction with the above-described findings, we conclude that FIV can be experimentally transmitted via milk to nursing kittens when the mother is infected in the early postpartum period.

Kittens that develop FIV infection have higher proportions of $CD4^+$ and Pan-T^+ lymphocyte subsets at birth than kittens that do not develop infection. While analyses of risk factors for vertical HIV transmission have mostly considered maternal factors such as viremia, immunologic status, clinical stage, and placental inflammation (18, 38), few have examined characteristics of the fetus or neonate that may influence individual susceptibility to infection. Evidence from adults and infants that have HIV-specific immune responses in the absence of infection (8, 36) suggests perhaps that not all individuals exposed to HIV develop infection. To investigate whether neonatal factors might be associated with susceptibility to FIV infection, we compared lymphocyte subset proportions in all kittens at birth by performing two-color flow cytometric analysis (11) of PBMC by using previously characterized monoclonal antibodies to feline lymphocyte surface antigens that define CD4, CD8, Pan-T, and immunoglobulin-bearing lymphocytes (42). Analysis was performed before any kittens were exposed to FIV.

Comparison of lymphocyte subsets revealed a difference in proportions of $CD4^+$ and Pan-T^+ cells at birth between kittens that developed FIV infection and those that did not (analysis included all positive and negative kittens, i.e., both kittens from the orally inoculated cohort and those that were nursed by infected mothers in both the positive and negative groups). In the immediate postpartum period, kittens that became infected with FIV had significantly greater proportions of these subsets than did the kittens that did not become infected (Table 3). While there was a tendency for the kittens that developed FIV infection to have a larger proportion of $CD8^+$ cells at birth, the difference was not statistically significant ($P = 0.06$). There was no difference at birth in the $CD4^+/CD8^+$ ratio or proportion of immunoglobulin-bearing cells (data not shown) between kittens that developed FIV and those that did not. Because analysis was performed when the kittens were less than 24 h old and before the mothers or the kittens were

TABLE 3. Comparison of lymphocyte subset populations in kittens that developed FIV infection and those that did not[a]

Kitten status	Mean ± SEM (range)[b]			
	% CD4 cells	% CD8 cells	% Pan-T cells	CD4/CD8 ratio
FIV negative ($n = 11$)	27.6 ± 3.3 (9.4–44.9)	7.5 ± 0.9 (3.6–12.5)	32.8 ± 5 (11–57.2)	3.83 ± 0.39 (3.71–5.52)
FIV positive ($n = 13$)	42.8 ± 4.4* (14.1–69.7)	10.9 ± 1.4 (7.8–26.7)	51.6 ± 4.2** (20.9–74.4)	4.48 ± 0.6 (0.77–8.89)

[a] Kittens were bled at birth for lymphocyte subset analysis either prior to receiving culture supernatant of FIV-infected FCD4E cells orally or prior to infection of the mothers. Data for all kittens of a given FIV status at 7 months of age were combined for preexposure analysis regardless of type of exposure. Results for three kittens (two in the FIV-positive group and one in the FIV-negative group) are not presented because the data were not available; FIV negativity was determined by the absence of the FIV *gag* segment in PCR-amplified lysates of PBMC and the absence of FIV antibodies as determined by ELISA and Western blotting; positivity was determined by PCR and antibody assays.

[b] Lymphocyte subsets were determined by double labelling PBMC with monoclonal antibodies to the various subsets (42) and analyzing the PBMC by two-color flow cytometry as described previously (11). In two cases, the difference between positive and negative kittens was statistically different by two-tailed Student's *t* test for unpaired samples. *, $P = 0.01$; **, $P = 0.008$.

inoculated with FIV, the observed differences cannot be ascribed to FIV exposure. Complete blood counts were not taken at this sampling, so it is unknown if the increased $CD4^+$ and Pan-T^+ proportions in the positive kittens were a result of greater absolute numbers of these lymphocyte subsets or smaller numbers of other subsets. Although the absolute numbers of these subsets are unknown, this finding is interesting in light of the report by English et al. (14) that described the highest level of provirus in the $CD4^+$ cells during the acute phase of infection with FIV-$NCSU_1$. The observation that kittens that developed FIV infection had greater proportions of $CD4^+$ and Pan-T^+ lymphocytes at birth than the FIV-negative kittens may indicate that differences in the immune system relate to differences in susceptibility to infection with FIV.

In this report we provide evidence for transmission of FIV from infected mothers to their offspring via milk. Three lines of evidence support lactogenic transmission of FIV-$NCSU_1$: (i) the presence of infectious virus in milk samples, (ii) a high rate of infection (62%) in kittens nursed by acutely infected mothers, and (iii) comparable rates of infection in orally inoculated kittens (45%). Published reports (6) of FIV infections in kittens have implicated milk as a route of infection, though in utero infection could not be excluded. Our results suggest that lactogenic transmission of FIV may be an important route of transmission during acute maternal infection, although the importance outside the experimental setting has yet to be determined.

The fact that all of the orally infected kittens were inoculated with cell-free virus, in addition to the finding that cell-free virus was recovered in the milk of one cat, suggests that cell-free virus in the milk is infectious to the neonate. These findings do not, however, exclude the possibility that cell-associated virus could be infectious to the neonatal kitten. Oral administration of PBMC from an FIV-positive cat to two negative adult (6 to 9 months of age) cats resulted in FIV infection in those two cats (29). The report by Moench et al. (29), in conjunction with our observation (unpublished) that one of two 15-week-old kittens orally administered cell-free FIV developed FIV infection, also raises the possibility that transmission of FIV across mucosal surfaces may not be age restricted. There are also cases of HIV in young children that are suspected to have resulted from an oral exposure to HIV beyond the infancy stage (10, 37).

Though horizontal transmission from the mothers to the kittens in this system cannot be completely excluded, the high rate of transmission observed in this study group is inconsistent with the extremely low rates of horizontal transmission observed among cats group housed with acutely infected cats in experimental settings by us and others (44, 49). Epidemiologic studies of large populations of cats in the United States and Japan indicate that casual contact such as grooming is an extremely inefficient mode of transmission (16, 48). The low rate of horizontal transmission may be attributed to small or undetectable amounts of infectious virus in the saliva of naturally and experimentally infected cats, especially during the acute stage of the infection (27, 48). Additionally, there was no evidence of horizontal transmission from orally infected kittens to their FIV-negative mothers despite prolonged and intimate contact between mothers and their nursing kittens.

The lack of vertical transmission of FIV in earlier studies (44, 49) may be related to the fact that cats with chronic infections were used. Vertical transmission of FIV appears to occur infrequently with cats with chronic infections (44, 49), perhaps because of neutralization of FIV by maternal antibodies in milk. Although neutralizing antibodies to FIV have not been described as being present in milk samples from FIV-infected cats, there are reports of high titers of serum FIV neutralizing antibodies in cats with asymptomatic infections (14). Alternatively, the observed differences in vertical transmission between our studies and those of others may be due to differences in pathogenicity among viral isolates. A study of HIV variants transmitted from mothers to their offspring demonstrated that only a limited number of maternal virus variants were passed to the children of infected mothers (47).

While summaries of epidemiologic surveys have put the overall vertical transmission rates for HIV at estimates of 14 to 40% (35), the transmission rate attributable to breast-feeding is debated. Some have reported transmission rates varying from 25 to 53% (45) in a developing country. The transmission rates may be lower among mothers with infection established antenatally than among those with infections acquired postnatally (30). Our findings support the contention that risk of transmission may be high if the maternal infection is established in the postnatal period. In this regard, the transmission rate we observed in acutely infected mothers is similar to that reported for milk transmission of HIV when mothers acquired their infections postpartum (45). In addition to maternal factors, individual characteristics of the neonate may also influence the outcome of exposure, as suggested by the differences in numbers of $CD4^+$ and Pan-T^+ cells between kittens that developed FIV and those that did not.

Though the route of viral passage across the mucous membranes is not known, several possibilities can be considered. Virus could enter the bloodstream or be exposed to susceptible lymphocytes through breaks in the mucous membranes. Second, virus may interact with dendritic cells of the squamous oral mucosa or with intestinal M cells, both of which are believed to mediate infection of T lymphocytes with HIV (3, 7). Lastly, virus could cross the gastrointestinal epithelium before physiologic gut closure occurs in the neonate. It is conceivable that these routes may be exploited by FIV and HIV during the postnatal period.

There are numerous reported similarities between HIV and FIV in viral structure, cell tropism, clinical disease, and induction of immunologic abnormalities (1, 4, 14, 31, 33, 43, 48, 49). These similarities have been extended by our finding that FIV can be transmitted via milk. Further studies are required to define the risk factors associated with transmission of FIV during the perinatal period and to determine whether transmission may occur via other routes. The FIV-cat model may prove useful in addressing some of the unanswered questions regarding vertical HIV transmission and pediatric HIV infection and in the development of strategies to prevent transmission of HIV and FIV across mucosal surfaces.

We thank Tedd Childers, Chris Parrish, Bob English, Doug Gebhard, Jim Rottman, and Paula Delong for technical assistance and Barbara Sherry and Fred Fuller for critical review of the manuscript. We also thank John Black, American BioResearch, Inc., Milton, Tenn., for the FIV-Black-infected CRFK cells.

This work was supported by NIH grant 1 RO1 AI32310.

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