Serum antibodies against feline oncornavirus-associated cell membrane antigen in cats bearing virally induced uveal melanomas

Jerry Y. Niederkorn, John A. Shadduck, Daniel Albert, and Myron Essex

The humoral immune response to a virally induced feline model of human uveal melanoma was studied by examining sera from cats bearing iridal and choroidal melanomas for antibody to the feline oncornavirus-associated cell membrane antigen (FOCMA). Anti-FOCMA antibodies appeared earlier and in higher titers in cats that ultimately expressed nonprogressing uveal melanomas. By 8 weeks after virus inoculation 56% of the cats with nonprogressing lesions had anti-FOCMA antibodies, whereas only 7% of the cats with progressive tumors produced demonstrable antibodies against FOCMA. Mean serum antibody titers were approximately 10 times higher in cats with nonprogressive lesions than in cats with progressive tumors. Similar trends were observed throughout the 28-week study period. Early appearance and a high titer of serum antibody against FOCMA correlated with nonprogressing uveal melanoma. The results indicated that uveal melanomas could be immunogenic and successfully managed by the host's immune system.

Key words: feline uveal melanoma, immune surveillance, indirect membrane immunofluorescence, chromium-51 release, feline oncornavirus-associated cell membrane antigen (FOCMA)

Humoral and cell-mediated reactivity against human ocular melanoma-associated antigens have been demonstrated. Results from immunofluorescence studies of sera from melanoma patients are of limited clinical use because of exceptionally high frequencies of false-positive results, and assays for cell-mediated reactivity against human ocular melanoma-associated antigens are presently unrefined for clinical use. Moreover, it is presently impossible to study accurately temporal events in the pathogenesis of human ocular melanomas, particularly in a prospective manner.

We recently have developed a feline model of uveal melanoma in which intraocular injection of kittens with the Gardner strain of feline sarcoma virus (GFeSV) results in the production of a spindle-cell pigmented lesion that closely resembles human spindle-cell melanomas. The in situ transformation and the viral etiology of this uveal melanoma permit a wide range of experimental manipulations.
specifications useful for studying the immune surveillance of pigmented ocular neoplasms.

The feline oncornavirus-associated cell membrane antigen (FOCMA) is a tumor-specific cell surface marker expressed specifically on tumors induced by feline leukemia virus (FeLV) or feline sarcoma virus (FeSV). Cats with high antibody titers to FOCMA are resistant to FeLV- and FeSV-induced tumors. Antibodies to FOCMA have been demonstrated not only by membrane immunofluorescence but also by complement-dependent lysis of FOCMA-bearing FL-74 target cells. Close correlation of tumor rejection and detection of lytic antibodies suggest that immune surveillance of feline lymphoid tumors is mediated through lysis by complement-dependent antibodies.

In the present study we examined the serum of cats bearing FeSV-induced uveal melanomas for the presence of antibodies to FOCMA and the relationship between FOCMA antibody titers and tumor growth and behavior.

Materials and methods

Infection of cats. Random-bred domestic cats were acclimated to the laboratory environment for 3 to 6 weeks, examined clinically, and vaccinated against respiratory diseases and feline panleukopenia. Peripheral blood leukocytes were examined by fixed-cell immunofluorescence for feline leukemia viral antigens. Cats were induced into estrus by light cycling, bred, and housed in isolation until parturition. Kittens, 10 to 15 days old, were injected with 0.05 ml of a concentrated suspension of GFeSV (Electronucleonics Inc., Bethesda, Md.) via a 30-gauge needle inserted at the limbus near the filtration angle. Serum was collected immediately before inoculation with GFeSV and at weekly intervals thereafter. The mean induction time for these melanomas is about 4 to 6 weeks. The size and progression of the lesions were recorded by measuring the vertical and horizontal dimensions of the tumor nodules as they were recorded in photographs. The product of these two dimensions was calculated, and the size of the lesions were graphed against this arbitrary scale.

Cells. Three mink cell lines were used as targets in immunofluorescence tests. Line 64F3CL7 is FeSV-transformed, a nonproducer, FOCMA-positive and negative for viral proteins (p30 and gp70). CCL-64A is FeLV-infected, nontransformed, FOCMA-negative, gp70- and p30-positive and releases virus. Normal mink lung cells (CCL-64) do not express FOCMA or viral structural proteins and were used as controls. All mink cell lines were maintained in Dulbecco's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum. The FL-74 cell line, derived from a lymphoid neoplasm from a FeLV-infected cat, has abundant FOCMA and was used for titering serum antibodies against FOCMA. Suspension cultures were grown in McCoy's 5a medium containing 20% heat-inactivated fetal calf serum as previously described.

Serologic dilutions. Sera were heat-inactivated (56°C/30 min) and used undiluted and in doubling serial dilutions of 1:10, 1:20, through 1:1280. Titers were expressed as the arithmetical mean. Negative results were recorded as titer of zero.

Indirect membrane immunofluorescence (IMI) test. The IMI test for FOCMA antibody has been described in detail elsewhere. Normal mink cells (CCL-64), 64F3CL7 mink cells, and CCL-64A mink cells were removed from culture flasks by gentle trypsinization. FL-74 cells were concentrated by centrifugation from suspension culture. All cells were washed twice with Hanks balanced salt solution, and the volumes were adjusted to 5 x 10⁵ cells/ml. Cells were then tested separately by incubation (37°C, 45 min) with 0.1 ml of each serum dilution. Cells were washed four additional times and incubated (37°C, 45 min) with fluorescein-labeled goat anti-cat IgG (Cappel Laboratories, Cochranville, Pa.) diluted 1:30. Cells were washed twice and examined by fluorescence microscopy. Samples were scored positive when 50% or more of the cells fluoresced.

3¹Cr-release test. A 2 hr 3¹Cr-release assay for detecting complement-dependent cytotoxic antibodies against FOCMA has been described. Maximum release from labeled FL-74 cells was determined by lysis of cell samples by repeated (three times) freezing and thawing. Spontaneous release was determined in samples containing normal cat serum and rabbit serum as a source of complement. Percent of specific release was calculated as follows:

\[
\text{Percent specific release} = \frac{\text{Experimental count} - \text{spontaneous count}}{\text{Maximum count} - \text{spontaneous count}} \times 100
\]

Lysis was considered to have occurred in test sample dilutions when specific release exceeded spontaneous release by more than 10%.
Table I. Relationship between tumor behavior and the appearance of FOCMA antibodies

<table>
<thead>
<tr>
<th>Tumor status (no. of cats)</th>
<th>Geometric mean antibody titer*</th>
<th>No. of cats never positive by IMI or ¹⁵⁹Cr tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;8 weeks after virus inoculation</td>
<td>&gt;8 weeks after virus inoculation</td>
</tr>
<tr>
<td>Progressive tumors (13)</td>
<td>IMI = 1:1.6 (7%)</td>
<td>IMI = 1:1.5 (23%)</td>
</tr>
<tr>
<td></td>
<td>¹⁵⁹Cr = 1:4 (30%)</td>
<td>¹⁵⁹Cr = 1:50 (38%)</td>
</tr>
<tr>
<td>Nonprogressive tumors (16)</td>
<td>IMI = 1:14 (56%)</td>
<td>IMI = 1:55 (75%)</td>
</tr>
<tr>
<td></td>
<td>¹⁵⁹Cr = 1:43 (69%)</td>
<td>¹⁵⁹Cr = 1:120 (75%)</td>
</tr>
</tbody>
</table>

¹⁵⁹Cr-release assay using rabbit complement and a 2 hr incubation period.

* Percent of cats in parentheses; cat sera were scored positive when the antibody titer was 1:10 or greater.

Table II. Comparison of ¹⁵⁹Cr-release and immunofluorescent antibody titers of all serum samples using FL-74 target cells*

<table>
<thead>
<tr>
<th>Tumor behavior</th>
<th>No. of samples</th>
<th>No. positive by either IMI or ¹⁵⁹Cr tests</th>
<th>No. positive by IMI test only</th>
<th>No. positive by ¹⁵⁹Cr test only</th>
<th>No. positive by both IMI and ¹⁵⁹Cr tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonprogressive</td>
<td>86</td>
<td>53 (61%)</td>
<td>5 (9%)</td>
<td>4 (22%)</td>
<td>44 (83%)</td>
</tr>
<tr>
<td>Progressive</td>
<td>71</td>
<td>26 (36%)</td>
<td>5 (19%)</td>
<td>14 (54%)</td>
<td>7 (27%)</td>
</tr>
</tbody>
</table>

* Data recorded as either positive or negative with serum diluted 1:10 and disregarding absolute antibody titer.

Results

Tumor induction and progression. Cats with progressive lesions (13 of 29 cats) had tumors that began as small, flat to slightly elevated, hyperpigmented lesions at the base of the iris. The lesions typically were first detectable when the kittens were 35 to 49 days old and enlarged steadily from that time. The lesions continued to enlarge and filled the anterior chamber. The eye often became severely buphthalmic, and some cats had to be killed at this stage because of keratoconjunctivitis and other complications.

Nine of the 13 cats with progressing tumors developed neoplastic lesions outside the orbit. Skeletal muscle was the most common site for these secondary tumors. Tumors in both eyes from one cat were classified as late progressors. These tumors developed slowly over a period of several months. They ultimately enlarged enough to severely distort the anatomy of the filtration angle, iris, and ciliary body; however, they did not fill the anterior chamber, nor did they result in buphthalmus. This cat did not develop secondary tumors.

Cats with tumors classified as nonprogressive (16 of 29 cats) had small, flat, pigmented plaques on the iris near the site of injection. The lesions enlarged slowly and in proportion to the growing eye. In the first 5 to 7 weeks after injection it was not possible to distinguish between cats whose flat pigmented plaques would remain static and cats that would develop progressive tumors. By 8 to 10 weeks the clinical course could be predicted. Cats whose lesions were discrete, flat, and discoid with heavy concentrations of...
Fig. 1. Independent fluctuation of immunofluorescent and cytotoxic (⁵¹Cr-release) antibody titers during uveal melanoma progression. Serum was collected at weekly intervals after virus inoculation and examined by IMI and ⁵¹Cr-release tests using FL-74 target cells. ○, Complement-dependent cytotoxic antibody titers (⁵¹Cr-release); ●, immunofluorescent antibody titers.

pigment did not develop progressive tumors, and no secondary tumors were detected.

Immunofluorescent and cytotoxic antibody studies. There was a strong correlation between tumor behavior and the appearance of serum antibodies against FOCMA (Table I). Fourteen of 16 (88%) cats with nonprogressive uveal tumors produced demonstrable immunofluorescent or cytotoxic antibodies during the entire study period, but eight of 13 (61%) cats with progressive tumors had no antibody in undiluted sera.

The first appearance of anti-FOCMA immunofluorescent or cytotoxic antibodies correlated with subsequent tumor behavior. By 8 weeks after virus inoculation only one of the 13 (7%) cats with progressive tumors produced antibodies detected by IMI test. Four of the 13 (30%) cats with progressive tumors had measurable levels of cytotoxic antibodies. By contrast, nine of the 16 (56%) cats with nonprogressive tumors had antibodies detected by IMI test, and 11 of the 16 (69%) cats had cytotoxic antibodies by 8 weeks.

Immunofluorescent and cytotoxic antibody titers were significantly higher than those found in cats bearing progressive uveal tumors (Table I). By 8 weeks after virus inoculation, the mean immunofluorescent antibody titer was 1:1.6 in cats with progressive tumors, but the mean titer of the nonprogressive tumor group was 1:14. The mean cytotoxic antibody titer in cats with progressive tumors was 1:4, and that of nonprogressive tumor cats was 1:43.

The mean fluorescent antibody titer for cats with progressive uveal tumors remained approximately the same throughout the entire study period, but the mean cytotoxic antibody titer rose from 1:4 at 8 weeks after virus inoculation to 1:50 in the latter weeks. Cats with nonprogressive uveal tumors had consistently high cytotoxic antibody titers ranging from a mean titer of 1:43 at 8 weeks.
Table III. Relationship of secondary tumors, anti-FOCMA antibody, and eye tumor behavior*

<table>
<thead>
<tr>
<th>Tumor behavior</th>
<th>IMI</th>
<th>51Cr</th>
<th>Secondary tumors†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive</td>
<td>+</td>
<td>+</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>1/8</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>−</td>
<td>0/11</td>
</tr>
<tr>
<td>Nonprogressive</td>
<td>+</td>
<td>+</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>−</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>−</td>
<td>0/2</td>
</tr>
</tbody>
</table>

IMI = indirect membrane immunofluorescence test; 51Cr = 51Cr-release tests.
*With FL-74 target cells.
†No. of cats with secondary tumors/no. of cats.

Table IV. Immunofluorescent detection of antibodies against FOCMA and viral structural proteins

<table>
<thead>
<tr>
<th>Tumor behavior (No. of cats)</th>
<th>Mean antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FL-74B 64F3CL7C CCL-64A</td>
</tr>
<tr>
<td>Nonprogressive (5)</td>
<td>20  24  4</td>
</tr>
<tr>
<td>Progressive (5)</td>
<td>2    4    0</td>
</tr>
</tbody>
</table>

Indirect membrane immunofluorescence test.
*FOCMA pos; gp70+ and p30+.
*FOCMA pos; gp70− and p30−.
*FOCMA neg; gp70+ and p30+.

after virus inoculation to 1:120 at the end of the study period.

When FL-74 cells were used as targets and the IMI and 51Cr-release test results for all 157 serum samples scored positive or negative without consideration of titer, there was a strong correlation (83%) between these two tests in cats demonstrating nonprogressive tumors but only a slight correlation (27%) in the cats with progressive tumors (Table II).

The disparity in 51Cr-release and immunofluorescent antibody titers for individual serum samples from cats with progressive tumors was further reflected by the independent fluctuation in titers during the study (Fig. 1).

Anti-FOCMA antibodies and appearance of secondary tumors. The subsequent appearance of secondary tumors correlated with the behavior of the uveal tumor (Table III). None of the cats presenting nonprogressive uveal tumors developed secondary tumors, but nine of the 13 (69%) cats with progressive uveal tumors did. Four of nine cats with progressive tumors and secondary tumors had cytotoxic antibodies that appeared rapidly when compared with the antibody profiles of cats with nonprogressive uveal tumors. Three of the four cats with progressive tumors and cytotoxic antibodies had secondary tumors. One cat with progressive uveal tumors had cytotoxic antibodies but no secondary tumors and was classified as a late progressor. Moreover, it cannot be assumed that this cat was free of secondary tumors, since neoplastic foci might have escaped observation at necropsy.

Detection of anti-FOCMA and antiviral antibodies. FL-74 cells express viral structural proteins (gp70 and p30) as well as abundant structural antigens. To distinguish between anti-FOCMA and antiviral antibodies, cat sera were tested by IMI tests using FL-74, 64F3CL7, CCL-64, and CCL-64A cells.

Table IV shows that cat serum containing antibodies against FL-74 cells also reacted strongly with FOCMA-bearing 64F3CL7 cells and, to a lesser extent, with CCL-64A cells, which express viral structural proteins but no FOCMA. Immunofluorescent antibody titers against FOCMA were always higher than antibody titers against viral structural proteins. Antiviral antibodies were never detected in serum lacking anti-FOCMA antibodies. Immunofluorescent antibodies were not demonstrated in normal mink cells (CCL-64).

Discussion

The purposes of this study were to determine whether cats bearing GFeSV-induced uveal melanomas produced antibodies against FOCMA and whether there was a correlation between FOCMA antibody and tumor behavior.

The results indicate that antibodies detected by IMI and 51Cr-release tests were largely against FOCMA. There was a close correlation between the initial appearance and titer of antibody and subsequent tumor...
behavior. Antibodies measured by immunofluorescence and $^{51}$Cr-release appeared sooner and in higher titers in cats that ultimately had uveal melanomas which did not progress. By contrast, 61% of the cats bearing progressive tumors never produced detectable antibody titers.

The poor correlation in $^{51}$Cr-release and immunofluorescent antibody titers in many of the sera from cats bearing progressive uveal tumors might be ascribed to the rabbit complement we used in a standard $^{51}$Cr-release test. More accurate correlation between IMI and cytotoxicity tests for anti-FOCMA antibodies can be derived if autologous (i.e., cat) serum is used as a complement source. We have subsequently examined available sera by IMI and $^{51}$Cr-release tests and have demonstrated improved correlation in the results of these two tests when cat serum was used as a complement source (data not shown).

The absence of secondary tumors in cats bearing nonprogressive tumors suggests that immune surveillance was operative. In the present system the roles of antibodies against viral structural proteins (gp70 and p30) and anti-FOCMA antibodies in this surveillance are unclear. Antiviral and/or anti-FOCMA antibodies might act independently or in concert. An effective anti-FOCMA response might lyse intravascular metastases or metastatic foci at peripheral sites. Antibodies against viral structural proteins might have prevented viremia and subsequent transformation at secondary sites. The latter suggestion is less likely because cats that resist the development of FeSV-induced progressive fibrosarcomas and produce high titers of serum antibodies against FOCMA may still maintain persisting viremia. It also should be noted that immune surveillance by cellular modalities has not been examined in this model system. Nonetheless, there was a close correlation between the presence of anti-FOCMA antibodies and the absence of secondary tumors.

Three cats with progressive uveal melanomas and secondary tumors also had cytotoxic antibodies. A combination of factors might have culminated in the production of cytotoxic antibodies in cats harboring progressive uveal melanomas. Since FeSV is defective and is always accompanied by helper-FeLV, all our GFeSV intraocular inocula contained excess FeLV. FeLV is immunosuppressive and may have induced transient immunosuppression in some cats. It has been demonstrated that primary presentation of antigen into the anterior chamber resulted in a modulated immune response in which cell-mediated functions were transiently suppressed, due possibly to the production of enhancing antibodies or suppressor T-lymphocytes. It is possible that the initial presentation of uveal tumor antigens via the anterior chamber resulted in a deviated cellular immune response. This anterior chamber–associated immune deviation may have acted in concert with or apart from FeLV-induced immunosuppression to permit tumor progression. Secondary tumors appearing as a consequence of metastasis and/or extraocular viral transformation then might have stimulated antibody formation. This "slipping through" hypothesis, which has been offered in other tumor systems, would suggest that secondary tumors were either intrinsically more immunogenic than intraocular tumors or that the extraocular milieu provided a setting more conducive for inducing cytotoxic antibody production. An overwhelming intraocular and secondary tumor burden would negate the effectiveness of cytotoxic serum antibodies in cats with progressive tumors. More persistent immunosuppression may be the reason for the absence of cytotoxic antibodies in the remaining cats expressing secondary tumors.

With the feline analog of human uveal melanoma, we found that neoplastic uveal cells, transformed in situ, stimulated the host's systemic immune response in spite of the immunologically unique nature of the anterior chamber of the eye. Moreover, complement-dependent antibodies produced in response to these uveal melanomas exercised an immune surveillance function that could be demonstrated in vitro and observed as a prognostic indicator in vivo. The occurrence...
and significance of ancillary immune modalities such as cytotoxic T-lymphocytes, natural killer cells, or tumoricidal macrophages has not been explored in this system. Nonetheless, the results indicate that virally induced uveal melanomas can be immunogenic and successfully managed by the host’s immune system.

We thank Mr. Warren Benoy, Ms. Mary Jean Geroulo, Mr. Ron Hervey, and Ms. Mary McGee for excellent technical assistance and Mrs. Mary Kay Douglass for secretarial help.

REFERENCES


