Biological Behavior of Tumors and Associated Retroviremia in Cats Inoculated with Snyder-Theilen Fibrosarcoma Virus and the Phenomenon of Tumor Recurrence After Primary Regression

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The fate of tumors and associated retroviremia was studied in 111 cats infected with the Snyder-Theilen strain of feline sarcoma virus (FeSV). Tumors appeared at the site of inoculation within 7 to 10 days. A retroviremic, due mainly to the associated feline leukemia virus helper virus (FeLV-helper), developed at the same time as tumors. Of the cats, 44 developed progressively growing tumors that were killed, and 67 developed tumors that regressed. There was a strong correlation between the persistence of the accompanying retroviremia and the growth of the tumors. Of the 44 cats with progressively growing fibrosarcomas, 11 of 44 (FeSV) remained retroviremic until death. Conversely, 53 of the 67 cats with solitary, regressing tumors were only transiently retroviremic. Tumor regression in these cats paralleled the disappearance of retrovirus from the blood. The fate of tumors and retroviremia was not always the same. Twelve cats remained persistently retroviremic after all signs of gross tumors disappeared. Two other kittens became nonviremic within 20 days after inoculation, yet tumors continued to grow and even metastasize for another 3 to 5 weeks before regressing. Fibrosarcomas recurred 3 weeks to 8 months later in 8 of 12 persistently retroviremic cats with regressed tumors. Although the blood and bone marrow from these cats contained predominantly FeLV-helper, tumor cells yielded both FeSV and FeLV-helper. Of 53 animals, 3 developed recurrent fibrosarcomas 5 weeks to 8 months after all signs of tumors and retroviremia had disappeared. Cultures from these tumors appeared initially like normal fibroblasts and were virus nonproducers. After one to three passages in culture, however, cells became malignantly transformed and replicated both FeSV and FeLV-helper. Cultures of the bone marrow from these and other nonviremic cats with regressed tumors yielded only FeLV-helper.

The Snyder-Theilen strain of feline sarcoma virus (ST-FeSV) readily induces fibrosarcomas in cats. Tumors in neonatal cats grow rapidly and metastasize, whereas tumors in older kittens grow slowly and frequently regress (19). Tumor regression is immunologically mediated; good immunity results in tumor regression, and poor immunity results in progressive tumor growth (4, 12). The immunity in cats with regressed tumors is presumably solid and long lasting. These conclusions are based, however, on experiments lasting only 3 to 7 weeks, the time it takes for tumors to kill the animal or to disappear. This period is too short to detect tumors that would recur later. The first indication that tumor regression may not be permanent has been observed by Aldrich and Pedersen (1). They have observed the reappearance of a fibrosarcoma in a cat in which a primary, virus-induced tumor had regressed several weeks earlier.

The emphasis of ST-FeSV research has been mainly on tumor induction and tumor immunity. ST-FeSV inocula, however, also contain a 10-fold excess of a nontransforming feline leukemia virus (FeLV) helper virus (FeLV-helper) (17). Cats inoculated with FeSV are infected, therefore, with two very different viruses at the same time. Unlike FeSV infection, FeLV infection is systemic and causes illness months or years later (8, 9). Although it has never been specifically studied, the fate of the FeLV-helper infection has been assumed to be the same as that of the tumors. In an earlier study, however, FeLV-helper was isolated from two cats with tumors which had previously regressed, and both FeSV and FeLV-helper were isolated from a third animal (1). Recently, deNoronha et al. (3) and Grant and co-workers (7) have also described persistent retrovirus infections in cats with previously regressed ST-FeSV-induced fibrosarcomas.

For the experiments reported here, we were concerned with the long- and short-term effects of ST-FeSV infection on 111 cats. Observations made over periods ranging from 4 weeks to 3 years demonstrated that tumor immunity was not always complete or long lasting. The fate of the accompanying FeLV-helper infection was also not inextricably tied up with tumor immunity, as has been assumed.

MATERIALS AND METHODS

Experimental animals. FeLV-negative kittens were obtained from the conventional and specific pathogen-free breeding colonies of the Feline Leukemia Research Laboratory, University of California, Davis. ST-FeSV-infected and noninfected animals were housed in isolation quarters of the Animal Resources Service, University of California, Davis.

FeSV challenge. Kittens were inoculated subcutaneously between the shoulder blades with $10^6$ to $10^7$ ST-FeSV-transformed feline fibroblasts. Tumors appeared at the site of inoculation within 6 to 10 days. Two cell lines, designated FF64/ST-FeSV and FF90/ST-FeSV, were used for inducing the fibrosarcomas. These cell lines were derived from normal feline dermal fibroblasts that were transformed in vitro with cell-free ST-FeSV (20) and were maintained as suspension cultures. Both cell lines produced an excess of transforming FeSV (8 × 10⁴ focus-forming units ml⁻¹) to nontransforming FeLV-helper (5 × 10⁴ tissue culture infective doses per ml). Freeze-thaw-disrupted cells contained...
only 0.1% of the amount of transforming and nontransforming viruses found in culture supernatants. The helper in ST-FeSV stocks has been identified previously as a subgroup B FeLV (17). ST-FeSV transformed cells were harvested from tissue culture fluids by low-speed centrifugation, washed several times in Hank's balanced salt solution, and resuspended at a concentration of 10^7 to 10^8 cells per ml. 

Serology. Levels of FeLV p27 were measured by a double-sandwich enzyme-linked immunosorbent assay with a battery of monoclonal mouse anti-FeLV p27 immunoglobulin G's (14). This test accurately measured FeLV p27 levels as low as 1 ng/100 μl. The amount of whole FeLV in the blood was ca. three times that of FeLV-p27, as determined by measurements of FeLV-p27 levels in sodium dodecyl sulfate-disrupted samples containing known weights of purified FeLV.

Retrovirus identification. Transforming and nontransforming retroviruses were differentiated from each other by cell culture and animal inoculation studies. Blood buffy coat cells, bone marrow, or tissue culture supernatants were exposed to adult feline urethral (7U) cells. When infected with FeSV, the 7U cell monolayers developed distinct foci of transformation within 5 to 10 days, and the numbers of foci were directly proportional to the amount of infectious transforming virus present in the initial inoculum. Nontransforming FeLV-helper titers were also measured in feline 7U cells. Serial 10-fold dilutions of culture supernatants, buffy coat-rich plasma, or bone marrow aspirates were overlaid on 7U cell monolayers for 24 h and then replaced with fresh medium. Tissue culture supernatants were assayed after 10 days for FeLV p27, and foci of transformed cells were counted. FeLV expression in the absence of sarcomagenic transformation was typical of FeLV-helper, whereas sarcomagenic transformation in the absence of FeLV p27 expression was typical for infectious but replication-incompetent FeSV. Sarcomagenic transformation with FeLV p27 expression was indicative of a dual FeSV–FeLV-helper infection.

Cells infected with several virus isolates were also tested for their ability to induce sarcomas when injected into kittens or nude mice. Tumor induction only occurred when the cells were morphologically transformed.

Identification of latent retrovirus infections. The presence of latent FeLV infections was detected by modification of a previously reported procedure (16). Bone marrow (0.05 to 0.1 ml) was taken from nonviremic cats with regressed tumors; cats had been infected 4 to 18 weeks earlier with ST-FeSV. Control bone marrow samples were obtained from specific pathogen-free cats and from animals with persistent FeLV infections. Bone marrow aspirates were immediately diluted in 2 ml of tissue culture medium containing 25 U of heparin sulfate. The diluted marrow was allowed to stand in a test tube for 10 min, and the erythrocyte-rich supernatant was discarded. Fragments of bone marrow settling to the bottom of the tubes were suspended in 5 ml of tissue culture medium (Eagle minimal essential medium-Liebowitz 15 [1:1]) with 10% fetal bovine serum and placed in disposable flasks (15 cm²). Cultures were incubated at 37°C in an atmosphere of 5% CO₂. One half of the culture medium was replaced every 3 to 7 days. A sample of the culture medium was assayed every 7 days for 6 weeks for the presence of FeLV p27. Latent retrovirus infection, when present, usually expressed itself within 3 to 5 weeks.

Latent FeLV infection in cultured tumor cells was detected as described above, except cells were derived from explants of finely minced tumor tissue.

RESULTS

Fibrosarcomas were induced by allogeneic ST-FeSV-transformed fibroblasts in 111 kittens. The kittens were from 16 different experimental groups (Table 1) and varied in ages from 12 to 24 weeks old. Differences in the clinical outcome after inoculation were apparent from group to group. These differences were probably due to factors such as age at the time of inoculation, source of animals, and variations in genetic susceptibility in randomly bred animals. Tumors usually appeared at the site of inoculation within 6 to 10 days. The tumors grew progressively larger in 44 kittens (Table 1), and at death, about one-half of the kittens had

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals in group</th>
<th>Cats with primary sarcomas</th>
<th>Cats with regressive sarcomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. with persistent retroviremia</td>
<td>No. with transient retroviremia</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>5</td>
<td>0</td>
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<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1</td>
<td>0</td>
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<tr>
<td>5</td>
<td>5</td>
<td>2</td>
<td>0</td>
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<tr>
<td>6</td>
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<td>2</td>
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<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>2</td>
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</tr>
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<td>10</td>
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<td>2</td>
<td>0</td>
</tr>
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<td>11</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>14</td>
<td>2</td>
<td>0</td>
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<td>15</td>
<td>8</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ Maximum tumor size was determined at the time of death for cats with progressively growing tumors and at the time of peak growth for cats with regressive tumors.

² Primary tumors continued to grow and metastasize for several weeks after the systemic retrovirus infection was no longer detectable. No metastatic lesions were seen in other cats with regressing tumors and tumor regression always occurred soon after termination of retroviremia. The total tumor burden in these two cats would have been considerably larger had metastatic lesions been included.
metastatic lesions in various organs. A total of 67 kittens developed regressing tumors that did not metastasize. Regressive tumors usually attained only a fraction of the maximum size attained by progressively growing tumors (Table 1) and then disappeared in 1 to 3 weeks.

There was a strong correlation between the persistence of retroviremia and progressive tumor growth. All 44 kittens with progressively growing primary tumors were still viremic at the time of death, 4 to 6 weeks after inoculation (Fig. 1). Conversely, 53 of 67 cats with solitary, regressing tumors were only transiently viremic. Tumor regression usually began around 15 to 28 days postinoculation, whereas viremia was evident only from days 7 to 21 (Fig. 2). Two exceptions to the above patterns occurred. First, 12 kittens remained persistently viremic after tumors disappeared (Fig. 3). Second, two kittens became aviremic by day 10, and yet the primary tumors continued to grow and metastasize for another 3 to 5 weeks. At that time, both primary and metastatic tumors underwent gradual but complete involution. There were no differences in the degree of retroviremia between the 12 viremic cats with tumors which regressed and the 44 viremic cats that died of progressive tumors. Both groups of animals maintained retrovirus levels of 20 to 60 ng/100 µl in the blood during the period of tumor regression or progression.

Characteristics of systemic retrovirus infection. We were interested in identifying the retrovirus present in the blood of FeLV p27-antigenemc cats with progressive and regressive fibrosarcomas. Buffy coat-rich plasma and bone marrow obtained from such cats were cocultivated with normal feline urethral (7U) cells. These cells were susceptible to both retrovirus infection and FeSV transformation. Specimens were taken from five cats with progressed tumors 1 to 2 weeks before death, whereas samples from four persistently viremic cats with regressed tumors were taken 2 to 6 weeks after tumors had disappeared. Virus levels in the serum of these cats were always between 0.7 and 1.8 µg/ml. Virus isolates were infectious but not transforming for feline 7U cells and induced foci on clone 81 S+L- cells. It was assumed, therefore, that all of the virus isolates were essentially FeLV-helper.

Fate of cats with regressed fibrosarcomas. In 65 cats, primary tumors at the inoculation sites regressed, and as far as it was possible to discern, the cats were grossly free of metastatic lesions. Within this group, there were 53 cats that had rejected tumors and eliminated their FeLV-helper viremia, and 12 cats with previously regressed tumors remained FeLV-helper viremic (Table 1). Fibrosarcomas reappeared in distant sites in 8 of 12 cats in this latter group, and the time to tumor recurrence was from 3 weeks to 8 months (mean, 5 months). Recurrent tumors were usually solitary; two developed in mouths, one in a ureter, one in a vertical ear canal and dermis of the face, one in the dura of a lower spinal cord, one in a brain, one in the parenchyma and capsule of a kidney, and one consisted of small multiple nodules under the skin of a chest wall. Almost pure cultures of productively transformed fibroblasts were obtained from such tumors. All of the tumors were highly invasive or located in inoperable sites, and as a result, the animals had to be killed.

Of 53 cats that had recovered from both sarcomas and retroviremia, 3 developed recurrent fibrosarcomas. In one cat, a recurrent tumor appeared as a small growth at the base of the tail, 8 months after primary tumor regression. Even though the tumor tissue appeared to extend beyond the surgical margins, the tumor did not reappear, and the cat has been free of tumors for over 1 year. A second cat developed a hemangioma or atypical fibrosarcoma in the retroperitoneal space 5 weeks postregression and died suddenly of an intra-abdominal hemorrhage. A third cat developed a diffuse fibrosarcoma in the medial thigh muscles 6 months after primary tumor regression. About three-fourths of the tumor was removed at the time of initial biopsy. After 2 to 4 weeks, the remaining tumor slowly regressed and could no longer be palpated. A biopsy taken just before the tumor regression showed only a granulomatous inflammation. Just after the thigh tumor disappeared, a fibrosarcoma was detected in the sublingual muscles. This tumor grew very rapidly; the cat eventually had to be killed after three unsuccessful attempts to surgically remove the tumor. Metastatic fibrosarcomas in the lungs and kidneys were apparent at necropsy.

Fibrosarcomas that recurred in persistently retroviremic cats differed somewhat in biological behavior and morpholo-
FeLV p27 expression coincided with the appearance of infectious virus by other tests. Bone marrow cultures from the retrovirus-unexposed, specific pathogen-free cats did not yield viruses over the same period, whereas cultures from persistently viremic cats produced viruses from the time they were initiated.

**DISCUSSION**

Kittens inoculated with allogeneic ST-FeSV-transformed fibroblasts developed both sarcomas and systemic retrovirus infections. The systemic retroviremia was due mainly to FeLV-helper present in the ST-FeSV inoculum. The predominance of FeLV-helper in the blood of ST-FeSV infected cats has also been reported by Grant and co-workers (7). In the experiments of deNoronha et al. (3), sarcomagenic virus was detected in the blood of relatively few infected cats, and then only late in the disease course when tumors were large and widespread. In contrast, FeLV-helper was easily isolated from the blood during all stages of the infection. Paradoxically, cell extracts of ST-FeSV-induced tumors are known to contain a 10-fold excess of FeLV-helper to FeSV (17), but the ratio of FeLV to FeSV in the blood of these cats appeared to be higher than this. This could have resulted from the dilutional effect of FeLV-helper produced by nontransformed cells in other areas of the body. It is also possible that the production of FeSV by tumor cells is considerably lower than the production of FeLV by nontransformed cells. In our hands, FeSV-infected Ff64 cells produced about 50-fold less retrovirus in vitro than the same cells infected solely with FeLV-helper, and most of the virus was transforming rather than nontransforming.

Tumor growth and systemic retrovirus infection seemed to be related in most of the cats. Progressive tumor growth was always associated with a persistence of the retroviremia, and tumor regression usually occurred concurrently with the disappearance of virus from the bloodstream. There were exceptions, however, mainly in the group of cats with regressing tumors. Tumor regression in the face of a persistent retroviremia occurred in 12 of 111 cats. This phenomenon was first reported a decade ago in three cats by Aldrich and Pedersen (1) and later by Schaller and associates (18). Tumor regression in the face of a sustained retroviremia in this group of cats could not be explained by quantitative differences in the levels of retrovirus in the blood. These cats maintained retrovirus levels in the blood that were as high as those in the blood of cats with progressively growing tumors.

**TABLE 2. FeLV p27 antigen expression by cultured bone marrow cells**

<table>
<thead>
<tr>
<th>No. of cats</th>
<th>Disease status</th>
<th>FeLV p27 antigen in serum (ng/100 µl)</th>
<th>Virus expression in cultures</th>
<th>Days in culture before FeLV p27 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>Nonviremic, regressed FeSV tumors</td>
<td>0</td>
<td>16</td>
<td>24.5 ± 8.7</td>
</tr>
<tr>
<td>4</td>
<td>Viremic, progressed FeSV tumors</td>
<td>47.8 ± 14</td>
<td>4</td>
<td>3a</td>
</tr>
<tr>
<td>4</td>
<td>No previous FeSV exposure</td>
<td>0</td>
<td>0</td>
<td>No FeLV p27 expression after 49 days in culture</td>
</tr>
</tbody>
</table>

a) Three days was the earliest time of testing. Actual expression of virus was probably much sooner.
Tumors recurred within 1 to 8 months in three-fourths of the cats that remained retroviremic after primary tumor regression. Tumors have also recurred in mice with regression after primary tumors (13). Unfortunately, the retrovirus status of the mice that remained retroviremic after primary tumor virus or transformed cells persisted in the cats after initial immunological containment. The failure of this study to identify FeSV in the blood of retroviremic cats with regressed tumors seemed to rule out FeSV persistence. In an earlier study, however, FeSV was isolated from a retroviremic cat with regressed tumors (1).

The paucity of FeSV in the blood and bone marrow of retroviremic cats with regressed tumors suggested that FeSV- and tumor-specific immunity were related, while immunity to FeLV-helper was not. This could only be FeSV- and tumor-specific immunity were related, while A antigens, and human lymphocyte-A antibodies will neutralize such particles (2). It was possible, therefore, for virus particles budding from transformed cells to carry tumor-specific antigens, whereas FeLV-helper budding from non-transformed cells in other tissue could not. Immunity to FeSV and tumors could then exist independently of FeLV-helper immunity. Alternatively, sarcoma cell-specific immunity might destroy the transformed cells in which the FeSV genome is selectively replicated.

The appearance of virus-negative fibrosarcomas in cats that were once infected with FeSV resembles the situation described for FeLV infection. About one-third of feline lymphosarcomas are not associated with retroviremia. Many of these, however, have been statistically linked to previous FeLV exposure (11). In our studies, cells cultured from recurrent fibrosarcomas in aviremic cats did not express viral proteins during initial passage. After several passages in vitro, however, FeSV and FeLV-helper were detected in culture supernatants. This suggested that virus production was being suppressed in vivo and that this suppression was negated during in vitro passage. In vivo suppression of retrovirus expression has been observed in other systems. ST-FeSV-induced fibrosarcomas in marmosets are negative for virus, but when explants of tumors are cultured in vitro, virus production occurs after several passages (21). Fetal puppies, which are not yet immunocompetent, develop virus-producing fibrosarcomas (6). In contrast, neonatal puppies, which are immunocompetent, develop virus-negative fibrosarcomas. Cells from ST-FeSV-induced fibrosarcomas in neonatal puppies will produce virus, however, when cultured in vitro. The suppression of virus production in heterologous versus homologous species and in neonatal versus fetal puppy tumors suggests that host immunity is involved with in vivo virus suppression. Retrovirus immunity in cats is also associated with the conversion of an active, productive infection to a latent, nonproductive one (17). Likewise, most of our cats which recovered from FeSV-FeLV-helper infections had latent retrovirus infection in their bone marrow. In three cats in this study, FeLV-helper was latent in bone marrows and tumors, while FeSV was present as a latent infection only in the tumor cells. This observation was additional evidence for a dichotomy in FeSV and FeLV-helper immunity within the same host.

The rate of tumor recurrence was much higher in virus-positive cats than in virus-negative cats (75 versus 6%), indicating that persistent retroviremia somehow enhanced tumor recurrence. This enhancement may have involved the facilitation of FeSV infection of normal cells by FeLV-helper, or it may have involved some immunosuppressive effect of the FeLV-helper infection on established FeSV-related immunity. Such an immunosuppressive effect could have resulted from nonspecific interactions between the virus and different lymphoid cell populations, or it could have resulted from specific immunosuppressive effects of FeLV proteins such as p15E (15). Indeed, FeLV p15E will enhance tumor growth if it is injected into cats before FeSV challenge (15).

The immunology of recurrent tumors is an interesting area of study. From what is known about the cats reported here, a few assumptions can be made. First, immunological events leading to suppression of retrovirus replication are separate from those involved in tumor regression. This conclusion is identical to that of deNoronha and co-workers (3), who also worked on ST-FeSV-induced sarcomas. Two of the cats reported in this study developed tumors and a transient retrovirus infection after FeSV challenge. The tumors continued to grow and metastasize for 2 to 6 weeks after all retrovirus expression was suppressed, again suggesting a dichotomy in tumoral and viral immunity. Tumors also recurred in three nonviremic cats with regressed tumors. Although it is remotely possible that these tumors were spontaneous or due to virus activation of new oncogenic genes, we believe that they were somehow related to the original ST-FeSV inoculation. The new tumors could have resulted from reinfection of fibroblasts by virus that persisted in the body. The recurrence of tumors was not associated, however, with a systemic activation of a latent retrovirus infection, even though such a latent infection was present in all of these animals.

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