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In Vitro Activation of Feline Complement by Feline Leukemia Virus

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Incubation of normal feline serum with purified feline leukemia virus (FeLV) at 37°C for 30 min resulted in the activation of the complement system via the classical pathway as demonstrated by consumption of the C1, C4, C2, C3, and, to a lesser extent, the later C components. A similar finding was observed when normal human serum was substituted for normal cat serum. In contrast, complement-dependent lysis of FeLV with normal feline serum as assayed by the release of ribonucleic acid-dependent deoxyribonucleic acid polymerase was one-third that of complement-dependent FeLV lysis with normal human serum. The levels of total hemolytic complement and neutralizing antibody in individual feline sera were also not proportional to the degree of virolytic activity. These observations indicate that the inefficient virolysis of FeLV by normal cat serum may be one of the factors contributing to the high incidence of leukemia observed in cats.

Feline leukemia virus (FeLV), a type C retrovirus, causes several diseases in cats: lymphosarcoma (LSA), nonregenerative anemia, thymic atrophy, myeloproliferative diseases, and a panleukopenia-like syndrome (1, 12). Despite the immunosuppressive effect of FeLV infection on cell-mediated immunity in cats, the humoral antibody response to this virus apparently remains intact after FeLV infection. Some cats when exposed to low doses of FeLV develop protective titers of neutralizing antibodies directed against virion envelope antigens (11). When FeLV infection results in cellular transformation, there appears on the cell surface a tumor-specific nonstructural antigen (FOCMA) (4). Production of antibody to FOCMA represents an immunological response to tumor development since high antibody titers to this antigen prevent tumor formation (5). Antibodies from healthy cats exposed to FeLV lyse feline lymphoma cells slowly in vitro with cat complement (8). Complement-mediated and apparently antibody-independent lysis of type C retroviruses, including FeLV, occurs after incubation of the virus with serum from primates, including normal human serum (NHS) (6, 19). Normal feline serum (NFS), however, was found to be less effective in virolysis, exhibiting only 10 to 30% of the lytic activity of NHS (6, 19), indicating that the inefficiency of virolysis of FeLV by feline serum may be a contributing factor to the high incidence of leukemia observed in cats.

In a recent study, we demonstrated that LSA-diseased cats persistently infected with FeLV were hypocomplementemic. C1q, C4, and C2 were markedly diminished (14). In this study, we show that although purified FeLV activates normal feline and human complement in vitro to a similar extent, virolysis with NFS is highly inefficient when compared with virolysis with NHS. Furthermore, levels of complement and neutralizing antibody were not proportional to the extent of viral lysis.

MATERIALS AND METHODS

Serum samples. (i) NFS. Sera from pet cats seen at the Animal Medical Center and the ASPCA Hospital, New York, were used for this study. Blood was drawn from the jugular vein, allowed to clot for 1 h at room temperature, and centrifuged at 2,000 × *g* at 4°C for 15 min to collect serum. Serum was stored in aliquots at −80°C and thawed only once.

(ii) NHS. A pool of serum from 15 healthy volunteers was prepared from whole blood after clot formation and centrifugation as described above. The aliquots were stored at −80°C.

IFA assay for the detection of FeLV infection. To determine whether individual cats were productively infected with FeLV, an indirect immunofluorescence antibody (IFA) assay was performed to detect the presence of FeLV internal group-specific antigens in peripheral blood leukocytes and platelets (9, 10).

FeLV neutralization test. The presence of FeLV neutralizing antibody in cat serum was determined as follows. Samples (0.05 ml) of a feline lung fibroblast (FLF) cell suspension (10^5 cells/ml) in Eagle minimal essential medium (MEM) were added to each well of an immunofluorescence microscope slide and incubated for 16 to 18 h in a 37°C humidified CO_2 incubator. After the FLF cells had attached to the slide,

the tissue culture medium was removed from each well, and 0.05 ml of diluted test serum was added. The sera to be tested were heat inactivated at 56°C for 30 min and diluted in MEM. Equal volumes of the diluted test serum and FeLV were mixed and incubated for 50 min in a humidified CO_2 incubator before the serum was added to the cells. After incubation for 60 min at 37°C, the FeLV-containing serum within each well was replaced with an equal volume of MEM, and incubation was continued for 3 days at 37°C. After washing and fixation of the cells in acetone, the IFA assay was performed. The foci positive by immunofluorescence were counted and compared with a negative control, consisting of FeLV plus MEM, and a positive control, consisting of FeLV plus cat serum which contained neutralizing antibody. The presence of foci positive by immunofluorescence indicated a lack of neutralizing antibody in the test serum.

Determination of total 50% hemolytic complement (TCH_{50}) and complement components in cat serum. Glucose-gelatin-Veronal-buffered saline, pH 7.2, containing 1.0 mM Mg^{2+} and 150 μM Ca^{2+} and gelatin-Veronal-buffered saline were prepared as described previously (15). TCH_{50} was determined by a modified method described previously (3), using immunoglobulin M hemolysin-sensitized ovine erythrocytes (10^8 cells/ml). Titration of complement components C1, C4, and C2 was performed by using intermediate cells $EAC1^{sp}$ for C4, $EAC1^{sp}4^{hu}$ for C2, and $EAC4^{hu}$ for C1. Guinea pig complement diluted 1:25 in 0.04 M ethylenediaminetetraacetic acid (EDTA) was used as a source of the later components C3 through C9. Components C3 through C7 were each titrated by using $EAC1^{sp}4^{hu}$ and guinea pig or human components obtained from Cordis Laboratories, Miami, Fla. C8 and C9 were titrated by using stable intermediate cells $EAC1^{sp}4\text{-}7^{hu}$ (Cordis Laboratories). Factor B was titrated according to a modified method described previously (7). Serially diluted samples were reacted with purified cobra venom factor (*Naja naja*) (2) for 30 min at 37°C in the presence of 1.0 nM Mg^{2+}. Human serum diluted 1:25 in 0.04 M EDTA and glutathione-treated human erythrocytes (10^8/ml) (2) were then added. The reaction was allowed to proceed for 1 h at 37°C. The degree of erythrocyte lysis was determined by measuring the release of hemoglobin at 412 nm in a Micromedic Systems spectrophotometer. A detailed method for the detection of cat complement has been recently published (14). Purified cobra venom factor was prepared as described previously (2).

Materials for complement titrations. Sheep erythrocytes were purchased from the Colorado Serum Company Laboratory, Denver, Colo.; sheep hemolysin was purchased from BBL Microbiology Systems, Cockeysville, Md.; guinea pig complement was from Texas Biological Laboratories, Ft. Worth, Tex.; and cobra venom factor was from Silver Springs Reptile Institute, Silver Springs, Fla.

Preparation of FeLV. FeLV was obtained from the tissue culture fluid of normal FLF infected with FeLV serotype AB, which was originally isolated from a cat with LSA (13). Cultures were grown as monolayers in roller bottles and fed once weekly with MEM supplemented with glutamine (2 mM), penicillin (250 U/ml), nonessential amino acids, and 15% heat-inactivated fetal calf serum (FCS). Culture fluids were collected and clarified by centrifugation at 2,000 × *g* for 30 min, and the supernatants were collected and centrifuged at 29,000 × *g* for 135 min. The pellet was suspended in phosphate-buffered saline, pH 7.2 (PBS), layered onto a 15 to 60% (wt/vol) sucrose gradient containing 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, 10 mM NaCl, and 1.5 mM EDTA, and centrifuged for 3 h at 100,000 × *g*. Material banding at a density of approximately 1.16 g/ml consisted mainly of FeLV serotype AB, as determined serologically. After dilution in PBS, the FeLV was concentrated by centrifugation for 1 h at 100,000 × *g*, and the resulting viral pellet was dissolved in either MEM or PBS to a final concentration of 0.1% (wt/vol).

Preparation of FeLV serotype AB grown in the absence of FCS. Productively infected FLF cells were seeded into roller bottles and grown for 3 days in MEM containing 15% heat-inactivated FCS. Three successive 24-h harvests in serum-deficient MEM were then collected, pooled, clarified, and centrifuged as described above.

Complement activation studies. Equal volumes (25 μl) of sucrose density gradient-purified FeLV (0.1%, wt/vol) and NFS were incubated at 37°C for 30 min. The serum-virus mixture was then cooled to 0°C and serially diluted for complement component titrations. Functional hemolytic titrations of all complement components were also performed on heat-inactivated FCS at appropriate concentrations to rule out the possibility that a heat-stable component of FCS present would significantly alter the results of titrations.

RDDP assay. Ribonucleic acid-dependent deoxyribonucleic acid polymerase (RDDP) activity released by viral lysis was quantitated by using a modification of a previously described method (18). The assay mixture consisted of 50 mM Tris-hydrochloride, pH 7.8, 60 mM KCl, 0.2 mM manganese acetate, 2 mM dithiothreitol, 0.4 A_{260} (absorbancy at 260 nm) units of polyriboadenylate-oligodeoxythymidylate primer (P-L Biochemicals), 0.01 mM thymidine triphosphate (dTTP), and 0.01 mM [^{3}H]dTTP (Schwarz/Mann, Orangeburg, N.Y.; 5 μCi). After incubation of 0.025 ml of serum and 0.025 ml of purified FeLV for 30 min at 37°C, an equal volume of assay mix was added to each sample and incubation was continued for 60 min at 37°C. RDDP activity is expressed as picomoles of [^{3}H]dTTP incorporated per milliliter of serum. Because we found that [^{3}H]dTTP was nonspecifically trapped on the glass fiber filters as a linear function of serum concentration, the assay was routinely performed with serum diluted 1:8. Pilot studies indicated that diluted sera did not differ from undiluted sera in virolytic potential. In addition, each feline serum was tested for its effect on the RDDP assay by incubation with Triton X-100-disrupted FeLV. Any serum having significant inhibitory activity (<10%) was eliminated from the study.

RESULTS

Activation of feline and human serum complement by FeLV. Fifty microliters of

FeLV (50 μg) and an equal volume of a pool of serum obtained from five healthy cats were incubated at 37°C for 30 min. Residual TCH_{50} values and levels of all nine complement components as well as factor B were then determined and compared with untreated serum containing 50 μl of buffer (Table 1). (Multiple reaction mixtures for each experimental point were used.) TCH_{50} levels decreased by 79% after incubation with FeLV. This was also reflected in decreased levels of C1, C4, C2, C3, and C5 by 79, 38, 69, 42, and 42%, respectively. The later components C6 through C9 were decreased to a lesser or insignificant extent. In contrast, factor B was decreased by only 8%. Incubation of NHS with FeLV under the same conditions also resulted in a marked decrease in both TCH_{50} (83%) and C1, C4, C2, and C3 components of the classical pathway (69, 69, 60, and 63%, respectively) (Table 2). C6 through C9 were decreased to a lesser extent, and factor B hemolytic activity remained unchanged. To determine that the effect we were observing was due to FeLV and not to cellular components, we included control experiments using noninfected cells processed in an identical manner as that used for the purification of FeLV. Our results showed that uninfected cell preparations did not activate the complement system (data not presented).

Lysis of FeLV NFS and NHS. To determine whether the observed activation of feline complement by FeLV resulted in viral lysis, the release of RDDP activity from serum-treated virions was determined. Incubation of NFS with FeLV resulted in a release of RDDP which was considerably less than that observed when FeLV was incubated with NHS under identical conditions. Serum from 25 healthy cats individually tested for FeLV virolysis had 29.1 ± 20.9% of the activity of NHS (data not presented). The activity of released RDDP in either serum was linear with time up to at least 90 min (Fig. 1). The rate of [^{3}H]dTTP polymerization, however, was significantly lower when virus was incubated in NFS than with NHS.

The length of time required for maximal disruption of FeLV by feline or human serum was

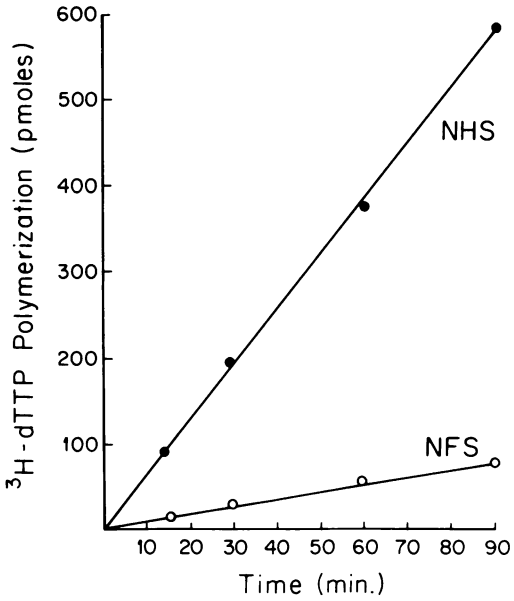

FIG. 1. *Kinetics of [^{3}H]dTTP polymerization by RDDP released after incubation of FeLV in human or feline serum. Equal volumes of virus and serum were incubated at 37°C for 30 min, cooled at 0°C, and divided into two equal volumes. The RDDP assay mix was then added to both samples and incubated at 37°C. Equal portions were removed at timed intervals for determination of acid-insoluble radioactivity. The mean of the two values obtained is plotted. Symbols: ●, NHS; ⊙, NFS.*

TABLE 1. *Effect of FeLV on total hemolytic complement and complement components in feline serum*[a]

Serum	TCH_{50}	C1	C4	C2	C3	C5	C6	C7	C8	C9	Factor B
NFS[b]	74	73,500	16,000	555	500	2,000	1,000	1,450	13,700	21,000	153
NFS + FeLV	16	15,250	9,900	169	290	1,160	810	1,250	13,600	21,000	140
% Decrease	79	79	38	69	42	42	19	14	1	0	8

[a] Values are recorded as mean CH_{50} units per milliliter of serum.
[b] Pool of serum obtained from five healthy cats. Incubation of serum and FeLV was at 37°C for 30 min.

TABLE 2. *Effect of FeLV on total hemolytic complement and complement components in human serum*[a]

Serum	TCH_{50}	C1	C4	C2	C3	C5	C6	C7	C8	C9	Factor B
NHS[b]	90	219,000	165,000	2,175	875	4,700	4,600	4,000	80,000	17,200	100
NHS + FeLV	15	68,000	51,500	885	325	3,375	3,850	2,800	78,000	13,600	100
Percent Decrease	83	69	69	60	63	28	16	31	2	21	0

[a] See Table 1.
[b] Pool of serum obtained from 15 healthy volunteers. Incubation of serum and FeLV was at 37°C for 30 min.

determined by incubating virus and serum at 37°C for various periods of time and then quantitating the RDDP released. The two sera yielded approximately parallel curves for viral disruption, with NFS exhibiting about threefold less virolysis than NHS (Fig. 2).

Maximal release of RDDP was achieved by a 20-min incubation. Longer incubations led to a reduced level of detectable RDDP activity. In additional experiments, it was shown that RDDP activity in both sera required an exogenous template and Mn^{2+} for activity.

The dependence of FeLV virolysis on a functional complement system is illustrated in Table 3. Heating pooled human or feline serum at 56°C for 30 min reduced virolysis to background levels. To determine whether the serum-mediated viral lysis was partly due to an FCS-derived factor adsorbed to FeLV (17), FeLV was grown in tissue culture medium in the absence of serum. After purification, this viral preparation was incubated with either NFS or NHS at 37°C for 30 min. The results did not differ from those with serum-grown virus. Both NFS and NHS lysed this viral preparation, with the former serum having approximately 33% of the virolytic activity of the latter (data not presented).

Comparison of FeLV lytic activity in serum from normal, leukemic, viremic, or nonviremic cats. Thirty-nine cats were classified by an IFA assay and histological diagnosis into four categories: (i) normal, not infected with FeLV; (ii) normal, productively infected with FeLV; (iii) LSA, not productively infected, and (iv) LSA, productively infected (Table 4). Sera from all cats were individually tested for FeLV lytic activity. No significant differences were detected in serum-mediated virolytic activity between these groups with the possible exception of the LSA, nonviremic group, where there was only a borderline statistically significant difference from LSA, viremic sera, using the two-tailed Student *t* test.

FeLV neutralizing antibody status or complement levels and serum-mediated lysis of FeLV. Sera of cats were tested for the presence of circulating FeLV neutralizing antibody to determine whether a relationship exists between virolytic activity and antibody status. Of the four groups of cats described above, specific antibody was detected only in normal nonviremic animals. The six normal cats screened for the presence of neutralizing antibody to FeLV had titers ranging from 1:2 to 1:10. No association was found between serum virolytic activity and neutralizing antibody titer over this range (data not presented). To determine whether a relationship existed between total hemolytic complement and the ability of feline serum to lyse FeLV, analyses of TCH_{50} levels and virolytic activity for each individual cat serum were performed; no relationship was observed (Table 5).

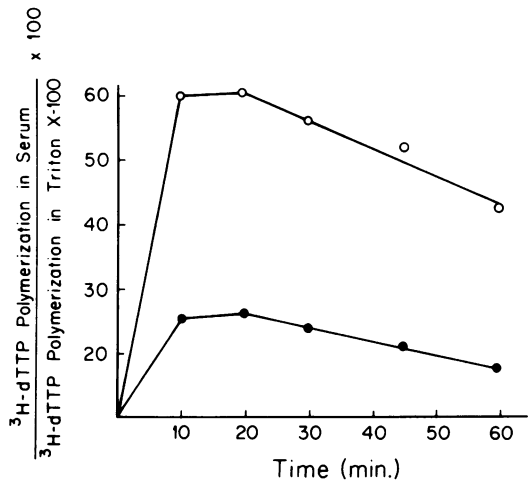

FIG. 2. *Kinetics of FeLV virolysis by human or feline serum. Equal volumes of FeLV and either human (⊙) or feline (●) serum were incubated at 37°C. At timed intervals, aliquots were removed and assayed for RDDP activity. The results are expressed as percentage of total available activity at the different times.*

TABLE 3. *Virolysis of FeLV by human and feline serum*

Sample	Virolytic activity (%)	
	NHS	NFS
No treatment	100.0[a]	30.7
Heat inactivation (56°C, 30 min)	11.7	13.1

[a] RDDP activity released by the NHS pool was set at 100% for comparison with other sera.

DISCUSSION

In an earlier study, we found that cats productively infected with FeLV and having LSA were hypocomplementemic and that activation of the complement system had occurred via the classical pathway (14). This activation could have resulted from the interaction of complement with circulating immune complexes. We have preliminary evidence that cats belonging to this group do have high levels of circulating immune complexes (N. K. Day, unpublished observations). In the present study, we show that incubation of purified FeLV with either NFS or NHS that did not contain immune complexes or virus-neutralizing antibody also re-

TABLE 4. *Feline serum-mediated release of RDDP activity from FeLV*

Diagnosis	Viral status	No. tested	pmol of [^{3}H]dTTP polymerized/ml per h[a]
Normal	−	10	33.1 ± 4.5 (25.0–39.3)
Normal	+	11	29.4 ± 8.2 (11.2–39.7)
LSA	−	7	23.6 ± 12.6 (5.0–44.3)
LSA	+	11	37.3 ± 7.9 (19.0–47.8)

[a] Triton X-100 treatment (100% viral lysis) released 250 pmol of [^{3}H]TTP polymerized per ml per h of RDDP activity. RDDP activity is expressed as the mean value ± 1 standard deviation. Numbers in parentheses give range.

TABLE 5. *Comparison of TCH_{50} levels and virolytic activity of feline serum*

Class	Viral status	No. tested	TCH_{50} (U/ml)	pmol of [^{3}H]-dTTP/ml per h[a]
Normal	−	10	84 (66–130)	33.3 (25.0–39.3)
Normal	+	11	46.2 (10–80)	29.4 (11.2–39.7)
LSA	−	5	73.8 (41–110)	19.5 (5.0–44.3)
LSA	+	3	≤10	42.9 (29.2–47.8)

[a] Complement levels and RDDP activity are expressed as mean values, with range in parentheses.

sulted in marked activation of the classical complement pathway. Thus, the virus itself appears to be capable of initiating the complement cascade. However, despite the FeLV-induced activation of the feline and human complement to apparently the same extent, virolysis of FeLV by feline serum is far less efficient than virolysis of FeLV by human serum. Sera of 25 individual healthy cats exhibited 29% of the virolysis found in NHS; in fact, some cat sera had only 5% of the lytic activity of fresh NHS. This inefficiency in FeLV virolysis by feline serum may contribute to the relative ease with which horizontal FeLV infection occurs in cats.

Sera from primates, including humans, lyse retroviruses in the apparent absence of antibody as a result of complement activation (16, 19). Therefore, it has been suggested that in higher mammals complement provides a natural defense mechanism which inhibits or interferes with retrovirus infection and replication. In addition, it was recently shown that the relative efficiency of complement-mediated virolysis correlated with primate phylogeny (16). In contrast, sera from lower mammals such as rats and mice or from chickens are unable to lyse retroviruses. The inefficiency of feline sera to lyse FeLV indicates that this species also fits into the latter category.

In the present studies, no differences were found in the degree of serum virolytic activity between normal nonviremic, normal viremic, LSA nonviremic, and LSA viremic cats. Furthermore, TCH_{50} and neutralizing antibody levels in all animals tested also did not parallel serum virolytic capacity. The lack of correlation may be due to the low level of efficiency for virolysis in feline sera.

Many possibilities exist for this inefficiency of feline sera in virolysis. Recently, we have shown that titration of complement components as observed by lysis of sheep erythrocytes cannot be performed by using the later cat complement components supplied in EDTA but can be performed with EDTA-treated human or guinea pig serum (14). It is possible that an inefficiency of C3 or C5 convertase formation necessary for activation of later components may be involved in limiting both erythrocyte and viral lysis or that the inefficient lysis may be due to the insusceptibility of cat complement to sheep erythrocytes. Studies relating to these questions are currently being done.

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