Circulating Levels of Feline Leukemia and Sarcoma Viruses and Fibrosarcoma Regression in Persistently Viremic Cats

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ABSTRACT

Eighty specific-pathogen-free kittens 3 to 4 months old received a single s.c. injection of Snyder-Theilen feline sarcoma virus; this isolate contained helper feline leukemia virus (FeLV) at a sarcoma/leukemia ratio of 1:1.5. Recipients developed fibrosarcomas 13 to 15 days later at the virus injection site; then, for 10 days, the tumors grew rapidly in all animals and reached a mean diameter of 2.7 cm. Tumors subsequently regressed in 45% of the cats and disappeared by 40 days; regressor animals then remained tumor free. Tumors progressed in all other cats, and death occurred between 30 and 90 days of disseminated secondary deposits. Although circulating FeLV was detected in the blood from all cats before or at tumor appearance, Snyder-Theilen feline sarcoma virus was detected only in tumor regressors during the last 2 weeks of life. Peak FeLV burdens exceeded 1000 infectious particles and 2.0 μg of protein with a molecular weight of 30,000 per ml blood, but no correlation was found between tumor progress and leukemia virus load. One-half of the tumor regressor group also eliminated their viruses infections. In these animals, virus-neutralizing antibody to both FeLV and Snyder-Theilen feline sarcoma virus was detected at high titer in serum. In remaining regressor cats, the tumors disappeared, but virusemia persisted, and no virus-neutralizing antibody activity to either virus was detected. Cytotoxic antibody to feline oncornavirus-associated cell membrane antigen was detected in high titer only in sera from the tumor regressor and the FeLV-negative group of cats, indicating that fibrosarcoma rejection and virus immunity are mediated by distinct mechanisms.

INTRODUCTION

Solitary, slowly growing fibrosarcomas account for about 20% of skin and oral cavity tumors of older cats (>5 years old), but these are rarely associated with retroviral infection. In younger cats, multicentric fibrosarcomas are relatively rare, but most (>80%) are virus associated (18, 33). Isolation of transforming FeSV from tumor tissue coincides with isolation of nontrans-
were withdrawn for infectious and transforming virus assays. Sera were obtained following centrifugation of nonheparinized samples 2 to 5 hr after clotting, and these were number coded and stored at -85° prior to assay for FeLV p30.

Preparation and Titration of FeSV:FeLV Stock. Methods have been detailed elsewhere (14). Briefly, the virus stock was prepared from a cell-free filtrate of a pooled homogenate of fibrosarcomas induced by Snyder-Theilen FeSV in five 2-week-old kittens. This virus isolate was tested for infectious FeLV using CCB81 S+L- cells (11) and for FeSV using CCL64 (19, 29) as detailed below, and it was found to contain 1.35 x 10² FFU FeLV per ml and 8.4 x 10⁶ FFU FeSV per ml. The isolate was also tested for FeLV infectivity and FeSV tumorigenicity in 8-week-old SPF kittens, and 50% inhibitory dose dilutions from stock were 10⁻⁴ and 10⁻⁶, respectively. All cats were challenged with 0.5 ml of a 1:50 dilution of this stock preparation.

Determination of Infectious FeLV in Cat Blood. Infectious FeLV particles (FFU/ml blood) were assayed using the virus isolation test described previously (6). A total of 2 ml of heparinized cat blood was layered over 2 ml of Ficoll-Isoaque and was centrifuged for 30 min at 400 x g to remove RBC. Theuffy coat layer and plasma were removed and remixed, and 0.2 ml was inoculated onto Petri dishes containing monolayers of CCB81 S+L- cells (11) previously treated with 2 μg Polybrene. After a 1-hr incubation at 37°, the monolayers were washed, and fresh medium was added. Medium changes were made at 3-day intervals; after 12 days, numbers of foci were scored.

Determination of Transforming FeSV in Blood. Transforming FeSV was assayed using the helper-independent CCL64 focus assay (29). The assay was performed in a similar manner to that described for FeLV, except that the buffy coat cells and plasma were layered over the mink CCL64 line (MV1Lu cells). Optimal focus formation occurred on Day 7 when the plates were scored.

Assays for VNA. Test serum dilutions were assayed for their capacity to neutralize FeLV and FeSV contained in the Snyder-Theilen FeSV:FeLV stock preparation prior to infection of CCB81 S+L- cells or CCL64 cells. Serum dilutions that inhibited the appearance of 50% or more of foci detected in control plates were scored as positive.

ELISA for FeLV p30 Using Monoclonal Antibodies. Three MAbs directed against the species-specific determinants of FeLV p30 were produced and characterized as described elsewhere (21). These MAbs each recognized distinct epitopes of FeLV p30, but they did not react with the analogous protein from unrelated retroviruses including murine retroviruses and baboon and cat RD114 endogenous virus (21). The 3 MAbs (designated MAbs 1, 2, and 3) were used in a double-antibody, solid-phase immunosorbent assay (ELISA) which is described in detail (21) and briefly as follows. One μg of MAb 1 in 100 μl of 0.05 M carbonate buffer, pH 9.6, was incubated for 3 hr at 37° to coat wells of micro-ELISA Immunon-1 plates (Dynatech, Inc., Alexandria, Va.). After a washing to remove unbound antibody, test sera samples (100 μl) were added to each well. The samples were each diluted 1:8 in Tris-buffered 0.9% NaCl solution, pH 7.4, containing 0.1% Tween 20; in order to measure circulating free p30 without disrupting intact virus, the same buffer was used but without detergent. After incubation for 30 min at 37°, unbound protein was washed out, and subsequently a pool of MAbs 2 and 3 which conjugated to horseradish peroxidase (36) was added. After incubation for 30 min at 37°, the wells were washed; the o-phenylenediamine substrate was then added; after incubation in the dark, the reaction was stopped by addition of acid. The reaction product was read in a Stasar II spectrophotometer equipped with a microcuvet dark, the reaction was stopped by addition of acid. The reaction product was then added to a DEAE-Sephadex column (0.9 x 15 cm) equilibrated with the same buffer. The column was washed with PBS, and bound protein was eluted with a linear gradient of 0.01 to 0.5 M NaCl. Fractions containing a single protein with a molecular weight of approximately 30,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were pooled, aliquoted, and stored at -70°. Protein concentration in the standard p30 preparation was determined by the method of Lowry et al. (20) by using bovine serum albumin as standard.

Assay for CDA to FOCA. This assay was performed as detailed previously (14, 16, 17) using ³¹Cr-labeled FL74 feline lymphoma cells (35) as targets.

RESULTS

Appearance and Growth Characteristics of FeSV-induced Progressing and Regressing Fibrosarcomas. A total of 92 cats received FeSV:FeLV as a single s.c. injection on the left flank. Twelve cats were excluded from subsequent considerations: 4 because they died of causes unrelated to the experiments; and 8 because they remained free from both virus infection and tumor throughout the study.

Eighty cats developed palpable s.c. fibrosarcomas at the injection site; these tumors were first detected 15 ± 2 (S.D.) days after inoculation. Some cats developed a single tumor mass, and others developed several discrete lumps that subsequently coalesced. Tumors progressed in 44 cats, and these animals died of disseminated secondary deposits at a wide variety of body sites. Tumors in 36 of the cats regressed completely, and these animals remained tumor free; absence of tumor was confirmed at necropsy of each cat performed between 100 and 120 days after virus injection.

During the first 9 days after tumor appearance, there was no difference in growth rates of tumors destined to progress or to regress. In both groups, mean tumor diameters doubled from 1.0 to 2.0 cm between Days 17 and 22, and on Day 24, the mean diameters of progressor and regressor tumors were both 2.7 ± 1.2 cm. On Day 26, the mean tumor diameter of progressors had increased to 3.4 ± 1.7 cm, but that of regressors had decreased to 2.2 ± 1.3 cm. Subsequently, tumor regression occurred rapidly; by Day 33, the mean diameter was 0.8 ± 0.9 cm; by Day 44, all tumors in this group had disappeared completely. Cats with progressing tumors died of disseminated secondary deposits between Days 31 and 92: 60% of the progressors died between Days 38 and 56. In this group, primary fibrosarcomas grew rapidly until they reached diameters of 4 to 5 cm; after this, growth slowed, although no decreases in tumor size were noted.

Infectious Virus and Virus Core p30 Levels in Sera from Tumor Progressor and Regressor Cats. Sera samples were removed from all cats at intervals after virus infection, and these samples were assayed for infectious FeLV by CCB81 S+L- focus formation and for virus p30 antigen by ELISA. Results

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from these assays are compared in Chart 1, A–C.

In the early stages after virus injection, we limited the minimum time between removal of blood samples to 10 days because the kittens used were small. At 6 days, 12 cats were bled; between 9 and 11 days after virus injection, a further 13 animals were bled. No virus or FeLV protein was detected in the samples removed at 6 days; at 9 to 11 days, low levels (<100 FFU; <40 ng FeLV p30) were detected in samples from 77% of the tested cats. The remaining 55 cats were bled on Day 15 when tumors first became palpable, and these data are shown in Chart 1A.

Infectious FeLV was detected in 95% of the samples, and these were found in progressors and regressors. Sixty-six percent of the cats were FeLV viremic, and 33% exhibited circulating virus p30. All regressors were found to be free of detectable virus and p30. Four regressors were free of infectious FeLV but remained positive in tests for virus core protein.

Detection of FeSV in Blood from FeSV:FeLV-injected Cats. All blood samples included (Chart 1, A–C) were assayed for FeSV by CCL64 target cell transformation, and this test was performed in parallel with quantitation of FeLV using CCC81 S+L– focus formation. A qualitative comparison of results from both assays is shown in Table 1. Although infectious FeLV was detected in blood from nearly all of the regressor cats early after virus injection, transforming FeSV was never detected in the same blood samples. Transforming virus was also absent from the blood of regressors at 15 days when FeLV was detected in nearly all the animals. At 30 days after injection, 7 of the 44 sera from regressor cats contained FeSV, and these positive samples were all from cats destined to die in the 31- to 39-day period.

Of the 27 tumor progressor cats that survived longer than 45 days after virus injection, 18 were bled at least once between the 30-day point and the terminal sample. From these samples (not shown), we found that FeSV was first detected in blood 9 ± 6 days prior to death. Terminal blood samples revealed that all cats were FeLV viremic, but only 66% exhibited circulating FeSV.

Titers of FeLV and FeSV in blood samples from 3 representative progressor cats are compared in Table 2. Generally, we detected an increase in transforming virus titer between first detection of virus and death, but in 90% of these animals, FeSV titers remained lower than the corresponding titers of FeLV.

Failure to Detect Antibody to FeSV in Persistently Viremic Tumor Regressor Cats. All fibrosarcoma regressor cats had FeLV and virus p30 detectable in their blood before tumor regression became evident, and one-half of these animals re-
mained persistently viremic but tumor free thereafter. Since antibody to FOCMA has been implicated in fibrosarcoma regression (8, 9, 31) and because FOCMA has been shown to be packaged in FeSV pseudovirus (28, 30), we attempted to detect antibody which would specifically neutralize FeSV and thereby account for the elimination of transforming virus in the presence of FeLV viremia.

Sera from normal SPF cats and from both fibrosarcoma progressor and regressor cats were each diluted serially before adding to a constant dilution of the stock Snyder-Theilen FeSV:FeLV isolate. After incubation, the virus:serum mixtures were plated in duplicate on appropriate target cells; subsequently, foci were quantitated which resulted from target cell infection with nonneutralized virus. The results are summarized in Table 3, and they reveal that VNA was detected at high titer only in the group of fibrosarcoma regressors which also eliminated FeLV infection. No antibody was detected with the property of specifically recognizing and neutralizing the sarcoma virus component of the stock virus isolate.

Failure to Detect FOCMA Antibody in Persistently Viremic Tumor Regressor Cats by the Lytic CDA Assay. Levels of lytic CDA in sera from FeLV-exposed cats have been shown to correlate with FOCMA antibody levels as measured by immuno-fluorescence (16), and high titers of FOCMA antibody have been shown to protect cats from FeLV-associated leukemias (8, 16, 17) and FeSV-associated fibrosarcomas (8, 9). Lytic CDA titers were determined in sera removed from cats during the last 10 days before death or experimental termination, and results are summarized in Table 4. Antibody levels remained low (<1:4) to undetectable in 97% of the tumor progressor cats and in 94% of the tumor regressor animals that remained persistently viremic. In contrast, 64% of the cats which eliminated both virus

### DISCUSSION

After infection of SPF kittens with Snyder-Theilen FeSV:FeLV, the course of tumor progression or regression was studied with regard to blood-borne virus loads and humoral immunity. Large burdens of circulating FeLV did not correlate with tumor progression, and FeSV was detected only in the blood of tumor progressor cats during the terminal stages of malignant disease. Two subgroups of fibrosarcoma regressors were found: in one, both tumor and viremia disappeared; and in the other, fibrosarcomas regressed permanently, but FeLV viremia persisted. Antibodies (VNA and CDA) appeared at high titers only in sera from regressor cats that also became virus free.

Appearance of viremia about 9 days after virus injection is consistent with data obtained by others (14, 27). Because FeSV was detected in blood from progressor cats during the terminal stages of malignant disease only (Table 2), almost all viruses represented in Chart 1, A and B are FeLV. Between 15 days (Chart 1A) and 30 days (Chart 1B), there was a marked increase in the level of FeLV p30, but there was no corresponding increase in infectious virus titer. In order to determine total p30 levels in the samples (data not shown), the ELISA was carried out using buffer containing Tween 20 which stripped the envelope from intact virions to reveal core protein. To measure free circulating p30, detergent was eliminated from the buffer (data not shown). Comparisons of the same samples diluted in one or the other buffer revealed that most of the increase in p30 titers was attributable to free antigen rather than to p30 found in intact but

### Table 1

<table>
<thead>
<tr>
<th>Tumor behavior</th>
<th>Time (days) after FeLV:FeSV injection</th>
<th>% of cats with infectious or transforming virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regressors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>24</td>
<td>92</td>
</tr>
<tr>
<td>30</td>
<td>36</td>
<td>86</td>
</tr>
<tr>
<td>100–120 (at necropsy)</td>
<td>36</td>
<td>50</td>
</tr>
<tr>
<td>Progressors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>31</td>
<td>97</td>
</tr>
<tr>
<td>30</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>Terminal (48 hr)</td>
<td>38</td>
<td>100</td>
</tr>
</tbody>
</table>

### Table 2

| Cat | Day of death | Virus | Infectious or transforming particles/mL of blood after virus injection
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 15</td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>FeLV</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeSV</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>FeLV</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeSV</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>58</td>
<td>FeLV</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeSV</td>
<td>0</td>
</tr>
</tbody>
</table>

*ND, not determined.*
noninfectious FeLV particles.

Termination of viremia, which occurred in almost one-half of the regressor cats, was probably under way at the 30-day point, for in Chart 1B a group of cats can be discerned with low levels of circulating infectious FeLV and p30. At this time, all cats were tested for neutralizing antibody, but no activity was detected. At later intervals, it was apparent that only those cats which eradicated both fibrosarcomas and viremia developed high levels of VNA (Table 3); findings that support the hypothesis that development of VNA limits virus infection in immune cats (15, 21, 27).

We were surprised to find FeSV in the blood of regressor cats only during terminal stages of disease, for FeSV was injected at a 1:1.5 ratio with FeLV, and FeLV appeared rapidly (9 to 15 days) in the circulation of both regressor and regressor cats. It might be argued that the cell transformation assay used to detect FeSV lacked sensitivity, but this is not consistent with the detection of high titers of FeSV in the original virus inoculum and in sera removed from regressor cats immediately prior to death (Table 2). The early detection of high titers of circulating FeLV is explained readily, because FeLV replication in blood lymphoid cells commences rapidly after infection (15, 18, 27). Replication of FeSV, however, is confined to fibroblasts concomitantly infected with helper FeLV. Presumably, spillover of free FeSV into the blood is limited to the later phases of disease when large volumes of primary and secondary fibrosarcoma tissue are engaged in FeSV replication.

Failure to detect VNA specifically directed to FeSV in tumor regressor but persistent FeLV viremic cats was not surprising, because FeSV is pseudotyped by FeLV and therefore shares envelope antigens. Rationale for the experiment lay in the reports that FOCMA was present in FeSV pseudotypes (31), that FOCMA antibody correlated strongly (16). Previous work showed that cats could be immunized against progressing fibrosarcomas but that this immunity did not necessarily correlate with CDA levels (14). In the presence of cat complement, CDA lyses virus-producing lymphoid cells (15–17), but we have not been able to demonstrate lysis of FeLV-infected or FeSV-transformed fibroblasts under the same conditions. Because CDA and VNA were both detected in virus-free tumor regressors, although these antibodies were essentially absent from the viremic regressor cats, it would appear that the antibodies function to destroy virus-infected lymphoid cells and to neutralize free virus, respectively. In leukemias, CDA functions to mediate immune resistance by destroying virus-positive tumor cells (15–17), but present results suggest that it is less likely that CDA mediates immunity to fibrosarcomas. Sera from some FeLV-positive tumor regressor cats were also studied for antibody to the feline transformation-associated FeSV gene products expressed specifically by transformed cells (2) by radioimmune precipitation and sodium dodecyl sulfate:polyacrylamide gel electrophoresis, but no specific activity to this polyprotein was detected.6

Feline fibrosarcomas undergoing regression are heavily infiltrated with lymphocytes (34) but, despite this indication that cellular immunity is important in regression of feline fibrosarcomas, 2 problems have made a direct study difficult. One logistical problem is a lack of inbred cats and the resulting difficulties imposed by histocompatibility restriction; the other is that feline fibrosarcomas have proven to be refractory to cell culturing efforts presumably because of their extraordinary content of mucin material (32). Indirectly, however, specific T-cell-mediated immunity has been demonstrated to play a central role in resistance of cats to autochthonous fibroblasts transformed by FeSV. Assuming an effector role for cellular immunity in regression of feline fibrosarcomas, it is apparent from present data that tumor rejection occurs in cats which remain persistently viremic. In the murine system, however, mice carrying persistent Moloney murine leukemia virus infections fail to reject M-MSV-induced fibrosarcomas, and the explanations advanced for this inhibition of T cell-mediated immunity include: (a) generation of suppressor cells which inhibit effector cell function (24); (b) reduction in the generation frequency of virus-specific effector cell precursors (4); and (c) inhibition of effector cell function by circulating a viral glycoprotein with a molecular weight of 71,000 (7). It is apparent that, in the cat system, high titers of circulating virus do not inhibit or suppress the tumor rejection process (cf. Ref. 23).

Given a relatively inefficient replication of FeSV cf. FeLV, development of a strong cellular response to the virus-transformed cells would serve to eliminate the tumor and therefore the source of sarcoma virus. Coincident appearance of a humoral response, involving both neutralizing antibody and antibody lytic for virus-infected lymphoid cells (15–17), would terminate FeLV infection. An efficient cellular response in the absence of an effective humoral response (Tables 3 and 4) may explain the category of fibrosarcoma regressors that remained persistently infected with leukemia virus.

REFERENCES


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