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Tumorigenic Potential of a *myc*-Containing Strain of Feline Leukemia Virus In Vivo in Domestic Cats

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The oncogenic capacity of a *myc*-containing strain of feline leukemia virus (FeLV), termed LC-FeLV, has been examined after inoculation of the virus into neonatal kittens. Like other *myc*-containing strains of FeLV, LC-FeLV may induce with relatively short latency, but does not necessarily induce, thymic lymphosarcoma in viremic animals. Naturally occurring and experimentally induced tumors are T-cell lymphomas which contain clonally integrated LC-FeLV proviral DNA and which cannot readily be cultivated in vitro in the presence or absence of exogenously supplied interleukin-2. Acquisition of *myc* by FeLV decreases the period of latency before the appearance of tumors but does not expand the spectrum of tumors induced by FeLV alone.

Feline leukemia virus (FeLV) is a family of horizontally transmissible type C retroviruses identified as causative agents of a spectrum of lymphoid malignancies, termed leukemia-lymphosarcoma complex, which affects domestic cats. FeLV is a representative of the slowly transforming retroviruses, since malignant disease occurs after a prolonged viremic period (mean = 2 years) and since the virus does not contain an oncogene (5, 6). Numerous oncogene-containing isolates of FeLV have been identified, however, including several which contain the oncogene *myc* (10, 12, 13). In fact, FeLV is the only naturally occurring mammalian retrovirus known to contain *myc* as an oncogene. One of the *myc*-containing strains of FeLV, termed LC-FeLV, was originally isolated as proviral DNA from a naturally occurring thymic lymphosarcoma of a domestic cat (10). The LC-FeLV provirus, 5.5 kilobase pairs (kbp) in length, contains a spliced version of the c-*myc* gene, 1.34 kbp in length, which has replaced the *pol* gene and part of the *gag* and *env* genes of the FeLV parent from which it was derived (2). Infectious LC-FeLV particles have been rescued by replication in the presence of FeLV as helper, and an examination of the biological activity of LC-FeLV(FeLV) in vitro in early-passage feline cells has been recently reported (1). Infection of early-passage leukocytes from peripheral blood, spleen, or thymus or of fibroblasts from neonatal kittens did not immortalize these cells or alter them morphologically. In contrast, infection of embryonic fibroblasts with the virus rendered these cells morphologically altered, only loosely adherent to the substrate, and capable of apparently infinite proliferation. In view of its ability to immortalize fibroblastic cells in vitro and of its original isolation from a thymic lymphosarcoma, we wished to determine the oncogenic potential of LC-FeLV in vivo in domestic cats.

Inocula for such a study were obtained from the supernatants of cultured cells productively infected, as described previously (1), with (i) LC-FeLV in the presence of helper FeLV-B/Gardner-Arnstein [=LC-FeLV(FeLV-B)], (ii) helper FeLV-B/Gardner-Arnstein alone (=FeLV-B), or (iii) helper FeLV-A/Glasgow-1 alone (=FeLV-A), kindly provided by Jennifer Rojko. Supernatants were collected from cells 24 h after feeding and were concentrated 25-fold by centrifugation through Centricon filters (Amicon). The infectious titer of the helper virus component of each concentrate was determined by using the clone 81 feline cell assay (7). Inocula were prepared to contain 2×10^5 infectious units each of helper FeLV-A and FeLV-B (=FeLV-A+B), with or without LC-FeLV, in a 0.5-ml total volume and were introduced intraperitoneally into FeLV-free kittens at 9 to 14 days after birth (Table 1). Weekly physical examinations and biweekly hematological examinations were performed during the course of the study and included complete hemogram and assay of plasma for the presence of FeLV *gag* antigens. For the latter purpose, a commercially supplied enzyme-linked immunosorbent assay kit (Leukassay F, Pitman-Moore) was employed. Upon evidence of neoplasia or other clinical symptoms (Table 1), animals were euthanized by barbiturate overdose and submitted for complete necropsy and histopathological examination.

Twelve animals from four different litters were examined in the study. In each case, animals were inoculated at 9 to 14 days after birth with LC-FeLV(FeLV-A+B) or with helper FeLV-A+B alone or were mock-infected by inoculation with culture medium. All animals except those which were mock-infected became viremic between 2 and 4 weeks after inoculation, as documented by an enzyme-linked immunosorbent assay for FeLV *gag* antigens in peripheral blood. In the case of mock-infected animals, cat 585-1 became viremic at 10 weeks after inoculation, apparently as a result of horizontal transmission from the littermate with which it was housed. Viremia was never demonstrated in 586-5, the other mock-infected animal, during the 12 months of study (Table 1).

Of eight animals inoculated with LC-FeLV(FeLV-A+B), three developed thymic lymphosarcoma with a relatively short latency of 4 to 6 months after inoculation. Tumors were detected upon euthanasia, which was performed when tumors reached a size sufficient to cause dyspnea, anorexia, or other symptoms apparent to the attending veterinarian. It is not known at what point before symptoms tumor induction was initiated. By comparison, littermates inoculated with helper virus alone demonstrated no evidence of FeLV-induced disease at the time of necropsy, 10 to 11 months

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TABLE 1. Viremia and subsequent pathology observed in cats inoculated with LC-FeLV(FeLV)

Litter	Animal	Inoculum[a]	Age (days) at inoculation[b]	Time (wks) to viremia[c]	Histopathology	Time (mos) of necropsy[d]
1	585-1	Mock	11	10[e]	Complex thymic lesions	12
	585-2	LC-FeLV(FeLV-A+B)	11	4	Transient lymphadenopathy[f]	12
2	586-1	LC-FeLV(FeLV-A+B)	11	4	Thymic necrosis, anemia	2
	586-2	LC-FeLV(FeLV-A+B)	11	4	None	12
	586-3	LC-FeLV(FeLV-A+B)	11	3	None	12
	586-4	FeLV-A+B	11	3	Peritonitis (FIP)[g]	10
	586-5	Mock	11	—	None	12
3	J5-1	LC-FeLV(FeLV-A+B)	14	2	Thymic lymphosarcoma Hypercalcemia Mineralization	4
4	981-1	FeLV-A+B	9	3	None	11
	981-2	LC-FeLV(FeLV-A+B)	9	3	Thymic lymphosarcoma	6
	981-3	LC-FeLV(FeLV-A+B)	9	3	Thymic lymphosarcoma Hypercalcemia	5
	981-4	LC-FeLV(FeLVA+B)	9	3	None	11

[a] Indicated are the particular inocula introduced into each animal: LC-FeLV(FeLV-A+B), LC-FeLV(FeLV) complex with helper FeLV of subtypes A and B; FeLV-A+B, helper FeLV of subtypes A and B; mock, inoculum prepared from the supernatant of uninfected cells.

[b] Pregnant queens were obtained commercially from a closed colony free of FeLV and other feline infectious diseases. Animals received no vaccinations by the supplier or subsequently and were maintained before and after FeLV inoculation in strict isolation. Littermates were housed with the dam until weaned (7 to 8 weeks) and for 3 to 4 months thereafter until the constraints of size required separation. Indicated is the number of days after birth at which the animal was inoculated intraperitoneally.

[c] Number of weeks after inoculation at which viremia was first detected by enzyme-linked immunosorbent assay. —, No viremia detected during the course of the study.

[d] Animals were treated symptomatically at the discretion of the veterinarian but were euthanized if symptoms were nonresponsive or progressive, if evidence of neoplasia was obtained, or for other reasons described in the text. Indicated is the number of months after inoculation at which necropsy was performed.

[e] Horizontally infected by exposure to littermate, 585-2.

[f] Generalized lymphadenopathy was observed by physical examination for 1 month between weeks 4 and 8 after infection.

[g] FIP, Feline infectious peritonitis caused by a coronavirus.

after inoculation (Table 1). In two of the animals, lymphosarcoma was associated with serum hypercalcemia and consequent mineralization of a variety of tissues. In no case did hematological examination demonstrate abnormal cells; in fact, biweekly hemograms of experimental and control animals were unremarkable. The frequency and kinetics of tumor induction observed in this study are comparable to those reported from a related study in which kittens were inoculated within 48 h of birth with either of two *myc*-containing strains of FeLV different from the strain examined here. In that study, five of seven animals inoculated with one strain and three of three animals inoculated with the other strain developed thymic lymphosarcoma within 3 to 5 months after inoculation (14). The higher frequency of tumor induction observed, as compared with the present study, may be a function of the virus strains used or may result from inoculation at 0 to 2 days after birth.

DNA preparations from the tumors of animals J5-1, 981-2, and 981-3 were examined by Southern blot analysis for the presence of integrated proviruses of LC-FeLV (Fig. 1A). Also included for comparison in this analysis was DNA from thymic lymphosarcoma 1110, the tumor from which LC-FeLV was originally derived (10). Digestion of tumor DNA with *Kpn*I yields a single 5.5-kbp fragment from LC-FeLV proviral DNA, since recognition sites for *Kpn*I occur only in the long terminal repeats (LTRs) of that provirus (10). In fact, such a fragment is evident after hybridization of a *myc*-containing probe with *Kpn*I-digested DNAs of tumors 1110, 981-2, and 981-3. In tumor J5-1, a slightly smaller strain of LC-FeLV predominates in which the *Kpn*I fragment measures 5.2 kbp (Fig. 1A, lane c). A smaller strain which contains a *Kpn*I fragment of 4.8 kbp is observed in the DNA of tumor 981-2. On the basis of intensity of hybridization, the

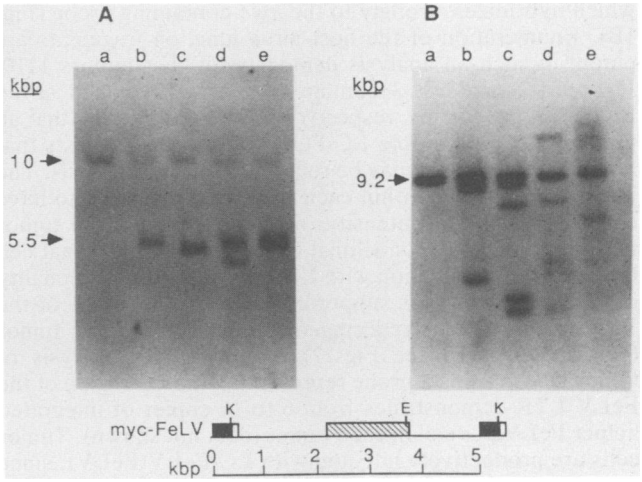

FIG. 1. Southern blot analyses of DNA from the kidney of a cat not infected with FeLV (lanes a) and from the following thymic lymphosarcomas of cats: 1110 (lanes b), J5-1 (lanes c), 981-2 (lanes d), and 981-3 (lanes e). DNA samples (10 μg) were digested with *Kpn*I (A) or *Eco*RI (B), electrophoresed in an agarose gel, and transferred to nitrocellulose. Blots were hybridized as previously described (1) to a radiolabeled probe representing avian v-*myc* (17). Molecular sizes were determined by comparison with the migration of *Hin*dIII-digested lambda DNA and *Hae*III-digested ϕX174 DNA electrophoresed in parallel. Indicated are fragments representing the feline c-*myc* gene (10 kbp in panel A, 9.2 kbp in panel B) and LC-FeLV proviral DNA (5.5 kbp). Below is a partial restriction enzyme site map of LC-FeLV proviral DNA (10) demonstrating positions of the recognition sites for *Kpn*I (K) and *Eco*RI (E). Symbols: ■ and ▭, U3 and U5 regions, respectively, of the LTRs; ▨, *myc* substitution.

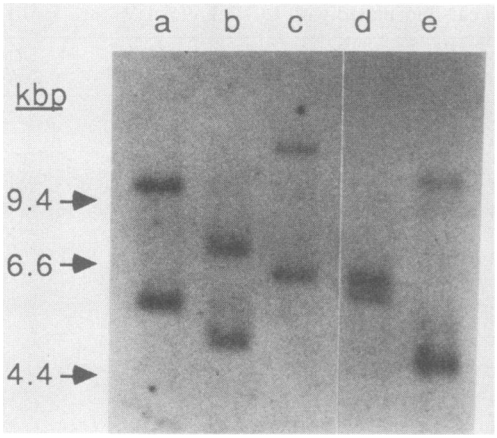

FIG. 2. Southern blot analysis of DNA from feline kidney (lane a) and from thymic lymphosarcomas of the following cats: 1110 (lane b), J5-1 (lane c), 981-2 (lane d), and 981-3 (lane e). DNA samples (10 μg) were digested with *Sac*I, electrophoresed in an agarose gel, and transferred to nitrocellulose. Blots were hybridized as previously described (1) to a radiolabeled probe representing the gene encoding the β chain of the murine T-cell receptor. Molecular sizes were determined by comparison with the migration of *Hin*dIII-digested lambda DNA and *Hae*III-digested φX174 DNA electrophoresed in parallel.

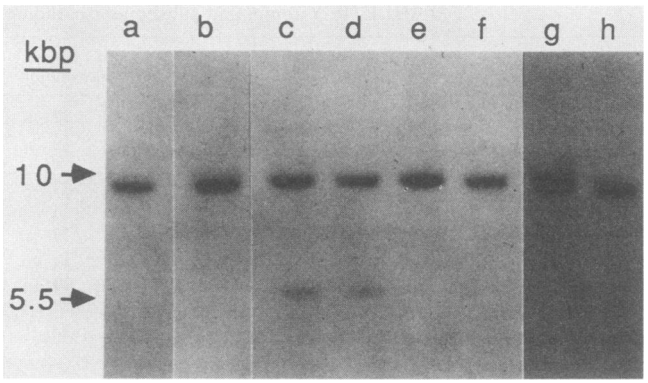

FIG. 3. Southern blot analysis of DNA from peripheral blood leukocytes of cats 585-1 (lane a), 585-2 (lane b), 586-2 (lane c), 586-3 (lane d), 586-4 (lane e), 586-5 (lane f), 981-1 (lane g), and 981-4 (lane h). DNA samples (10 μg) were digested with *Kpn*I, electrophoresed in an agarose gel, and transferred to nitrocellulose. Blots were hybridized as previously described (1) to a radiolabeled probe representing avian v-*myc* (17). Molecular sizes were determined by comparison with the migration of *Hin*dIII-digested lambda DNA and *Hae*III-digested φX174 DNA electrophoresed in parallel. Indicated are fragments representing the feline c-*myc* gene (10 kbp) and LC-FeLV proviral DNA (5.5 kbp).

4.8-kbp strain occurs in tumor 981-2 with a frequency equal to that of the originally introduced species (Fig. 1A, lane d). LC-FeLV contains a single recognition site for *Eco*RI located 57 bp from the 3′ end of the feline v-*myc* sequences (2); thus, digestion of tumor DNAs with *Eco*RI yields from each LC-FeLV provirus a single host-virus junction fragment which hybridizes strongly to the *myc*-containing probe (Fig. 1B). Enumeration of the host-virus junction fragments apparent in such an analysis demonstrates that tumors 1110, J5-1, 981-2, and 981-3 contain two, four, five, and seven LC-FeLV proviruses, respectively. The observation that all tumors contain multiple LC-FeLV proviruses suggests that multiple integrations may be required for tumorigenesis. The junction fragments within each tumor are observed to have equal hybridization intensity, an indication that the tumor mass represents a monoclonal expansion of an original cell transformed by infection with LC-FeLV. The likely clonality of each tumor mass is supported further by analyses of the T-cell receptor gene rearrangements present in each tumor (described below; see Fig. 2). Southern blot analysis of tumor DNAs using a probe representing the U3 region of the FeLV LTR demonstrates from 6 to 16 copies of integrated helper FeLV per cell in each tumor (data not shown). Tumor cells are productively infected with LC-FeLV(FeLV), since infection in vitro of feline fibroblasts with a homogenate of tumor J5-1 resulted in expression in the fibroblasts of both LC-FeLV and FeLV, as measured by Northern (RNA) blot analysis (data not shown).

DNA samples from each tumor were also examined by Southern blot analysis using a probe representing the gene which encodes the β chain of the murine T-cell receptor (kindly provided by Edward Palmer). Southern blot analysis of *Sac*I-digested DNA demonstrates this gene to be rearranged in tumors 1110, J5-1, 981-2, and 981-3 as compared with its germ line organization in nonlymphoid feline tissue (Fig. 2). These results indicate that the tumor mass in each case is composed of T cells. At the time of removal from the animal, a single-cell suspension of each thymic tumor was placed into culture under conditions demonstrated previously to support the growth of primary- and early-passage feline T cells (1). Specifically, cells were deposited at a density of 10^6/ml in RPMI 1640–10% fetal calf serum–2 mM glutamine–5×10^{-5} M 2-mercaptoethanol–0.05 mg of gentamicin per ml. Concanavalin A at 10 μg/ml was included during the first 3 days in culture. Duplicate cultures were prepared from each tumor mass; to one culture was added human recombinant interleukin-2 (IL-2) (100 units/ml; Cetus Corporation). In neither the presence nor the absence of IL-2 did any of the cultures survive beyond 3 months. In contrast, cells isolated from thymic tumors induced by two different *myc*-containing strains of FeLV could be readily established in culture even in the absence of exogenous IL-2 (14). The properties which distinguish LC-FeLV in this respect from other *myc*-containing isolates remain a subject for investigation. It is noteworthy that feline thymic lymphosarcomas in general are not readily established in culture in the presence or absence of exogenously added growth factor (14).

Of the five animals inoculated with LC-FeLV(FeLV) which did not develop thymic lymphosarcomas (Table 1), cat 585-2 demonstrated a transient lymphadenopathy for 1 month beginning at 1 month after inoculation. The animal was healthy at the time of necropsy, 12 months after inoculation. Littermate 585-1, horizontally infected by exposure to 585-2, exhibited complex, possibly premalignant lesions in the thymus at the time of necropsy. A third animal, 586-1, died at 2 months after inoculation. Histopathological examination demonstrated both thymic necrosis and anemia. Southern blot analysis of DNA from peripheral blood leukocytes of these animals failed to demonstrate the presence of LC-FeLV (Fig. 3), although the genome of helper FeLV was apparent (data not shown). Thus, it cannot be determined with certainty whether the pathology observed resulted from the action of LC-FeLV or of the helper virus. It is noteworthy, however, that control animals inoculated with helper FeLV alone demonstrated no lymphadenopathy or abnormality of the thymus when examined as late as 11 months after inoculation (Table 1). The remaining animals inoculated

with LC-FeLV(FeLV), 586-2, 586-3, and 981-4, demonstrated no signs of disease at the time of necropsy (Table 1). Southern blot analysis of DNA prepared from peripheral blood leukocytes of these animals readily demonstrated integrated LC-FeLV proviral DNA in cats 586-2 and 586-3 (Fig. 3). An identical Southern blot analysis using a probe representing the U3 region of the FeLV LTR demonstrated integrated helper FeLV in the peripheral blood leukocytes of all animals except 586-5, a cat which was mock-infected (data not shown). The presence of LC-FeLV in peripheral blood leukocytes of cats 586-2 and 586-3 represents polyclonal infection rather than monoclonal expansion of infected cells, since Southern blot analysis with a v-*myc* probe after digestion of the DNAs with *Eco*RI did not reveal discrete host-virus junction fragments (data not shown). These results indicate that cats 586-2 and 586-3 were productively infected with LC-FeLV(FeLV) but developed no disease as a result. An inference from these observations is that LC-FeLV is relatively inefficient in inducing thymic lymphosarcoma or another thymic abnormality in infected kittens.

Two conclusions may be drawn from these studies. First, LC-FeLV may induce, but does not necessarily induce, malignant disease in infected kittens. Its low pathogenicity suggests that LC-FeLV may indeed not represent a proximal leukemogen. Second, the tumorigenic capacity of LC-FeLV is restricted to induction of T-cell lymphosarcoma of the thymus, the same tumor from which it was originally isolated. This is true as well of two independently isolated *myc*-containing strains different from LC-FeLV (14). The acquisition of v-*myc* does not expand the spectrum of tumors induced by FeLV alone, since FeLV infection is associated with induction of thymic lymphosarcoma after long latency (mean = 2 years [6]). In contrast, the acquisition of v-*myc* by avian leukosis virus greatly increases the tumorigenic spectrum of that virus. Evidence indicates that the broad transforming potential of the *myc*-containing avian retroviruses, including MC29 and its relatives, is determined at least in part by the avian v-*myc* gene (4, 8, 11). The failure of feline v-*myc* to expand the tumorigenic spectrum of FeLV beyond the induction of lymphoid tumors may reflect the close sequence relatedness between feline v-*myc* and c-*myc* genes. Feline v-*myc* as represented in LC-FeLV is identical to feline c-*myc* over that portion of the coding sequence which they share (2, 16). Neither of the feline v-*myc* genes examined carries a mutation equivalent to the codon 61 mutation exhibited by all avian v-*myc* genes examined (2, 15, 16). Alternatively, FeLV-encoded determinants may define the tissue tropism and thereby limit the oncogenic spectrum of LC-FeLV. In murine leukemia viruses, by comparison, enhancer sequences of the LTR are major determinants of thymotropism (3, 9). Regardless of the mechanism by which the oncogenic spectrum is restricted, it is intriguing that the rate of tumor induction is increased by the action of feline v-*myc* in all three *myc*-containing isolates examined (14; this report). These observations suggest the possibility that the relatively slow and inefficient induction of malignant disease by FeLV reflects the requirement for a second event involving activation of *myc* or of a functional relative.

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