

Infection and Immunization of Cats with the Kawakami-Theilen Strain of Feline Leukemia Virus¹ (40379)

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Feline leukemia is caused by an oncovirus, feline leukemia virus (FeLV), that is transmitted horizontally in nature and under laboratory conditions (1). The Kawakami-Theilen strain (2) of FeLV (KT-FeLV), commonly used in experimental investigations, was isolated from a case of spontaneous lymphosarcoma in a Persian cat and was shown to induce leukemia in newborn kittens (3). A lymphoid cell line, designated FL74, was established employing a solitary renal tumor of a cat that received KT-FeLV in the second cell-free virus passage (4). Recent studies of KT-FeLV virus, purified from the FL74 cell line, revealed that it was infectious and produced disease in cats inoculated when newborn (5, 6) but not at 5 months of age or older (7).

Studies in these laboratories were directed toward development of a cat model for assay of feline leukemia virus vaccine that might have relevance to the evaluation of possible vaccines against leukemia in man. The present report describes the findings in studies to measure the effects of viral dose, mode of transmission, and host age on the responses of cats to KT-FeLV virus and to evaluate a killed virus vaccine in cats.

Materials and methods. Animals. Outbred cats from the specific pathogen-free (SPF) colony established in our laboratories were used. The cats were housed in biocontainment cages. Hartley strain female guinea pigs were obtained from Buckshire Corporation, Perkasio, Pennsylvania.

Tumor Cells. FL74 feline lymphoid tumor cells (4) were propagated in Leibovitz medium employing 10% fetal calf serum and

neomycin. The cells were collected 6 days following transfer.

KT-FeLV virus. Fluid was harvested from seven-day-old FL74 cell cultures that were shedding KT-FeLV. Concentration and purification of virus were by centrifugation in a Beckman L3-40 ultracentrifuge at 28,000 rpm employing a 20-60% sucrose gradient in a CF32 rotor equipped with a 60,000g core at a flow rate of 2 liters/hr.

KT-FeLV vaccine. Purified virus containing 7.4×10^{11} virions (0.6 mg protein) per ml was treated with 1:4000 formalin for 21 days at 4° after which excess formalin was inactivated with sodium bisulfite. The vaccine was shown to be free of live virus when tested in 1.4 ml vol by the same assay as used to test for FeLV viremia (see below). The vaccine induced FeLV neutralizing antibody (50.8 geometric mean titer) in 5/6 guinea pigs that were given two doses 4 weeks apart, the first dose of which was in Freund's complete adjuvant.

Assay for FeLV viremia. Lymphocytes were concentrated from cat plasma by the Hypaque-Ficol gradient method (8). Two-tenths ml volumes of lymphocytes were inoculated into duplicate cultures of feline embryo fibroblast (FEF) seeded at 5×10^5 cells per 60 × 15 mm Falcon plate. The cultures were incubated at 36° in 5% CO₂ in air, refed at 3 days, and split 1:2 on days 7 and 14. A portion of the supernate collected on day 21 was tested for RNA-dependent DNA polymerase activity by a modification of the procedure of Verwoerd and Sarma (9). Cultures were considered positive for polymerase activity if the counts per minute (cpm) of incorporated tritiated thymidine triphosphate were at least twice those of uninoculated control cultures.

Assay for FL74 cytotoxic antibody. Cytotoxic antibody was assayed by the ⁵¹Cr release

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microassay described by Johnson *et al.* (10) using 5×10^5 ^{51}Cr -labeled FL74 cells, 0.025 ml of heat inactivated serum, and 0.05 ml of 1:5 rabbit complement per well. The ^{51}Cr release assay detects antibodies to feline oncovirus cell membrane antigen (11) and FeLV envelope glycoprotein (12) on the surface of the FL74 target cell. Titers were expressed as the greatest initial dilution of serum that gave twice the cpm of the complement control.

Assay for FeLV neutralizing antibody. Neutralizing antibody was assayed in a microtiter assay employing the FeLV-1 strain of FeLV virus (13) that contained subgroups A and B of FeLV virus. In the tests, 0.1 ml amounts of twofold serial dilutions of heat-inactivated serum were incubated with 100 TCID₅₀ of virus in 0.025 ml volume for one hour at room temperature in microtiter plates after which 10^4 FEF cells were added. The cultures were incubated and fed as in the virus assays described above and were tested for viral polymerase activity on day 7 or 8 of incubation. The titer was the highest initial dilution of serum that inhibited 100 TCID₅₀ of FeLV virus.

Results. Susceptibility and immunologic response to infection in 1- to 2-day-old kittens. One- to 2-day-old kittens were inoculated intraperitoneally (ip) with purified KT-FeLV virus in serial tenfold dilutions in 1 ml vol. The undiluted virus contained 10^{12} virions

per ml and $10^{5.3}$ TCID₅₀ per 0.2 ml. A portion of the kittens in 2 or more litters was employed in testing each virus dilution and the remaining kittens, together with the queens, served as cage-contact controls. Table I shows that a portion of cats that received 10^9 or more virions ($1 \times 10^{3.0}$ or more TCID₅₀) became moribund. None of the contact controls became ill or died. FeLV virus was isolated from the peripheral lymphocytes of all cats that became ill but not from inoculated animals that remained healthy. The thirteen cats that became ill presented evidence of disease with findings that included one or more of the following: Erythroid hypoplasia, thymus atrophy, lymphoid depletion, leukopenia and varying degrees of medullary osteosclerosis and lymphoblastic or lymphocytic leukemia. All cats were anemic with red blood cell counts ranging from 0.5 to $2.4 \times 10^6/\text{mm}^3$. Five of 13 cats had posterior paresis; 6/13 had dry bone marrow; 7/8 bone marrow specimens examined histologically showed lymphoblastic or lymphocytic leukemia or lymphosarcoma; and 3/3 femurs showed medullary osteosclerosis on histologic examination.

Table II presents the findings for development of cytotoxic antibody against FL74 cells among the cats that were described in Table I. Kittens that developed viremia following injection of virus failed to develop cytotoxic antibody. By contrast, 10/16 non-viremic in-

TABLE I. MORBIDITY AND VIREMIA IN CATS DURING NINE TO TEN MONTHS FOLLOWING INOCULATION WITH KT-FeLV VIRUS AT ONE TO TWO DAYS OF AGE.

KT-FeLV Virus	Morbidity		Virus isolation from cats			
	Kind of exposure	Dose (No. of virions)	No. moribund/Total no.	Mean survival days (Range)	Animals that became moribund ^a No. viremic/No. tested	Healthy animals ^b No. viremic/No. tested
Intraperitoneal injection of virus		10^{12}	4/5	128 (95-179)	4/4	0/1
		10^{11}	3/10	83 (78-92)	3/3	0/7
		10^{10}	5/8	201 (134-292)	5/5	0/3
		10^9	1/3	268	1/1	0/2
		10^8	0/5			0/5
		10^7	0/6			0/6
		None	0/5			0/5
Cagemates ^c of inoculated cats	—	0/45				0/41

^a The number of virus isolations from each cat varied from two to four beginning 1-2 months after inoculation.

^b The number of virus isolation attempts for each cat ranged from two to eight with an average of four to five beginning 1-2 months after inoculation.

^c The 45 exposed cats comprised 29 kittens and 16 nursing queens.

oculated cats and 6/15 non-viremic cats (3/10 kittens, 3/5 queens) in contact with viremic cats developed cytotoxic antibody. The contact cats developed cytotoxic titers of 1:4 to 1:128 4 to 7 months after initial exposure.

Susceptibility and immunologic response to infection in 17- to 24-week-old cats. As shown in Table III, 17- to 24-week-old cats were injected ip with concentrated KT-FeLV virus or subcutaneously (sc) with FL74 shedder cells. The infectivity titers of the virus used in the 17- and 22-week-old cats were $10^{5.0}$ and $10^{6.0}$ TCID₅₀/0.2 ml, respectively. In contrast to one- to two-day-old kittens, none of the

17- to 24-week-old cats became ill or developed viremia. However, nearly all of the inoculated cats developed cytotoxic antibody. None of the contact animals became ill and none developed viremia or cytotoxic antibody, and all isolated controls remained devoid of virus and antibody. These findings indicated lack of susceptibility to KT-FeLV virus in cats that had achieved 17 weeks of age.

Protection of kittens against FeLV by immunization of the queens. It was evident that cats developed spontaneous resistance to KT-FeLV virus at too early an age to permit primary vaccination with FeLV vaccine prior to virus challenge, and passive immunization was therefore attempted. In the study two SPF cats were given six doses of formalinized FeLV virus vaccine by the intramuscular route and three similar cats were given saline placebo. The first vaccine dose consisted of 1 ml of formalinized aqueous vaccine (7.4×10^{11} virions, 0.6 mg protein) emulsified with an equal volume of Freund's complete adjuvant. This was followed by five doses of the aqueous vaccine diluted 1:5 and given at monthly intervals. The queens were mated after the fourth vaccine dose. One of the vaccinated queens developed a neutralizing antibody titer of 1:4 and a cytotoxic antibody

TABLE II. THE RELATIONSHIP BETWEEN VIREMIA AND CYTOTOXIC ANTIBODY IN CATS EXPOSED TO KT-FeLV VIRUS BY INOCULATION OR BY CONTACT WITH INFECTED CAGEMATES (Table I).

Kind of exposure to KT-FeLV virus	Development of viremia	Cytotoxic antibody No. positive/No. tested
Intraperitoneal injection (10^9 to 10^{12} virions)	positive	0/10
	negative	10/16
Contact exposure (cagemate) with viremic cats with non-viremic cats (controls)	negative	6/15
	negative	0/25

TABLE III. VIREMIA AND CYTOTOXIC ANTIBODY IN CATS GIVEN KT-FeLV VIRUS OR FL74 CELLS AT 17-24 WEEKS OF AGE.

Age when inoculated (weeks)	Cat treatment			Virus recovery attempts ^a	Cytotoxic antibody ^b	
	Inoculum (virus or cells)	Dose of virions or cells/ml	Route of exposure	No. cats positive/No. tested	No. positive/No. tested	Titer range ^c
17	KT-FeLV virus (2 ml)	2.4×10^{12}	ip inoculation	0/4	4/4	
			Contact exposure (cagemate)	0/2	0/2	
22	KT-FeLV virus (1 ml)	4×10^{12} 4×10^{11} 4×10^{10}	ip inoculation	0/4	2/4	16-32
				0/4	3/4	8-32
				0/4	3/4	32-128
			Contact exposure (cagemate)	0/6	0/6	<4
24	FL74 cells (3 ml)	1.8×10^9	sc inoculation	0/4	4/4	32-128
			Contact exposure (cagemate)	0/4	0/4	<4
Isolated controls (17-24 weeks)	None	None	None	0/7	0/7	<4

^a Cats inoculated at 17 weeks of age were assayed for virus 0, 6, 11, 12, 14, 25, 41, and 53 weeks after exposure; cats inoculated at 22-24 weeks of age were tested 2, 4, 8, 11, 26, and 45 weeks after exposure.

^b Cats inoculated at 17 weeks of age were assayed for antibody 1, 4, 8, 11, 12, and 14 weeks after inoculation; cats inoculated at 22-24 weeks of age were tested 4, 6, 12, 26, 36, and 45 weeks after inoculation.

^c Titers were determined 4-6 weeks after inoculation.

titer of 1:32. The other failed to develop detectable neutralizing antibody but did develop cytotoxic antibody of low titer (1:2). Five kittens born to the vaccinated and eight kittens born to the placebo control queens were challenged ip with 1 ml of live KT-FeLV virus (9.8×10^{11} virion, $10^{5.3}$ TCID₅₀/0.2 ml) when newborn and the animals were observed for development of leukemia. Table IV shows that none of the passively immunized and four of eight of the control kittens became moribund with leukemia. Sacrifice at 10 months after inoculation revealed that one of five passively immunized and three of the four remaining control animals presented pathologic evidence of leukemia. None of these cats was viremic or anemic at the time of necropsy. The one passively immunized kitten that developed leukemia was from a litter of two kittens derived from the particular queen that failed to produce detectable neutralizing antibodies. The incidence of leukemia was 88% in the controls and was 20% in the kittens born of vaccinated queens. The numbers of animals were small, but the difference was significant statistically ($P < .10$).

Discussion. The findings in the present study show the striking age susceptibility of SPF cats given KT-FeLV virus. Half of the kittens inoculated with 10^9 to 10^{12} virions or $10^{3.0}$ to $10^{6.0}$ TCID₅₀ of KT-FeLV virus during the first or second day after birth became moribund. By contrast, none of 20 animals given KT-FeLV virus when 17, 22 or 24

weeks of age became ill or developed viremia. These findings are generally consistent with the reports that demonstrated KT-FeLV susceptibility in newborn cats (3, 5, 14) and KT-FeLV resistance in young adult cats (7, 14).

It was of considerable interest that none of the 1- to 2-day-old kittens that were persistently viremic and that became ill developed cytotoxic antibody. By contrast, more than half of the kittens that were nonviremic and remained healthy developed cytotoxic antibody suggesting that the cytotoxic antibody might have been the means for eliminating virus-infected cells and for protective immune response in the host. Importantly, most of the cats that were given the virus at 17–24 weeks of age also developed cytotoxic antibody suggesting that such antibody might be an important determinant in age-susceptibility to this infection in cats. Our findings are consistent with those of other workers who found that persistently viremic cats were usually less likely to have high antibody titers against FL74 cell membrane antigens (1, 14–16). By contrast, cats with high titers of cell membrane antibody were usually free of persistent viremia and disease (1, 14–16), although healthy viremic cats can develop high cell membrane antibody titers (16, 17).

Contact exposure of kittens to viremic cagemates that had been inoculated at one to two days of age did not result in illness or viremia. However, 3/10 such animals did develop cytotoxic antibody. It was evident, therefore, that some form of contact trans-

TABLE IV. PROTECTION OF KITTENS BORN FROM QUEENS VACCINATED AGAINST LEUKEMIA WITH FORMALINIZED FeLV VIRUS VACCINE.

Immunization of queens ^a	Moribund kittens ^b with leukemia			Surviving kittens with lymphoid leukemia when sacrificed at 10 months ^c	All kittens
	No. moribund/Total	Survival time (days)			No. with leukemia/Total
		Average	Range		
FeLV virus vaccine	0/5	—	—	1 ^d /5	1/5 (20%) ^e
Placebo control	4/8 ^f	99	68–163	3/4	7/8 (88%)

^a Cats were first vaccinated with formalinized KT-FeLV (7.4×10^{11} virions) in Freund's complete adjuvant. Five additional vaccinations consisting of aqueous vaccine diluted 1:5 were given at 4-week intervals. The cats were mated after the fourth vaccine dose was given.

^b Kittens were born 8–20 days after last dose of vaccine was given and were challenged (ip) at 1 day of age with 9.8×10^{11} KT-FeLV ($10^{5.3}$ TCID₅₀/0.2ml).

^c None of these cats was viremic or anemic at time of necropsy.

^d Also, had minimal focal myelofibrosis.

^e Level of significance $P < .10$.

^f Three of four cats had severe anemia, myelofibrosis and virus. All 4 had lymphoid leukemia.

mission of virus had taken place, but the kittens, by the time of effective exposure, were probably at least one month old and responded in the way of 17- to 24-week-old FeLV-inoculated animals. These findings agree with reports that cats in contact with FeLV-infected cats generally develop cell membrane antibody two to five months after exposure (15, 17, 18). The fact that 3/5 queens developed cytotoxic antibody by contact exposure to their infected kittens suggested that adult cats are susceptible to repeated exposure as reported by Essex *et al.* (19). Our observed 40% (6/15) incidence of KT-FeLV contact transmission as evidenced by development of cytotoxic antibody reflects the natural occurrence of antibodies to feline leukemia virus associated cell membrane antigens among cats in certain high risk urban environments (20). The frequency of horizontal transmission and the development of persistent viremia vary considerably in different studies using different strains of FeLV and different populations of cats and test conditions (15, 19, 21). Rates for persistent viremia following contact transmission range from 0% in the present study with KT-FeLV virus to 100% with FeLV-5 virus (21) and with a reported 20% with Rickard-FeLV virus (15).

The extreme early age dependence for susceptibility to the KT strain of FeLV precluded the development of a means for measuring protective immunization by vaccination of kittens that could be challenged at a later time and followed for development of illness. Protective efficacy of KT-FeLV vaccine was measured instead in passive protection tests in which the queens were immunized during pregnancy and the progeny were challenged with live virus. Protection in offspring was afforded by the KT-FeLV vaccine containing subgroups A, B, and C viral antigens of unknown quantities (22) against challenge with the homologous KT-FeLV virus strain. It was of special interest that protection was afforded to one of two of the progeny of one queen even though she had not developed detectable neutralizing antibody and showed only low level cytotoxic antibody. These findings are in disagreement with those of Hoover *et al.* (23) who reported that immunization of pregnant queens with 3-5 weekly injections of UV-inactivated sucrose

gradient-purified KT-FeLV failed both to induce antibody and to protect the progeny against leukemia induced by a heterologous strain of FeLV (Rickard). Our findings are in accord, however, with those of other studies (24, 25) in which protection of kittens was achieved by maternal vaccination with other strains of FeLV or feline sarcoma viruses. The differences in protective efficacy in these studies may be due to differences in homology of vaccine and challenge virus, to different vaccination regimens, and to differences in the age of kittens at challenge.

The present studies failed in their attempt to evolve a meaningful animal model that could be used to measure protection against virus challenge by previous immunization of the same individual. Such an assay would have important implications in the evaluation of vaccines against the still elusive and hypothetical leukemia virus of man. The extreme early age-susceptibility precluded direct oncogenicity tests of kittens and demonstration of protective efficacy. It was shown, however, that passive protection could be afforded by immunization of the pregnant queen. Presence of neutralizing and cytotoxic antibody in the queen appeared important in protecting the progeny against leukemia on challenge.

Summary. Injection of high doses of KT-FeLV feline leukemia virus produced both viremia and leukemia in a portion of 1- to 2-day-old kittens but not in 17- to 24-week-old animals. Contact of susceptible cats with infected cagemates did not result in viremia or disease. Development of cytotoxic antibody was coordinated with protection against viremia and disease in kittens challenged with FeLV virus. The rapid evolution of resistance to KT-FeLV virus precluded the development of a practical means for conventional vaccination and challenge experiments in cats. Killed vaccine given to pregnant queens did, however, afford protection against FeLV virus on challenge in the progeny.

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1. Essex, M., in "Advances in Cancer Research" (G. Klein and S. Weinhouse, eds.), Vol. 21, p. 175,

- Academic Press, New York (1975).
2. Kawakami, T. G., Theilen, G. H., Dungworth, D. L., Munn, R. J., and Beall, S., *Science* **158**, 1049 (1967).
 3. Theilen, G. H., Dungworth, D. L., Kawakami, T. G., Munn, R. J., Ward, J. M., and Harrold, J. B., *Cancer Res.* **30**, 401 (1970).
 4. Theilen, G. H., Kawakami, T. G., Rush, J. D., and Munn, R. J., *Nature (London)* **222**, 589 (1969).
 5. Hoover, E. A., Kociba, G. J., Hardy, W. D., Jr., and Yohn, D. S., *J. Nat. Cancer Inst.* **53**, 1271 (1974).
 6. Hoover, E. A., and Kociba, G. J., *J. Nat. Cancer Inst.* **53**, 1277 (1974).
 7. Jarrett, W., Jarrett, O., Mackey, L., Laird, H., Hood, C., and Hay, D., *Int. J. Cancer* **16**, 134 (1975).
 8. Boyum, A., *Scand. J. Clin. Lab. Invest.* **21**, 77 (1968).
 9. Verwoerd, D. W., and Sarma, P. S., *Int. J. Cancer* **12**, 551 (1973).
 10. Johnson, T. R., Massey, R. J., and Deinhardt, F., *Immuno. Commun.* **1**, 247 (1972).
 11. Grant, C. K., DeBoer, D. J., Essex, M., Worley, M. B., and Higgins, J., *J. Immunol.* **119**, 401 (1977).
 12. Salerno, R. A., Lehman, E. D., Larson, V. M., and Hilleman, M. R., *J. Natl. Cancer Inst.*, **61**, 1487 (1978).
 13. Jarrett, O., Laird, H. M., Hay, D., *Nature (London)* **238**, 220 (1972).
 14. Hoover, E. A., Olsen, R. G., Hardy, W. D., Jr., Schaller, J. P. and Mathes, L. E., *J. Nat. Cancer Inst.* **57**, 365 (1976).
 15. Hoover, E. A., Olsen, R. A., Hardy, W. D., Jr., and Schaller, J. P., *J. Nat. Cancer Inst.* **58**, 443 (1977).
 16. Pedersen, N. C., Theilen, G., Keane, M. A., Fairbanks, L., Mason, T., Orser, B., Chen, C. H., and Allison, C., *Amer. J. Vet. Res.* **38**, 1523 (1977).
 17. Stephenson, J. R., Essex, M., Hino, S., Hardy, W. D., Jr., and Aaronson, S. A., *Proc. Nat. Acad. Sci. U.S.A.* **74**, 1219 (1977).
 18. Mathes, L. E., Yohn, D. S., Hoover, E. A., Essex, M., Schaller, J. P., and Olsen, R. G., *J. Nat. Cancer Inst.* **56**, 1197 (1976).
 19. Essex, M., Cotter, S. M., Sliski, A. H., Hardy, W. D., Jr., Stephenson, J. R., Aaronson, S. A., and Jarrett, O., *Int. J. Cancer* **19**, 90 (1977).
 20. Rogerson, P., Jarrett, W., and Mackey, L., *Int. J. Cancer* **15**, 781 (1975).
 21. Jarrett, W., Jarrett, O., Mackey, L., Laird, H., Hardy, W., Jr., and Essex, M., *J. Nat. Cancer Inst.* **51**, 833 (1973).
 22. Sarma, P. S., and Log, T., *Virology* **54**, 160 (1973).
 23. Hoover, E. A., Schaller, J. P., Mathes, L. E., and Olsen, R. G., *Infect. Immun.* **16**, 54 (1977).
 24. Yohn, D. S., Olsen, R. G., Schaller, J. P., Hoover, E. A., Mathes, L. E., Heding, L., and Davis, G. W., *Cancer Res.* **36**, 646 (1976).
 25. Schaller, J. P., Hoover, E. A., and Olsen, R. G., *J. Nat. Cancer Inst.* **59**, 1441 (1977).

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