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Immunology

Remission of leukemia and loss of feline leukemia virus in cats injected with *Staphylococcus* protein A: Association with increased circulating interferon and complement-dependent cytotoxic antibody

(immunotherapy/humoral immunity)

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ABSTRACT **We have injected purified *Staphylococcus aureus* protein A intraperitoneally into leukemic cats infected with feline leukemia virus, into cats persistently infected with feline leukemia virus but without hematologic or cytologic abnormalities, and into healthy cats without feline leukemia virus infection. Pre- and post-treatment serum samples were evaluated sequentially for interferon activity and for complement-dependent cytotoxic antibody. Our results indicate that serum interferon increased dramatically (<3 to 324 units/ml) during treatment only in cats that responded to staphylococcal protein A therapy. Increase of interferon preceded or was closely associated with increasing levels of cytotoxic antibody, loss of viremia, and correction of cytological and hematological abnormalities of three leukemic cats. The cytotoxic antibody was shown to be specific for envelope glycoprotein gp70 of the feline leukemia virus. One persistently feline leukemia virus-infected cat without leukemia that became nonviremic also developed high levels of interferon and specific cytotoxic antibody. By contrast, interferon levels of cats not responding to treatment had levels of <3 to 27 units/ml. Normal healthy cats injected with staphylococcal protein A showed moderate transient increases of interferon but no detectable cytotoxic antibodies to FL-74 cells. These data suggest that interferon and cytotoxic antibody may play important, possibly complementary roles in inducing remission of leukemia and loss of viremia in cats treated with staphylococcal protein A.**

Remission of leukemia–lymphoma associated with disappearance of feline leukemia virus (FeLV) in cats treated by *ex vivo* immunoadsorption using *Staphylococcus aureus* Cowan I strain organisms (1, 2) and *Staphylococcus* protein A (SPA) bound to filters has been reported (2). The remission of leukemia with either treatment was associated with sustained high levels of a circulating complement-dependent cytotoxic antibody against a feline lymphoma cell line (FL-74) (3). Using monoclonal antibodies prepared against FeLV and FL-74 cells,† we demonstrated that the cytotoxic antibody was directed against the viral envelope glycoprotein gp70 (3). In the present report, we show that remission of leukemia and loss of viremia can frequently be induced in cats infected with FeLV with or without leukemia by intraperitoneal (i.p.) injections of SPA. Since SPA induces γ interferon (IFNγ) *in vitro* (4, 5), IFN activity and complement-dependent cytotoxic antibody were studied in sera of several cats that had been injected i.p. with SPA. It will be shown that regression of malignancy and loss of evidence of virus infection occurred in cats that experienced first an increase in IFN that was followed by appearance and usually progressive increase of a complement-dependent cytotoxic antibody directed against a virus-infected feline lymphoma cell line.

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MATERIAL AND METHODS

Animals. Pet cats were selected for treatment by *ex vivo* immunoadsorption (2, 3) or by injection with purified SPA (Pharmacia, Uppsala, Sweden). The SPA injections were given i.p. (20 μg/2.75 kg of body weight) twice weekly for 10–12 weeks. Bone marrow and peripheral blood smears were evaluated prior to treatments and after every 10th treatment. FeLV status was determined biweekly by indirect immunofluorescence assay (IFA) (6) and enzyme-linked immunosorbent assay (ELISA) as described (2). Pre- and post-treatment serum samples were stored at −70°C until assayed for IFN and cytotoxic antibody.

Feline IFN Assay. Feline IFN was assayed by a modification of the microplaque reduction method (7), using approximately 40 plaque-forming units (pfu) of vesicular stomatitis virus (VSV) per well on FFC-9 cells (fetal cat fibroblast cell line kindly provided by N. C. Pedersen, University of California, Davis). An IFN concentration of 1 unit/ml was defined as the concentration required to decrease the number of pfu per well by 50%. All serum samples were assayed in duplicate, and the IFN titers represent the average of the two determinations. For all IFN assays, *Staphylococcus aureus* enterotoxin A (SEA)-induced feline IFN and Newcastle disease virus (NDV)-induced feline IFN were used as reference feline IFNs for monitoring the assays.

***In Vitro* Induction of Feline IFNs by SEA or NDV.** SEA (Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, OH) and NDV (American Type Culture Collection, Rockville, MD) were used for the *in vitro* induction of IFNs (8, 9). For the preparation of SEA-induced IFN, normal cat peripheral blood lymphocytes, isolated by centrifugation of heparinized blood through a Ficoll/Hypaque gradient (Pharmacia), were incubated with SEA (0.5 μg/ml) at a concentration of 5×10^6 cells per ml in 35-mm Falcon culture dishes. Cultures were maintained at 37°C in culture boxes gassed with 10% CO_2/7% O_2/83% N_2 for 3 days on a rocking platform (8 cycles per min). The supernates from these cultures containing feline IFN were stored in aliquots at −70°C until used. For induc-

Abbreviations: IFN, interferon; NDV, Newcastle disease virus; SEA, *Staphylococcus aureus* enterotoxin A; pfu, plaque-forming units; FeLV, feline leukemia virus; gp70, glycoprotein of molecular weight 70,000; SPA, *Staphylococcus aureus* protein A; VSV, vesicular stomatitis virus; MAb, monoclonal antibody; i.p., intraperitoneal(ly).

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tion of feline IFN by NDV, FCC-9 cells were grown in minimal essential medium supplemented with 10% fetal calf serum (GIBCO) and Garamycin (GIBCO) at 40 μg/ml in 75-cm^2 plastic flasks (Falcon) until the monolayer became confluent. The culture medium was then replaced by 3 ml of minimal essential medium containing NDV (approximate titer, 2.5×10^4 50% egg-infection dose units). Virus was allowed to be adsorbed for 1 hr before addition of 15 ml of fresh culture medium. The monolayer was maintained for 24 hr. The culture medium was then centrifuged at 20,000 rpm for 45 min (Beckman JA-21 fixed-angle rotor) to remove the virus. The supernate containing feline IFN was stored in aliquots at −70°C until used.

Characterization of Serum IFNs in Cats. Feline IFNs were characterized on the basis of pH 2 and heat lability (56°C for 30 min) (8, 10). IFN preparations were adjusted to pH 2 with 1 M HCl, incubated at 4°C for 24 hr, and then adjusted to pH 7.0 with Hepes buffer (GIBCO) prior to the IFN assay.

Electroblot Analysis of Antibodies to FeLV Antigens in Cat Serum During SPA Treatment. Electroblot analysis of antibodies of FeLV antigens in cat serum during SPA treatment was carried out as described before (3). Cytotoxic antibody was determined as described before (3).

Inhibition of Cytotoxic Activity in Cat Serum. Monoclonal antibodies (MAbs) to FeLV and FL-74† were used as described (3) in studies of inhibition of cytotoxic antibody. Three of these MAbs (7.2, 73.5, and 6.5) were used in the inhibition of cytotoxicity assay as described previously (3).

Inhibition of Cytotoxic Activity in Cat Serum Against FL-74 Cells by Using an $F(ab')_2$ Fragment of Goat Antibody to FeLV gp70. $F(ab')_2$ fragment of goat antibody to FeLV gp70 was isolated by gel filtration on a Sephadex G-150 (Pharmacia) column (1.5 × 110 cm) as described previously (11). The $F(ab')_2$ fragment was concentrated and analyzed by NaDodSO$_4$/PAGE and electroblot for molecular weight determination as well as for reactivity with FeLV gp70 in purified FeLV. Inhibition of cytotoxicity assays by using the polyclonal $F(ab')_2$ fragment of anti-FeLV gp70 was performed as described above.

RESULTS

Responses of Persistently FeLV-Infected Cats with or Without Leukemia to SPA: Remissions of Leukemia and Loss of Viremia. Eight cats persistently infected with FeLV (three preleukemic, one erythroleukemic, and four without leukemia) and six healthy control cats not infected with FeLV were injected i.p. with purified SPA. Four persistently infected cats were classified by bone marrow cytology as follows: one cat (152) had frank erythroleukemia, and three cats (153, 154, 155) were preleukemic. Preleukemia is a syndrome characterized by a group of severe hematologic and cytologic abnormalities often associated with anemia, leukopenia, and thrombocytopenia. Some of these animals show a tendency to develop frank leukemia with time (12).

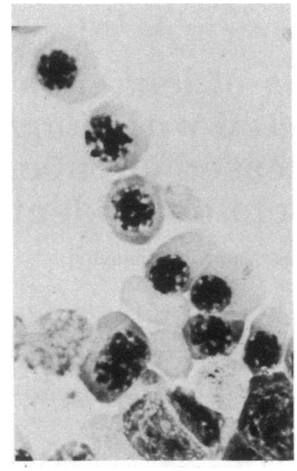

FIG. 1. High magnification (×800) view of numerous megaloblastoid rubricytes and metarubricytes present in pretreatment bone marrow aspirates of cat 153. These dysplastic forms were not present in marrow aspirates after the 10th treatment.

After treatment with SPA, the following results were obtained. Two cats with preleukemia (153 and 154) and one cat with erythroleukemia (152) went into long-term remission lasting more than 7 months to date. The remissions were characterized by complete correction of the cytological and hematological abnormalities and loss of viremia. Bone marrow before treatment of cats 153 (Fig. 1) and 154 showed erythroid dysplasia, megaloblastosis, and erythroid dyscrasia. These abnormalities were completely corrected after the 10th and 15th injections of SPA into cats 153 and 154, respectively. Cat 152, which suffered from frank erythroleukemia, went into complete and lasting remission of the leukemia after 20 injections of SPA (Fig. 2). All cats, including the healthy cats, tolerated the i.p. injections of SPA without fever or hematologic changes (other than the response of the leukemia) or manifestations of ill health.

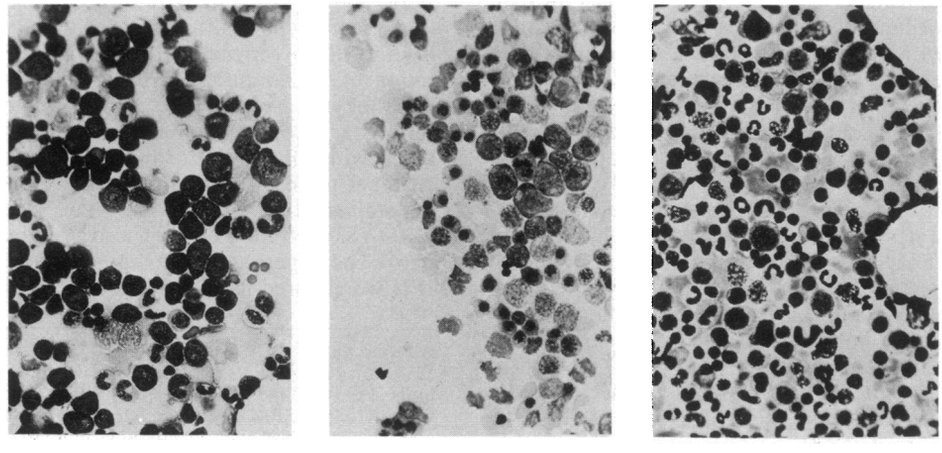

FIG. 2. Bone marrow cytology of cat 152 with erythroleukemia. (×360.) (*Left*) Pretreatment. Excessive numbers of rubriblasts and prorubricytes and inappropriately low numbers of more mature erythrocytic precursors are present. (*Center*) After 10 treatments. Numerous megaloblastoid rubricytes and metarubricytes are present and the blast population is reduced from pretreatment numbers. (*Right*) Post-treatment. A heterogenous population of cells is present without significant abnormalities.

Table 1. IFN activity in pet cats treated with SPA

Time after initial treatment, weeks	IFN activity, units/ml					
	Responder cats		Nonresponder cats*		Healthy cats†	
	Cat 154	Cat 155	Cat 160	Cat 161	Cat 109	Cat 110
1	<3	22	9	17	27	6
2	81	71	<3	<3	9	20
3	162	324	<3	<3	<3	19
4	7	72	<3	<3	9	27
5	9	14	<3	<3	<3	20
6	9	18	<3	<3	9	16
7	243	37	<3	<3	81	38
8	9	<3	<3	<3	9	11
9	<3	ND	<3	<3	<3	<3
10	<3	27	<3	<3	<3	6

ND, not done.
*Representative of 9 FeLV-infected cats with or without leukemia.
†Representative of 10 FeLV-negative healthy cats.

Serum IFN Activities of Healthy FeLV-Negative and Persistently FeLV-Infected Cats with and Without Leukemia After SPA Treatment. Two of the six healthy cats showed moderate transient elevations of IFN at one point in the successive twice-weekly series of injections of SPA (Table 1). In contrast, the responder cats that were cured or became virus negative regularly showed impressive and sometimes persistent elevations of serum IFN levels, illustrated for two representative cats (154 and 155) in Table 1. By contrast, the cats that did not become virus negative, represented by cats 160 and 161 (Table 1), had undetectable levels of IFN after SPA injections.

Characterization of Serum IFNs. The properties of serum IFN detected in pet cats during SPA injection (153, 154, 155) are presented in Table 2. Cat 101 with leukemia (Table 2), described previously (3), had been successfully treated by *ex vivo* immunoadsorption using SPA. This cat also developed high levels of serum IFN and was therefore included in this study. As shown, serum IFN from all cats (101, 153, 154, 155) was heat labile (56°C/30 min). A pH sensitivity test was performed on serum of cat 101 only, due to shortage of samples, and the IFN was shown to be sensitive. On the other hand, NDV-induced IFN was heat stable, while SEA-induced IFN lost its activity by approximately 60% after pH 2 or heat treatment. These data suggest that the serum IFN produced by these cats during treatment may have properties similar to those observed for SEA-induced IFN and hence likely to be IFNγ.

Relationships of Serum IFN and Complement-Dependent Cytotoxic Antibody, and FeLV Status of Cats Treated with SPA. Fig. 3 shows the relationships of serum IFN, cytotoxic antibody, and FeLV status of four cats (101, 153, 154, 155) that responded to treatment and/or displayed loss of viremia during SPA therapy (one by *ex vivo* immunoadsorption and three by injection). Whereas sera of cats 153 and 155 showed marked increase in IFN titers prior to appearance of free circulating cytotoxic antibody to FL-74 cells, the increase of IFN in sera of cats 101 and 153 was concomitant with increase of complement-dependent cytotoxic antibody (Fig. 3). Furthermore, increased IFN and increased cytotoxic antibody in sera of all cats appeared prior to clinical improvement and before disappearance of viremia. Cats that did not respond to therapy did not have increased IFN or cytotoxic antibody in their sera and remained infected with FeLV. Although the cat with frank erythroleukemia, which responded by undergoing remission after SPA injection, was not studied for IFN responses, a persistent elevation of the complement-dependent cytotoxic antibody occurred prior to regression of the leukemia.

Table 2. Effects of pH 2 and heat treatments on serum IFN activity in cats treated with SPA

Treatment	IFN activity remaining in sample, %					
	Cat 101	Cat 154	Cat 155	Cat 153	SEA-induced IFN*	NDV-induced IFN†
pH 2	20	ND	ND	ND	29	100
Heat	40	23	33	33	39	100
None	100	100	100	100	100	100

ND, not done.
*Normal cat peripheral blood lymphocytes at 5×10^6 cells per ml were incubated with SEA at 0.5 μg/ml for 3 days. Culture medium was measured for IFN activity by microplaque reduction assay.
†IFN was induced by incubating monolayer cell culture of feline fetal fibroblasts (FFC-9).

Electroblot Analysis of Antibodies of FeLV Antigens in Cat Serum During SPA Treatment. To further investigate the immune response of diseased cats against FeLV before and after SPA injection, we analyzed the antibodies developed against viral antigens in cat serum by the electroblot technique. The titers of antibodies to FeLV antigens increased dramatically with successive treatments in all responding cats, and this was concomitant with increases in cytotoxic antibody to FL-74 cells and associated with loss of viremia and remission of leukemia in most instances. In most responding cats tested, antibodies were first developed against FeLV proteins of molecular weights 70,000 and 58,000 and not to the major core protein, p27, until later in the course of treatment. Antibodies to FeLV antigens were either not demonstrable or showed no increase in titers during successive treatments in cats not responding to SPA therapy (figure not shown).

Inhibition of Cytotoxic Activity in Cat Serum Against FL-74 Cells by Using MAbs to FeLV or FL-74 Cells. MAbs 7.2, 73.5, and 6.5 were used in the assay of inhibition of cytotoxicity. The results of these assays are shown in Table 3. Incubation of FL-74 cells with cat serum (101, 155) in the presence of complement resulted in approximately 90% cell death. When the FL-74 cells were allowed to react with various dilutions of ascites fluid or its IgG fraction containing MAb 73.5 at 37°C for 30 min prior to the addition of cat serum positive for the cytotoxic antibody, complement-mediated lysis was strikingly abolished as determined by % cell death. Similarly, complement-dependent cytotoxic activities in serum of cats 153 and 155 were also dramatically diminished by prior incubation of FL-74 cells with MAb 73.5 (data not shown). By contrast, complement-dependent cytotoxic activity against FL-74 cells in the serum of one healthy FeLV-negative cat (249) could not be abolished by prior incubation of the cells

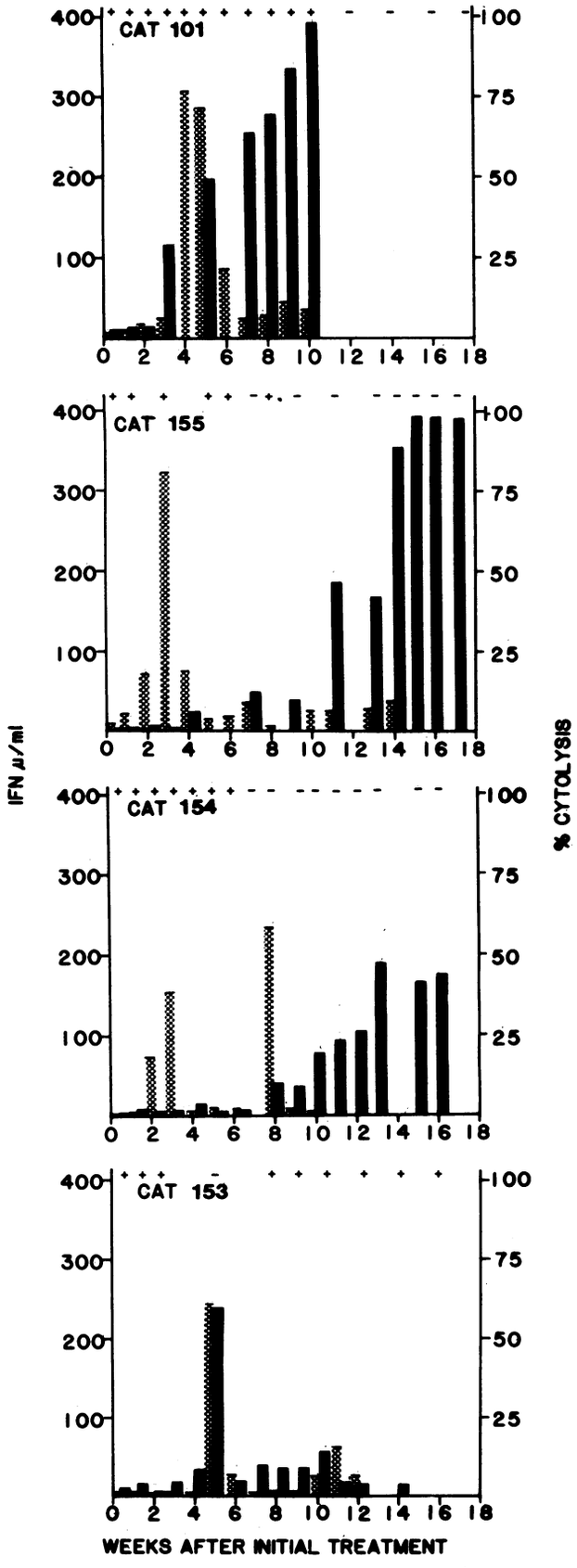

FIG. 3. Relationships of IFN and cytotoxic antibody in cats treated by SPA. The dotted bars represent serum IFN titers in units/ml. The closed bars represent the relative concentrations of cytotoxic antibody in serum. The + and − signs on top represent the viral status of the treated cats. Cats 153, 154, and 155 were treated by SPA injection. Cat 101 was treated by *ex vivo* immunoadsorption.

Table 3. Inhibition of complement-mediated cytotoxic activity in cat serum against FL-74 cells by using monoclonal and polyclonal antibodies

Cat serum	Anti-FeLV gp70	Dilution	Cytolysis,* %
101	MAb 73.5	0	95
	(ascites fluid)	1:50	27
		1:200	26
		1:500	34
		1:1000	34
		1:1500	80
155	MAb 73.5	0	91
	(IgG fraction)	1:500	26
		1:1000	31
155	Polyclonal	0	89
	$F(ab')_2$ fragment	1:20	28
		1:40	47
		1:200	71
		1:400	78
249†	MAb 73.5	0	49
		1:50	46
		1:100	43

MAb 7.2 against FeLV p27 core protein and MAb 6.5 against FL-74 cell membrane antigen failed to block cytotoxic activity of cat serum.

*Data represent average of duplicate measurements; variation between duplicates was less than 10%.

†Antibody reactive with normal feline cell antigens was detected in the serum of a healthy FeLV-negative cat. This cat had no antibodies against FeLV antigens.

with MAb 73.5 (Table 3). It was interesting to observe that the serum of this particular healthy cat was also cytotoxic to normal cat peripheral blood lymphocytes and normal cat erythrocytes in the presence of complement and that this cat had no demonstrable antibodies against FeLV antigens. MAbs 7.2 and 6.5 failed to abolish the cytotoxic activity against FL-74 cells of the serum of responding cats (101, 153, 155) (data not shown).

Inhibition of Cytotoxic Activity in Cat Serum Against FL-74 Cells by Using $F(ab')_2$ Fragment of Goat Antibodies to FeLV gp70. The $F(ab')_2$ fragment isolated from goat antiserum to FeLV gp70 was analyzed by $NaDodSO_4$/PAGE and electroblot technique for molecular weight determination as well as for reactivity with FeLV gp70 in a purified preparation of FeLV. The reactivity of this $F(ab')_2$ fragment with FeLV gp70 was also demonstrated (data not shown). The results of inhibition of cytotoxic activity against FL-74 cells in cat serum (155) by using $F(ab')_2$ fragment of anti-FeLV gp70 are shown in Table 3. Suppression of cytotoxicity was achieved in a dose-dependent manner when target cells (FL-74) were allowed to incubate with the $F(ab')_2$ fragment from goat antiserum to FeLV gp70 prior to reaction with cytotoxic antibody in cat serum.

DISCUSSION

Prior investigations have shown that, in several malignant diseases of experimental animals (1, 2, 13) or humans (14–16), regressions of disease may sometimes be produced by passing blood over columns containing SPA. In our studies *ex vivo* immunoadsorption with either whole organism *Staphylococcus* Cowan I protein A or SPA filters produced regressions in approximately 50% of pet cats suffering from FeLV-induced neoplasias. In approximately 18% of virus-infected leukemic cats, viremia also disappeared; 8% had sustained loss of viremia and appeared cured of leukemia (2, 3). Because leaching of staphylococcal products, SPA, or both was considered a possible mechanism in *ex vivo* immunoadsorption, the present studies were carried out by using twice-weekly i.p. injections of purified SPA (Pharmacia)

into FeLV-infected cats with and without leukemia and into healthy cats.

Two of three cats with preleukemia showed dramatic regression, and in one instance there was regression of a frank erythroleukemia. Most of the cats responding to i.p. injection of SPA also lost their viremia. Even in virus-infected cats without leukemia, loss of viremia occurred in one of four. Although definite conclusions are not yet possible, the data reveal that regression of neoplasia and loss of viremia occur in cats that respond with both transient increase of IFN and appearance in the blood of a complement-dependent cytotoxic antibody shown to be specific for envelope glycoprotein gp70 of the virus. The IFN at least in serum of one cat had characteristics of IFNγ. Future studies to determine this need to be carried out.

Neither the increased circulating IFN levels nor the cytotoxic antibodies appeared in healthy uninfected cats treated with SPA or cats that were nonresponsive to the SPA injections and maintained leukemia, viremia, or both in spite of the treatment. Just what role the IFN response plays in regression of the neoplastic disease and the virus infection and how its influence relates to the role of the cytotoxic antibody is unclear. Nonetheless, it is provocative to consider several properties of IFN that may be playing a role—i.e., its antiproliferative influences (17, 18), its capacity to activate macrophages and other effector cells (19, 20) that can inhibit neoplasia, and its increase of natural killer functions (21). Each of these possibilities needs investigation in future analyses. One of the most provocative aspects of the present investigations is that, once the neoplasms have regressed and a viral negative state has been achieved, the disease often to date has not recurred. This might be attributable to the antiviral or antitumor actions of the cytotoxic antibody or to both. These mechanisms, of course, would not exclude other mechanisms involving the cell-mediated immunities, which have not yet been studied.

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