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Pathogenesis of feline leukemia virus-induced erythroid aplasia: Hematologic, immunologic, and therapeutic studies

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PATHOGENESIS OF FELINE LEUKEMIA VIRUS-INDUCED ERYTHROID APLASIA: HEMATOLOGIC, IMMUNOLOGIC, AND THERAPEUTIC STUDIES

DISSERTATION

Presented in Partial Fulfillment of the Requirements of the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Philip M. Zack, D.V.M., Ph.D.

* * * * *

The Ohio State University

1987

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DEDICATION

To my father, for setting the standard.

To my mother, for helping me to see it.

To my wife, for helping me to reach it.

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I offer my thanks and acknowledgement to all who helped me during the previous three years: to my advisor and mentor, Dr. Gary Kociba, for his guidance, financial support and, most of all, friendship; to the U.S. Army Veterinary Corp. for providing me the opportunity to pursue these studies; to Dr. Charles Capen and the Department of Veterinary Pathobiology for offering a program of unparalleled excellence; to Drs. Maxey Wellman, Lawrence Mathes, Charles Brooks, James Blakeslee, Richard Olsen and Thomas Rosol for their technical assistance, advice, and philosophy; to Mr. Arthur McMillen and Mr. Joshua Halper for their technical assistance; to Ms. Jolene Reiter for her outstanding assistance, constant dependability and wonderful sence of humor; to Ms. Virginia Stump for her magical preparation of this and other manuscripts; and most of all, to my wife, Nancy, and daughters, Moira and Breanna, for their endless support, patience, understanding, and love throughout a very long journey.

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PUBLICATIONS

Lafrado L, Zack PM, Mathes L, Olsen R. (1987) Biological Effects of Staphylococcal Protein A Treatment of Feline Leukemia Virus-Infected Cats. Am. J. Vet. Res., In press.

DiBartola SP, Rutgers HC, Zack PM, Tarr MJ. (1987) Clinicopathologic Findings Associated with Chronic Renal Disease in Cats: 74 Cases (1973-1984). J. Am. Vet. Med. Assoc. 190:1196-1202.

Hultgren BD, Zack PM, Pearson EG, Kaneps AJ. (1987) Juvenile Granulosa Cell Tumor in an Equine Weanling. J. Comp. Path. 97:137-142.

Fossum TW, Sherding RG, Zack PM, Birchard SJ, Smeak DD. (1987) Intestinal Lymphangiectasia Associated with Chylothorax in Two Dogs. J. Am. Vet. Med. Assoc. 190:61-64.

Major Field: Veterinary Pathology

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CHAPTER I

ANTITHYMOCYTE GLOBULIN TREATMENT OF RETROVIRUS-INDUCED FELINE ERYTHROID APLASIA: *IN VIVO* AND *IN VITRO* STUDIES

INTRODUCTION

Feline leukemia virus (FeLV) is an exogenous type C retrovirus capable of inducing several cytoproliferative and cytosuppressive syndromes of the feline hemic-lymphatic system. The more common effects of the virus are severe immunosuppression, leukemia, lymphoma, myeloproliferative disease, and erythroid aplasia (EA) (2). There are three subgroups (serotypes) of FeLV, designated FeLV-A, FeLV-B, and FeLV-C. These subgroups are defined by virus-interference and neutralization, and reflect differences in the *env* gene product, gp70 (1). The presence or absence of specific subgroups alters the disease potential of a particular virus isolate. Only a few naturally occurring isolates are capable of inducing EA and these all contain FeLV-C (3,4,20). Although EA is associated with FeLV-C, biologically or molecularly cloned FeLV-C does not induce EA without the presence of FeLV-A except in newborn kittens (5,10,18). The Kawakami-Theilen strain of FeLV (FeLV-KT) contains a mixture of FeLV-A, B, and C subgroups. It consistently induces EA characterized by rapid depletion of erythroid progenitors without a corresponding

effect on the number of colony-forming units granulocyte-macrophage (CFU-GM) in the bone marrow (4,6,10,20). The pathogenesis of FeLV-induced EA is unknown. Possible mechanisms include direct effects of the virus on erythroid progenitors or critical accessory cells as well as immunologic elimination. EA caused by FeLV represents a unique model of virally induced selective inhibition of erythropoiesis.

Currently, 50% to 70% of human patients with aplastic anemia (AA) are successfully treated with antithymocyte globulin (ATG) (7,8,9). Additionally, there are several case reports describing successful treatment of pure red cell aplasia (PRCA) with ATG. (11,12,15,26). However, the mechanism of action of ATG is unknown, and attempts to establish pretreatment predictors for its success have, with the exception of one report, failed (13). Contrary to expectations the number of circulating lymphocytes, ratios of various subclasses of T-cells, or the presence of activated circulating lymphocytes are not useful in identifying patients likely to recover with ATG treatment (14,15). Although generally considered to exert its effect via immunosuppression or T-cell lysis, ATG may be able to stimulate hematopoiesis by augmenting release of growth factors from marrow accessory cells or by directly stimulating progenitors (16,17, 23).

The objective of these studies was to evaluate ATG prospectively as a treatment of PRCA in a reproducible model with an established retroviral etiology. We examined the effects of ATG <u>in vivo</u> on hematopoiesis in normal cats and in cats with FeLV-induced EA,

specifically quantifying erythrocytic and granulocytic progenitors, changes in marrow differential cell percentages, and alterations in hematologic parameters of the peripheral blood. Normal goat globulin (NG) was administered to anemic cats to evaluate the specificity of ATG-induced changes (21,22). In vitro studies were performed testing the ability of ATG or NG to enhance erythroid progenitor numbers from low density bone marrow mononuclear cells of cats infected with FeLV pre- and post-inoculation. Both continuous exposure incubation and short-term incubation with complement were used as treatments to attempt to reverse EA, to determine if autoreactive lymphocytes suppressing erythropoiesis are present, and to attempt prediction of responders prior to in vivo treatment.

MATERIALS AND METHODS

Animals. All cats were obtained at 6 to 8 weeks of age from a specific pathogen free (SPF) breeding colony maintained by the Department of Veterinary Pathobiology, The Ohio State University. This colony is uniformly free of infection and immunity to FeLV. The cats were tranquilized with intramuscular ketamine hydrochloride prior to bone marrow or blood collection and prior to treatment. Infected cats were pretreated with methyl prednisolone (5 mg/kg) to enhance their susceptibility to productive FeLV infection.

Preparation of Goat Anti-Feline-Thymocyte Globulin (ATG) and Goat Normal Globulin (NG). Thymuses from 4-6 month old SPF cats were collected asceptically, minced, and passaged through stainless steel mesh to obtain a single cell suspension. Thymocytes were washed three times in Hank's balanced salt solution (HBSS) (Gibco Laboratories, Grand Island, NY) and depleted of thymic macrophages and epithelial cells by incubation for one hour in a nylon wool column previously equilibrated with fetal bovine serum. Thymocytes were eluted and washed once with HBSS.

Pre-immunization serum was collected and pooled from two adult goats for preparation of NG. Each goat was injected intravenously with 10^7 thymocytes/kg on two occasions 14 days apart. The goat anti-feline thymocyte serum was collected 10 days following the second injection and pooled. The sera were heat-inactivated at 56°C for one hour and were fractionated by sequential precipitation with equal volumes of 80% and 90% saturated ammonium sulfate at pH 7.0 and

room temperature. The final precipitate was suspended in distilled water and dialyzed against 0.85% NaCl at pH 8 until ammonium sulfate could no longer be detected in the dialysate using barium chloride. Both ATG and NG were extensively adsorbed with packed feline red blood cells for *in vivo* use. Because of cytotoxicity for normal hematopoietic progenitors (25), portions of ATG and NG for *in vitro* experiments additionally were adsorbed against normal feline bone marrow mononuclear cells (BMMC) for *in vitro* experiments. Protein concentration of adsorbed ATG and NG was determined using the modified Biuret method (Dart Total Protein, Coulter Electronics, Inc., Hialeah, FL). Protein composition was determined by agarose electrophoresis (Corning Electrophoresis System, American Scientific Products, McGaw Park, IL). Both ATG and NG consisted of at least 85% gamma globulin.

ATG functional activity was tested using skin allografts and complement-dependent thymocyte cytotoxicity as previously described (24). ATG administered intramuscularly at 50 mg/kg daily for 5 days extended allograft viability to 20 to 24 days in two cats compared to 7 to 9 days in NG treated and untreated controls. Skin autografts were used as viability controls in all graft experiments. Cytotoxic activity was evaluated using serial twofold dilutions of ATG or NG in a microtiter plate, adding 5×10^4 heterologous feline thymocytes (prepared as described above), incubating for one hour, adding 10% rabbit complement in serum prescreened for low toxicity, and incubating at room temperature for 30 minutes. Trypan blue was added, 200 cells were counted, and the percentage of viable and dead cells

determined for each dilution. The specific cytotoxicity titer was defined as the inverse of the dilution at which 50% of thymocytes were killed. The cytotoxicity titer of ATG used *in vivo* exceeded 5120/mg. A titer of greater than 640 has been reported to be necessary for maximal immunosuppression (27). Two µg ATG adsorbed with BMMC consistently killed 100% of 1 x 10^5 feline thymocytes, while 100 µg NG failed to kill thymocytes in the cytotoxicity assay. Neither BMMC adsorbed ATG or NG suppressed normal bone marrow progenitor growth in clonal assays.

Viral Inoculum. The FeLV inoculum was prepared from equal volumes of serum and BMMC lysate pooled from cats with anemia induced by *in vivo* passage of FeLV-KT as described (20). The FeLV infectivity titer of the inoculum was assessed by the S+/L- focus induction assay as described previously (20). Cats were infected by intraperitoneal injection of 0.4 ml inoculum containing 1 x 10^5 FFU FeLV-KT.

Experimental Groups - *In Vivo* Studies. Six to 8 week old kittens from several litters were randomly assigned to one of four *in vivo* treatment groups. ATG group cats (n=4) were inoculated with FeLV-KT and treated with ATG after anemia developed; NG group cats (n=4) were inoculated with FeLV-KT and treated with NG after anemia developed; anemia control group cats (n=4) were inoculated with FeLV-KT and not treated; ATG normal group cats (n=3) were normal cats administered ATG. ATG or NG treatment of anemic cats was initiated between 4 and 6 weeks post-inoculation (PI) when an individual cat's packed cell volume (PCV) was less than 0.25. Both ATG and NG were administered intravenously at 50 mg/kg/day for 8 days followed by a

similar dosage once weekly for a total treatment time of 8 weeks. Transfusions of 20 ml of washed packed heterologous feline RBCs were administered when an individual cat's PCV was less than 0.10. No cat received more than two transfusions. Blood was collected pre-inoculation and weekly PI from each cat to perform complete hemograms (Coulter S plus IV, Coulter Electronics Inc., Hialeah, FL) and reticulocyte counts, and to evaluate circulating leukocytes for the presence of FeLV using an indirect immunofluorescence for FeLV p27 as modified by Hoover et al. (28). Bone marrow was aspirated pre-inoculation and biweekly PI and post-treatment from either the proximal humerus or the proximal femur. No bone was sampled more than twice and samples from the same bone were collected no less than 8 weeks apart. Samples were aspirated into a syringe containing 2 ml of 0.75% sodium citrate in phosphate buffered saline and placed immediately on ice.

Bone Marrow Cytologic Examination. Smears of bone marrow aspirates were made after each collection and stained with Wright-Giemsa stain. Five hundred nucleated cells were counted and designated as myeloid, erythroid, or lymphocytic. The erythroid precursors were quantified as the percentage of all nucleated cells counted excluding lymphocytes. Lymphocytes were counted as the percentage of all nucleated cells.

Low Density Bone Marrow Mononuclear Cell Separation (LDBMMC). Whole marrow cell suspensions were diluted to 20 ml with α-MEM (Gibco) in 16 x 95 mm polystyrene tubes and underlayered with an equal volume of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden)

adjusted to a specific gravity of 1.071. LDBMMC were collected from the interface after centrifugation for 30 minutes at 400 g, washed twice in HBSS, and resuspended in α-MEM.

In Vitro Studies. LDBMMC from cats infected with FeLV-KT were collected at preinoculation and two and four weeks PI prior to any in vivo treatments. For each continuous exposure study, 5 x 10^4 LDBMMC in 0.2 ml of α-MEM were incubated in 12 x 75 mm polystyrene tubes at room temperature for 1 hour with either 100 µg ATG, 100 µg NG, or α-MEM as a control. After incubation, cells were added to culture medium and plated to assay for erythroid progenitors as described below. The globulin concentration used was based upon preliminary studies indicating the maximum enhancement of erythroid progenitor numbers at 100 µg. For complement studies 5 x 10^4 LBDMMC in 0.2 ml of α-MEM were incubated in 12 x 75 mm polystyrene tubes at room temperature for 20 minutes with either 2 µg ATG, 2 µg NG, or medium as a control. Rabbit complement in serum prescreened for low toxicity was added to each tube to a final concentration of 10% and incubation continued for an additional 1/2 hour. The cells were then washed with 1 ml α-MEM and cultured for CFU-E and BFU-E as described below.

Progenitor Assays. The culture methods for colony forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) were similar to those described by Abkowitz et al. (19) for feline bone marrow. Briefly, 7.5 x 10^4 LDBMMC in 0.15 ml α-MEM were added to 1.35 ml of medium containing 1.3% methylcellulose (Dow-Corning, Midland, MI) in Iscove's Modified DMEM (Hazleton Research Products),

1% deionized bovine serum albumin (Armour Pharmaceuticals, Tarrytown, NY) heat-inactivated at 56°C for 30 minutes, 10^{-4}M beta mercaptoethanol, 30% fetal bovine serum (Armour Pharmaceuticals), sheep plasma erythropoietin (Step III, Connaught Laboratories, Swiftwater, PA) at one unit per milliliter, and 5% pokeweed mitogen conditioned medium from bone marrow mononuclear cells. The assays were performed in 35 mm cluster dishes (Costar, Cambridge, MA) with 5×10^4 cells in 1 milliliter of medium per well. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in nitrogen-enriched air. Erythroid colonies were counted on day 4 of culture as red colonies containing 8-50 cells (CFU-E) and on day 9 as red colonies composed of more than 200 cells, usually with closely associated subunits (BFU-E).

Colony-forming units-granulocyte/macrophage (CFU-GM) were quantified using a modification of our previously described method (20). Briefly, 2.5×10^5 LDBMMC in 0.1 ml α-MEM were added to 0.9 ml medium consisting of 0.5% methylcellulose in α-MEM, 0.4% bovine serum albumin, glutamine (2 mM), 55 U/ml penicillin, 55 µg/ml streptomycin, 33% horse serum (Gibco) heat-inactivated at 56°C for 30 minutes, and 11% pokeweed mitogen conditioned medium from bone marrow mononuclear cells. The cells were plated in 96-well culture dishes (Costar) with 5×10^4 cells in 0.2 ml medium per well. The cultures were incubated at 37°C in humidified air with 5% carbon dioxide. Colonies containing 40 or more cells were counted on day 7.

Statistical Analysis. Data from the in vivo experiment were evaluated using a two-way analysis of variance and the Newman-Keul's

follow-up test to identify significant differences between groups or times. Data from the in vitro experiment were analyzed using the Wilcoxon's Signed-Rank Test.

RESULTS

In Vivo Studies

Hematologic Findings. Cats first became viremic, as determined by indirect immunofluorescence for p27 of FeLV in circulating leukocytes, at two weeks PI. All cats which developed anemia remained positive for FeLV throughout the course of the experiment irrespective of treatment group. The onset of anemia was rapid, becoming evident by four weeks PI as demonstrated by significantly decreased packed cell volumes (Fig. 1). The anemia was progressive and all FeLV-positive cats required transfusion by 6 to 7 weeks PI (two weeks post-treatment initiation). Neither ATG nor NG had an effect on the progression of the anemia (Fig. 1). A peripheral regenerative response was absent in all FeLV-positive cats with most animals lacking detectable reticulocytes (data not shown). There was no significant change in mean PCV, and reticulocyte numbers remained within normal limits in the group of normal cats treated with ATG. Peripheral blood total and differential leukocyte counts, including lymphocytes, remained within normal limits for all four groups throughout the study (data not shown) as well as for the two cats used to demonstrate ATG protection of skin allografts.

Bone Marrow Cytology. In differential bone marrow cell counts of cats inoculated with FeLV-KT, erythroid precursors of bone marrow nucleated cells increased from 30% preinoculation to 45% at two weeks PI (Fig. 2). However, by four weeks PI, a rapid decline became evident. The decrease in erythroid precursors was progressive in the

anemia control and NG treated groups for the remainder of the experiment. ATG treatment increased erythroid precursors from 15 ± 2% ($\bar{X}$ ± SEM) to 35 ± 6% ($\bar{X}$ ± SEM) by two weeks post-treatment initiation ($p<.05$). This percentage of erythroid cells was similar to the preinoculation value and significantly increased above NG and anemia control group percentages at the same time interval. The effect of ATG was transient, however, since erythroid precursors decreased to 17 ± 3% ($\bar{X}$ ± SEM) by four weeks post-treatment initiation and steadily declined. Erythroid precursors were not significantly different in the FeLV-positive treatment groups at four, six, and eight weeks of treatment. ATG also increased the percentage of erythroid precursors in the marrow of normal cats 28 ± 1 ($\bar{X}$ ± SEM) pretreatment to 43 ± 7% ($\bar{X}$ ± SEM) at two weeks post-treatment initiation (Fig. 2). The effect in this group also was transient, declining to pretreatment levels by four weeks post-treatment initiation.

Before inoculation, the percentage of bone marrow lymphocytes for all groups had a wide range (1% to 29%, Fig. 3). In FeLV-positive cats, lymphocytes declined to between 0% and 4% (mean of 2 ± 0.4% SEM) by two weeks PI. ATG treatment of anemic cats caused the percentage of lymphocytes to increase significantly ($p<.05$) at two weeks post-treatment initiation to a mean of 12.3 ± 1% (SEM) and four weeks post-treatment initiation to a mean of 15 ± 5% (SEM). The percentage of lymphocytes subsequently decreased in the ATG treatment group and at six and eight weeks of treatment was similar for all FeLV-positive cats. Normal cats treated with ATG also experienced a significant increase in the percentage of lymphocytes from a mean of

11 ± 2% (SEM) pretreatment to a mean of 27 ± 7% (SEM) at four weeks post-treatment initiation ($p<.05$). Levels declined gradually at six and eight weeks post-treatment initiation.

Effects on Hematopoietic Progenitors. In the early stages of FeLV infection, the number of CFU-E increased 77% ($p<.05$) from 113 ± 15 per 5×10^4 LDBMMC ($\bar{X}$ ± SEM) at preinoculation to 200 ± 30 per 5×10^4 LDBMMC ($\bar{X}$ ± SEM) at two weeks PI (Fig. 4). By four weeks PI, CFU-E had declined ($p<.05$) and only sporadic CFU-E were seen regardless of treatment throughout the remainder of the experiment. In normal cats, ATG treatment induced a significant increase in CFU-E at four weeks of treatment ($p<.05$). The effect, however, was transient with levels returning to the normal range by six weeks of treatment.

In contrast to CFU-E, the number of BFU-E for all FeLV-positive cats was unchanged at two weeks PI (Fig. 5). Subsequently, BFU-E numbers declined ($p<.05$) from 31 ± 4 per 5×10^4 LBDMMC ($\bar{X}$ ± SEM) at preinoculation to 9 ± 5 per 5×10^4 LDBMMC ($\bar{X}$ ± SEM) by four weeks PI. Following treatment, BFU-E steadily declined despite treatment with ATG until they were seen only rarely by the fourth week of ATG treatment (8 to 10 weeks PI). Although BFU-E numbers were higher after two weeks of ATG treatment in normal cats, these changes were not statistically significant.

The number of CFU-GM in all FeLV-positive cats increased approximately 110% ($p<.01$) from 72 ± 4 per 5×10^5 LDBMMC ($\bar{X}$ ± SEM) at preinoculation to 150 ± 17 per 5×10^4 LDBMMC ($\bar{X}$ ± SEM) at two weeks PI (Fig. 6). Levels declined slightly by four weeks PI but remained significantly increased throughout the experiment with no differences

among treatment groups. ATG treatment of normal cats caused CFU-GM numbers to increase from 57 ± 3 ($\bar{X}$ ± SEM) at pretreatment to 117 ± 35 ($\bar{X}$ ± SEM) by week four of treatment ($p<.01$). Although numbers progressively declined at six and eight weeks following initiation of treatment, these levels were significantly higher than mean counts at preinoculation and at week two of treatment.

In Vitro Studies

CFU-E. Treatment of LDBMMC by continuous exposure to ATG in the cultures increased CFU-E to 300% of controls preinoculation ($p<.005$), and to 200% of controls two weeks PI ($p<.005$) and four weeks PI ($p<.005$) (Fig. 7). Continuous exposure treatment with NG similarly increased CFU-E to 300% of controls preinoculation ($p<.005$), 225% of controls two weeks PI ($p<.005$), and 170% of controls at four weeks PI ($p<.05$) (Fig. 7). The effect, however, was proportional to the untreated cell controls following the pattern evident for CFU-E in Fig. 4. When LDBMMC from four weeks PI were treated with ATG or NG, CFU-E were increased back to normal numbers (preinoculation), but this level was less than 33% of the CFU-E concentration achieved by continuous exposure of preinoculation bone marrow to either ATG or NG. Short-term incubation with ATG plus complement caused CFU-E to increase to 123% of controls preinoculation ($p<.01$) but had no effect PI. NG plus complement had no effect on numbers of CFU-E pre- or post-inoculation.

BFU-E. Continuous exposure treatment of LDBMMC with ATG increased BFU-E to 233% of controls preinoculation ($p<.01$), and two weeks PI ($p<.01$) and to 400% of controls four weeks PI ($p<.025$) (Fig.

8). Continuous exposure treatment with NG increased BFU-E to 300% of controls preinoculation ($p<.005$) and two weeks PI ($p<.005$) but had no effect at four weeks PI. Levels again were proportional to untreated cell controls following the pattern evident for BFU-E in Fig. 5. Although continuous exposure treatment with ATG increased BFU-E at four weeks PI, the level was only 33% of normal preinoculation numbers. Short-term incubation with ATG plus complement caused BFU-E to increase to 180% of controls preinoculation ($p<.05$) but, as with CFU-E, had no effect PI. NG plus complement had no effect on numbers of BFU-E pre- or post-inoculation.

DISCUSSION

In these studies, ATG was used to treat an acute virus-induced erythroid aplasia. In vivo ATG stimulated hematopoietic cells, including progenitor cells in normal cats, while enhancing more differentiated marrow precursors in anemic cats. In vitro, ATG and NG equally enhanced erythroid progenitors from normal and anemic cats when incorporated in the culture system. However, treatment with ATG plus complement had no effect on progenitor numbers from anemic animals, suggesting that EA is probably not T-cell mediated.

ATG is an important therapy for idiopathic AA and PRCA in man producing a survival rate approaching that for bone marrow transplantation. The fact that many patients have reversed helper/suppressor lymphocyte ratios, circulating activated suppressor lymphocytes, circulating and/or bone marrow γ-interferon, and evidence of lymphocyte associated marrow suppression supports an immune-mediated mechanism for at least some cases of AA and PRCA and may represent evidence of an uncharacterized viral etiologic agent (30). FeLV-infected cats have immunologic abnormalities including depressed cell-mediated immunity related to severe thymic atrophy, paracortical lymphoid depletion (31), and impaired T-helper cell function (32). Immunosuppression could contribute to the pathogenesis of FeLV-induced EA. ATG has been shown to be effective in some cases of immune-mediated hematopoietic suppression in man.

FeLV-KT infection of cats produced severe nonregenerative anemia by four weeks PI at which time PCV, reticulocyte numbers, marrow

erythroid precursors, and marrow CFU-E and BFU-E were significantly decreased. The extremely rapid reduction in PCV is partially attributable to shortened erythrocyte survival (33). A previously unreported effect of FeLV-KT seen in our studies was the significant increase in CFU-E and marrow erythroid precursors but not BFU-E or reticulocyte counts at two weeks PI, the time period when the cats first develop viremia. The reason for this increase cannot be explained by a lowered PCV because many of the cats have an increased packed cell volume at this time, nor by an increase in erythropoietin levels since previous studies have demonstrated an inverse relationship between PCV and erythropoietin production in FeLV-induced EA (34). One possible explanation is rapid destruction of circulating erythrocytes with an extremely brief period of regeneration and undetected reticulocytosis due to sampling frequency. Anisocytosis and macrocytosis without reticulocytosis have been described in cats with experimental EA (35). Alternatively, there could be direct viral effects on progenitors or accessory cells as suggested by the 100% increase in CFU-GMs and extensive reduction in bone marrow lymphocyte numbers seen also at two weeks PI. The increase in number of CFU-GM is maintained throughout the course of infection and, while significant initially, part of the increase is relative as the number of erythroid progenitors decreases with time. The lymphocyte percentage of bone marrow nucleated cells was highly variable before inoculation, an age-related phenomenon in weanling cats (3). FeLV infection significantly decreased bone marrow lymphocytes at two weeks PI with most marrow smears completely devoid of lymphocytes

irrespective of peripheral lymphocyte counts. The marrow lymphocyte percentage returned to normal levels by four weeks PI.

We chose to initiate in vivo treatment with ATG or NG between 5 and 7 weeks PI, a time when erythroid progenitors are severely but not completely suppressed. Our results indicate that ATG was not able to reverse FeLV-induced EA, although a transient regenerative response was produced in vivo by ATG at two weeks post-treatment initiation as demonstrated by an increased percentage of erythroid precursors from 15% to 35%. Additionally, ATG treatment increased the percentage of marrow lymphocytes at two and four weeks of treatment. Neither of these effects were observed in the NG or anemia control treatment groups.

There are few reports characterizing the in vivo effects of ATG on normal hematopoietic progenitors and precursors. One study described an increase in colony-forming units-culture (CFU-C) but no effect on colony-forming units-spleen (CFU-S) in a mouse splenic colonization system after antithymocyte serum treatment of donor animals (36). In vivo treatment of normal cats produced increases in the percentages of erythroid precursors and marrow lymphocytes as well as CFU-E and CFU-GM during the first four weeks of treatment. A modest but not significant increase in BFU-E was noted at two weeks post-treatment initiation. These data demonstrate that our ATG preparation had the capacity to stimulate hematopoietic progenitors, erythroid precursors, and marrow lymphoid cells.

In anemic cats, the lack of any increase in CFU-E after treatment with ATG may have been secondary to a deficiency of BFU-E or to

viral inhibition of their response to ATG. ATG also failed to enhance CFU-GM in FeLV-positive cats but the FeLV infection already had increased CFU-GM to levels comparable to those in ATG treated normal cats, possibly reflecting maximal stimulation. The increase in marrow lymphocytes is intriguing as it implies proliferation after stimulation by ATG, an unexpected effect, particularly in conjunction with FeLV infection. We do not know if these were null cells, B-cells, or T-cells. T-cell lineage may seem unlikely as immunosuppressive ATG reduced circulating T-cell numbers and with complement is cytotoxic to T-cells in vitro. However, ATG also has been shown to act as a T-cell mitogen in vitro associated with elevated interleukin 2 production (17). Our ATG preparation modestly enhanced T-cell blastogenesis of normal peripheral blood mononuclear cells but not of FeLV-infected cells (data not shown), however, this does not exclude the possible stimulatory effects of ATG on a more primitive marrow T-cell, or B- or null cells.

A second consideration is whether the marrow lymphocytes acted as accessory cells in ATG treated normal cats to stimulate CFU-E and CFU-GM or whether ATG had a direct effect on progenitors. In vitro coculture studies have shown the ability of T-cells, but not B-cells or monocytes, from normal subjects to increase CFU-E and CFU-GM (15,38). Experiments also suggested that ATG may stimulate erythropoiesis (16) and granulopoiesis (39) indirectly by enhancing release of growth factors from T-cells. Temporally in our study ATG began enhancing marrow lymphocytes two weeks prior to CFU-E and CFU-GM in normal cats, suggesting they could have acted as accessory cells.

Subsequently, all three cell populations were decreased after six weeks of treatment. Other reports have demonstrated the ability of ATG to bind with many cell types (40), to induce growth and differentiation of normal marrow after depletion of T-cells and macrophages (23), and to increase the sensitivity of CFU-E to low doses of erythropoietin (16). This latter effect may account for the increase in marrow erythroid precursors observed in both ATG treated FeLV-positive and FeLV-negative cats.

The effects of ATG on the marrow in both normal and anemic cats were transient, suggesting that stimulation waned over time. An ideal in vivo treatment regimen for using ATG in AA or PRCA patients has not been established. The dosage for these experiments was selected to achieve immunosuppression as previously described (40) and to limit serum sickness side effects. The total dose was higher than amounts used in many reports. The cats were treated for two months because four to six weeks generally are required to generate a positive response to ATG in patients with AA.

The in vitro studies demonstrated that ATG, but not NG, plus complement enhanced both BFU-E and CFU-E from normal LDBMMC, an effect independent of enrichment after lysis of marrow lymphocytes. ATG plus complement, however, had no effect on progenitors from marrow samples at two and four weeks PI and did not reverse EA. These results suggest that FeLV-induced EA is not associated with autoreactive T-cells to erythroid progenitors and probably is not T-cell mediated. This conclusion is supported by a recent report in which coculture studies showed that T-cells from cats with EA failed

to inhibit erythroid colony growth from normal or autologous marrow cells (41). Continuous exposure to ATG or NG without complement within the culture system enhanced progenitors dramatically at preinfection and at two weeks PI. The effect also was significant but more modest at four weeks PI for CFU-E and with ATG alone for BFU-E. The equivalent effects of ATG and NG are difficult to explain and contrary to a previous report using normal LDBMMC (16).

The failure of ATG to reverse FeLV-induced EA is significant because it suggests that an immunomodulating agent with the ability to stimulate hematopoietic cells and suppress T-cell function in vivo was not able to exert its effects in the presence of overt retroviral infection. Also, the persistence of granulocytic progenitors in cats infected with FeLV-KT indicates that an inadequate marrow reserve of pluripotential stem cells was not a critical factor in the failure to respond to ATG. The in vitro studies imply that the mechanism of EA does not include autoreactive T-cells to erythroid progenitors. A reason for the ineffectiveness of ATG remains speculative and dependent upon further studies elucidating the mechanism of its action and/or the pathogenesis of FeLV-induced EA.

SUMMARY

The Kawakami-Theilen strain of feline leukemia virus (FeLV-KT) was used experimentally to produce erythroid aplasia (EA) in cats. The *in vivo* effects of goat anti-feline-thymocyte globulin (ATG) on hematopoiesis were investigated in FeLV-negative normal and FeLV-positive anemic cats. Control groups included FeLV-positive anemic cats treated with normal goat globulin (NG) and FeLV-positive untreated anemic cats. Treatment was initiated in anemic cats between four and six weeks post-inoculation (PI) when erythroid progenitors are reduced to 10% of normal levels. During the first two weeks of treatment, ATG significantly increased the numbers of erythroid precursors in bone marrow from 15% at four weeks PI to 35% post-treatment in normal cats. Additionally, by four weeks after initiation of treatment with ATG, the percentage of marrow lymphocytes increased in anemic cats to 15% from 3% at four weeks PI, and increased to 27% from 11% pretreatment in normal cats. ATG stimulated a two-fold increase of CFU-E and a three-fold increase of CFU-GM in normal cats between two and four weeks after initiation of treatment but had no effect on CFU-E or CFU-GM in anemic cats. The *in vivo* effects of ATG were transient despite weekly treatment. Cats treated with NG were not significantly different from untreated anemic control cats.

In vitro studies were performed using low density bone marrow mononuclear cells (LDBMMC) from cats prior to *in vivo* treatment. Short-term incubation with ATG plus complement but not NG plus

complement significantly enhanced CFU-E and BFU-E from cats pre-inoculated but not after they became viremic. These results suggest that T-cells do not suppress erythroid progenitors in anemic cats. Continuous exposure to ATG or NG in cultures of LDBMMC from cats pre- and post-inoculation stimulated CFU-E and BFU-E to greater than 200% of controls except for BFU-E at four weeks PI which were selectively enhanced by ATG to 400% of controls. Although continuous exposure to ATG or NG returned CFU-E to normal levels at four weeks PI, BFU-E were increased to only 33% preinoculation cell control levels.

These data indicate that 1) ATG stimulates erythropoiesis, granulopoiesis, and possibly lymphopoiesis in vivo; 2) ATG enhances numbers of differentiated erythroid precursors and marrow lymphocytes but not erythroid progenitors in cats with EA, a cytopenia of known viral etiology; and 3) EA probably is not T-cell mediated.

Fig. 1. Changes in PCV (mean ± SEM) before and after treatment of normal FeLV-negative cats treated with ATG (ATG-N) (n=3), untreated FeLV-positive anemic cats (anemia-C) (n=4), and FeLV-positive anemic cats treated with ATG (n=4) or NG (n=4). All cats inoculated with FeLV-KT at week 0 became FeLV-positive at 2 PI and remained viremic through the course of the experiment. Globulin treatments were initiated between 5 and 7 weeks PI. Neither ATG nor NG improved PCV in FeLV-positive cats.

a. Decreased ($p<.05$) in FeLV-negative cats.

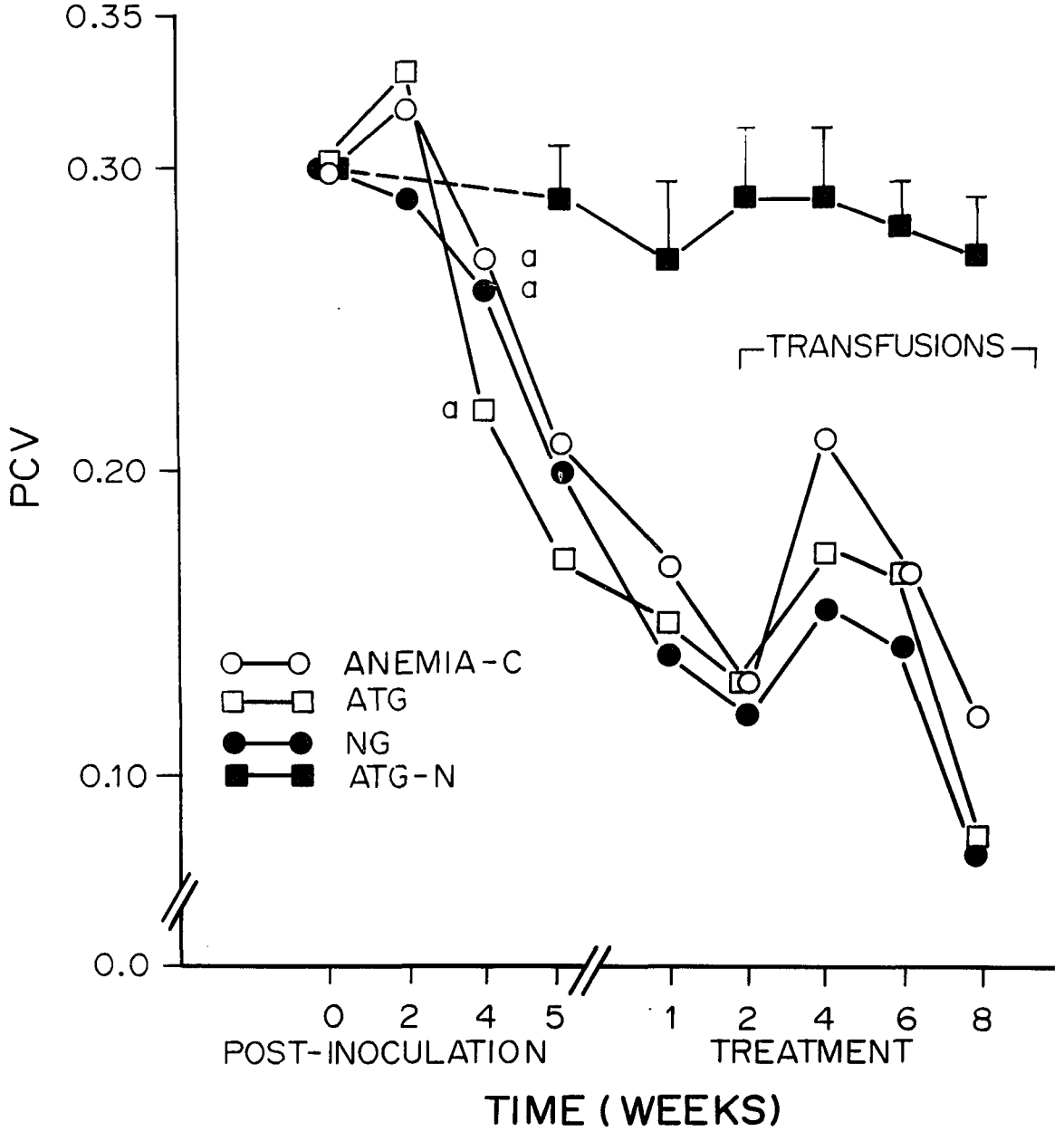

0.35
0.30
0.20
0.10
0.0
PCV
a
a
a
TRANSFUSIONS
ANEMIA-C
ATG
NG
ATG-N
0
2
4
5
1
2
4
6
8
POST-INOCULATION
TREATMENT
TIME (WEEKS)

Fig. 2. Changes in the percentage of erythroid precursors (mean ± SEM) of bone marrow cells of normal cats treated with ATG (ATG-N) (n=3), in untreated anemic cats (anemia-C) (n=4), and anemic cats treated with ATG (n=3) or NG (n=4).

a. Increased ($p<.05$) in FeLV-positive cats vs. 0 PI or 4 PI.

b. Decreased ($p<.05$) in FeLV-positive cats vs. 0 PI.

c. Increased ($p<.05$) vs. NG or anemia-C and vs. 4 PI.

d. Increased ($p<.05$) vs. 0 PI and 4 and 6 weeks of treatment.

*Lymphocytes were not included in the cell counts.

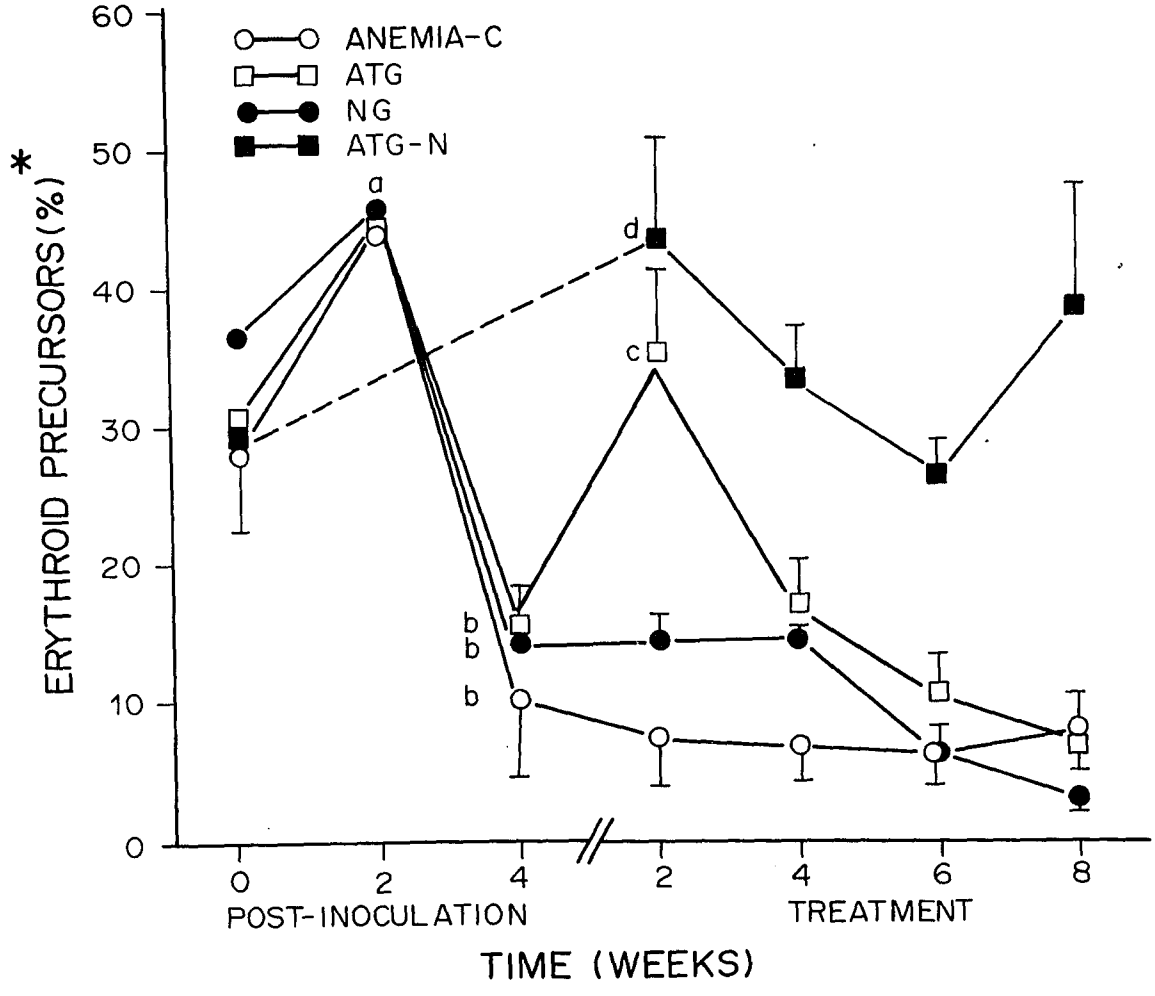

ANEMIA-C
ATG
NG
ATG-N
ERYTHROID PRECURSORS (%)*
60
50
40
30
20
10
0
a
b
b
b
c
d
0
2
4
2
4
6
8
POST-INOCULATION
TREATMENT
TIME (WEEKS)

Fig. 3. Changes in the percentage of lymphocytes (mean ± SEM) of bone marrow cells in normal cats treated with ATG (ATG-N) (n=3), untreated anemic cats (anemia-C) (n=4), and anemic cats treated with ATG (n=4) or NG (n=4). There was no significant variation in peripheral blood lymphocyte counts among groups.

a. Decreased ($p<.05$) in FeLV-positive cats vs. 0 PI.

b. Increased ($p<.05$) vs. NG or anemia-C and vs. 4 PI.

c. Increased ($p<.05$) vs. all other times.

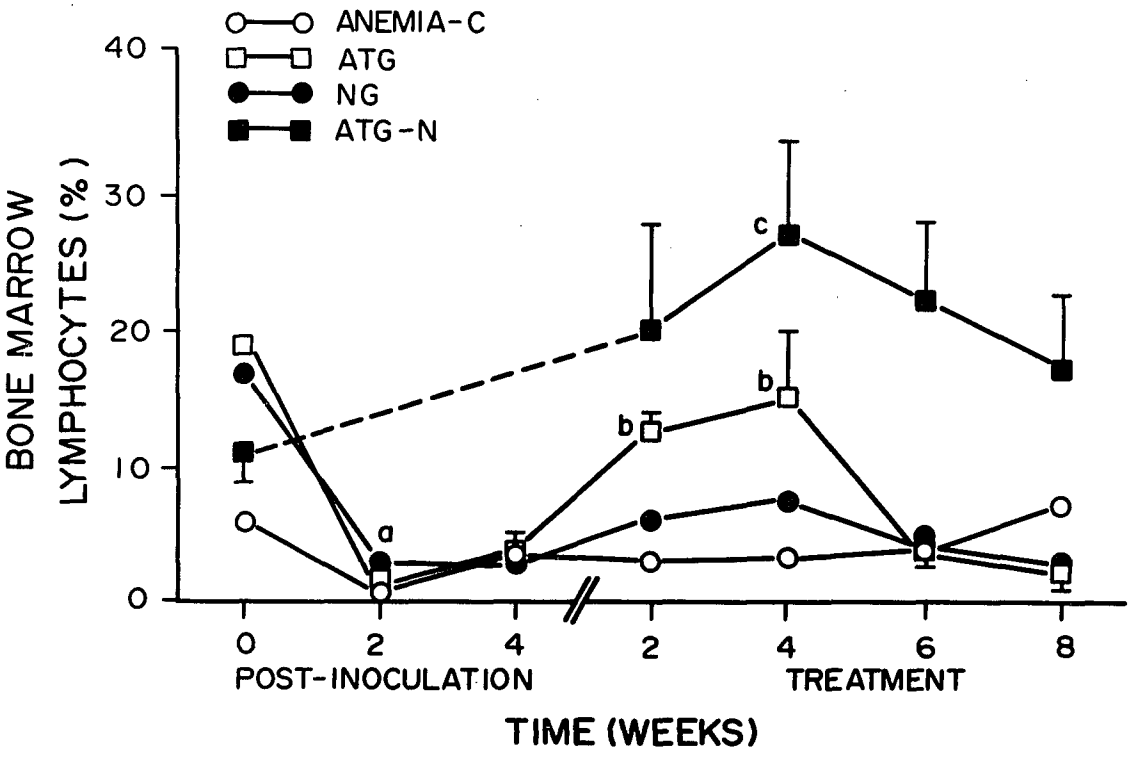

ANEMIA-C
ATG
NG
ATG-N
BONE MARROW LYMPHOCYTES (%)
40
30
20
10
0
a
b
b
c
0
2
4
2
4
6
8
POST-INOCULATION
TREATMENT
TIME (WEEKS)

Fig. 4. Effects of ATG, NG, or no treatment on numbers of CFU-E (mean ± SEM) vs. time in normal cats treated with ATG (ATG-N) (n=3), untreated anemic cats (anemia-C) (n=4), and anemic cats treated with ATG (n=4) or NG (n=4).

a. Increased ($p<.05$) in FeLV-positive cats vs. 0 PI.

b. Decreased ($p<.05$) in FeLV-positive cats vs. 0 PI.

c. Increased ($p<.05$) vs. 0 PI and 2 weeks of treatment.

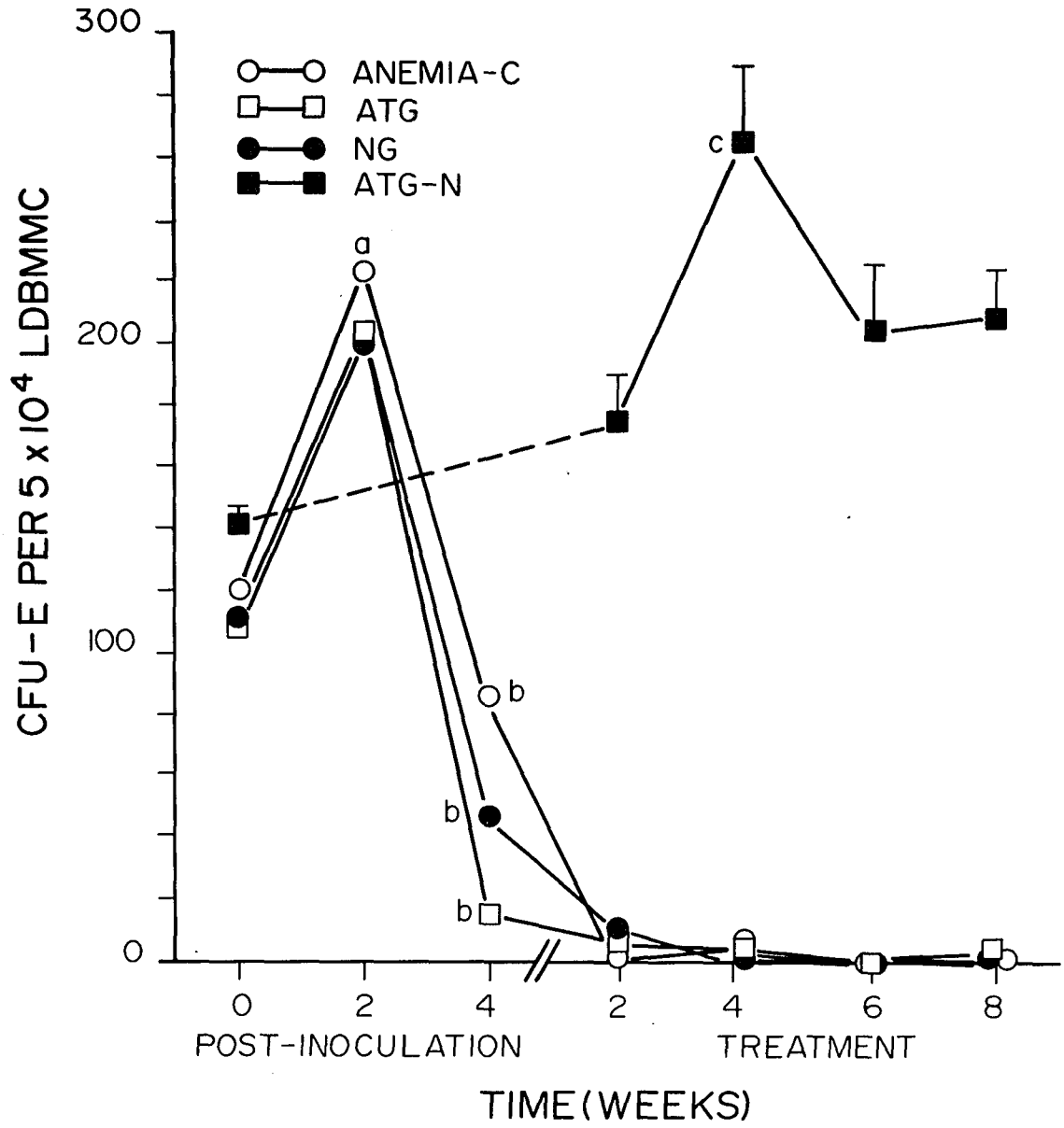

300
200
100
0
CFU-E PER 5 x 10⁴ LDBMMC
ANEMIA-C
ATG
NG
ATG-N
a
b
b
b
c
0
2
4
2
4
6
8
POST-INOCULATION
TREATMENT
TIME (WEEKS)

Fig. 5 Effects of ATG, NG, or no treatment on numbers of BFU-E (mean ± SEM) vs. time in normal cats treated with ATG (ATG-N) (n=3), untreated anemic cats (anemia-C) (n=4), and anemic cats treated with ATG (n=4) or NG (n=4).

a. Decreased ($p<.05$) in FeLV-positive cats vs. 0 PI and 2 PI.

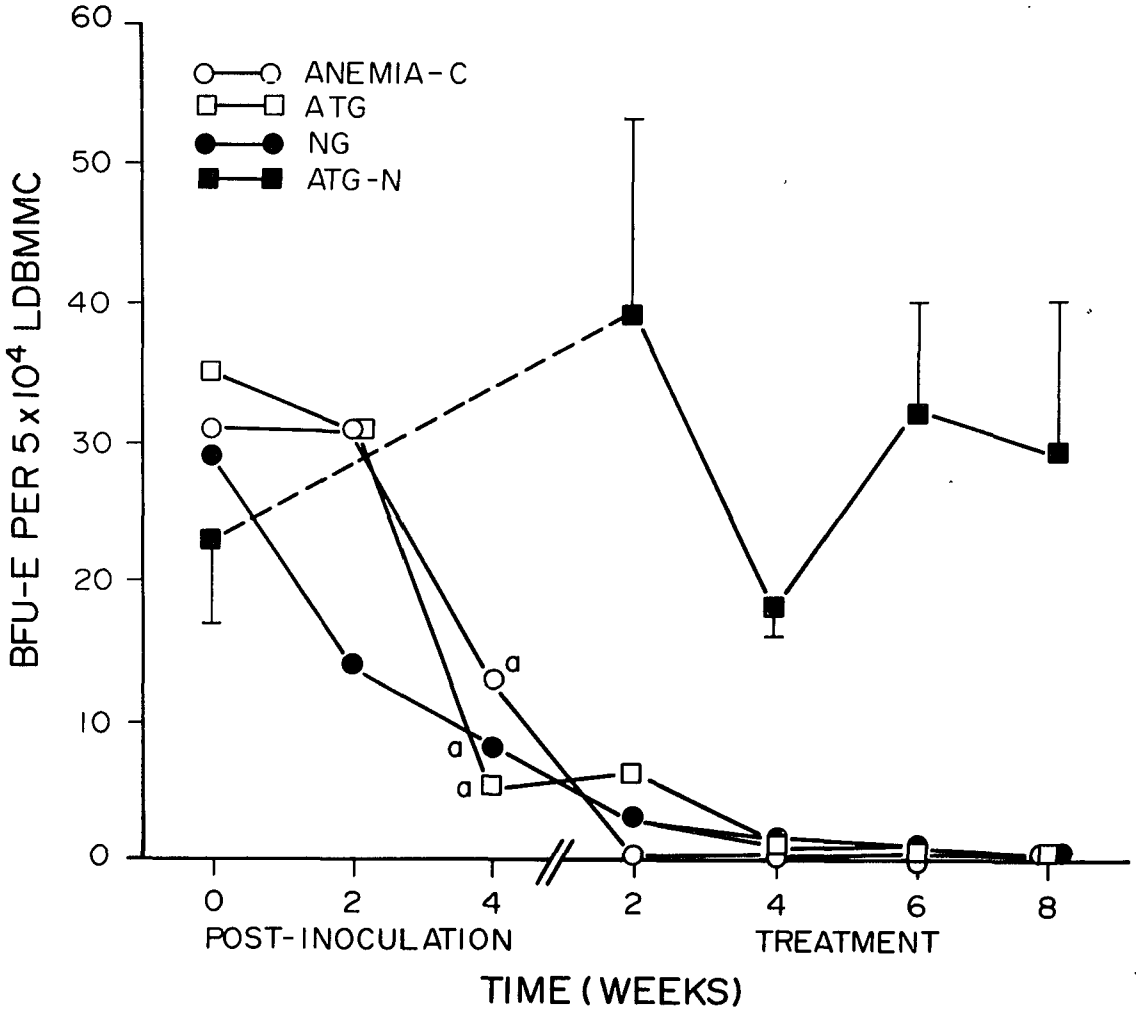

ANEMIA-C
ATG
NG
ATG-N
BFU-E PER 5 x 10⁴ LDBMMC
60
50
40
30
20
10
0
a
a
a
0
2
4
2
4
6
8
POST-INOCULATION
TREATMENT
TIME (WEEKS)

Fig. 6. Effects of ATG, NG, or no treatment on numbers of CFU-GM (mean ± SEM) vs. time in normal cats treated with ATG (ATG-N) (n=3), untreated anemic cats (anemia-C) (n=4), and anemic cats treated with ATG (n=4) or NG (n=4).

a. Increased ($p<.01$) in FeLV-positive cats vs. 0 PI. Numbers stay significantly higher through remaining time periods.

b. Increased ($p<.01$) vs. 0 PI and 2 weeks of treatment.

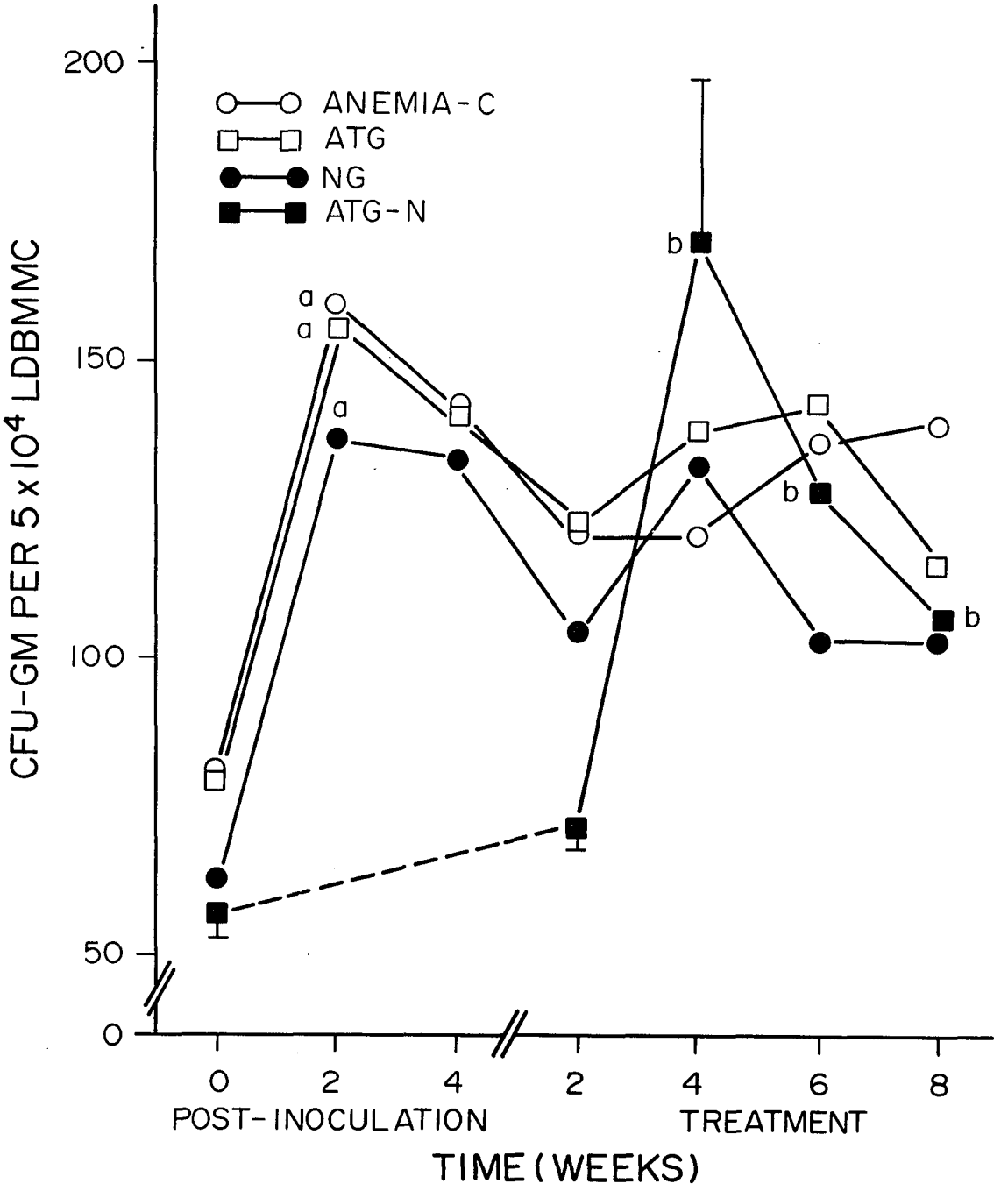

200
150
100
50
0
ANEMIA-C
ATG
NG
ATG-N
CFU-GM PER 5 x 10⁴ LDBMMC
a
a
a
b
b
b
0
2
4
2
4
6
8
POST-INOCULATION
TREATMENT
TIME (WEEKS)

Fig. 7. Effects of ATG or NG treatment in vitro on CFU-E (mean ± SEM) of LDBMMC from cats inoculated with FeLV (n=12) at 0, 2, and 4 weeks PI. For continuous exposure studies cells were incubated for 1 hour with media, ATG, or NG and added to the assay system without washing. For complement (C') studies cells were incubated for 20 minutes with media, ATG, or NG followed by addition of C' for 30 minutes. The cells were then washed, resuspended, and added to the culture system.

* = $p<.005$; ** = $p<.01$; $^{+}$ = $p<.05$.

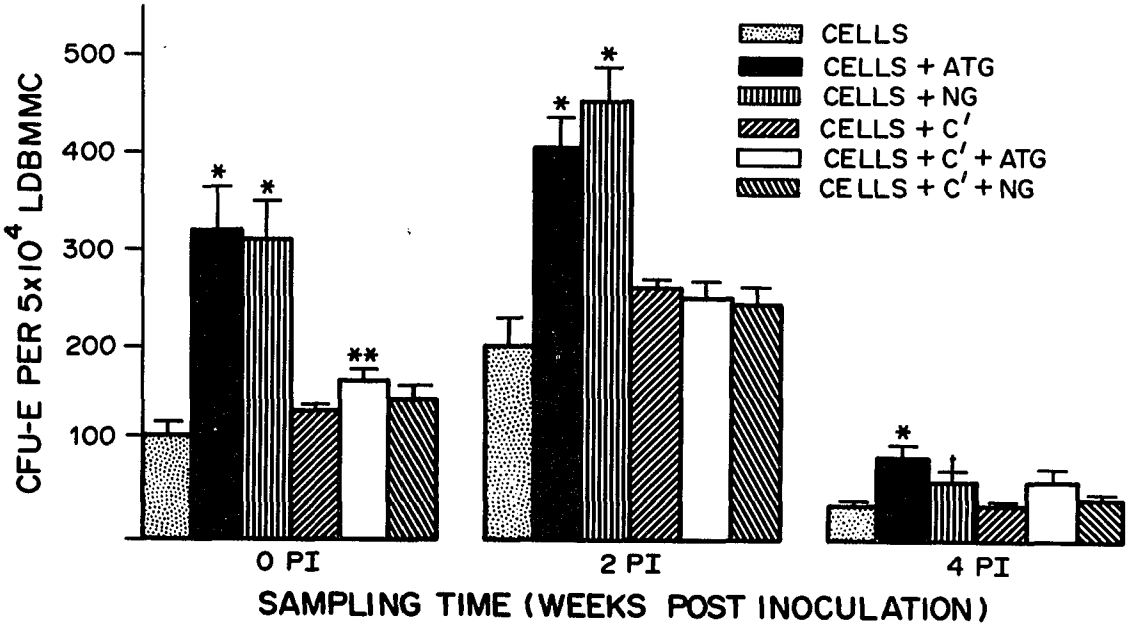
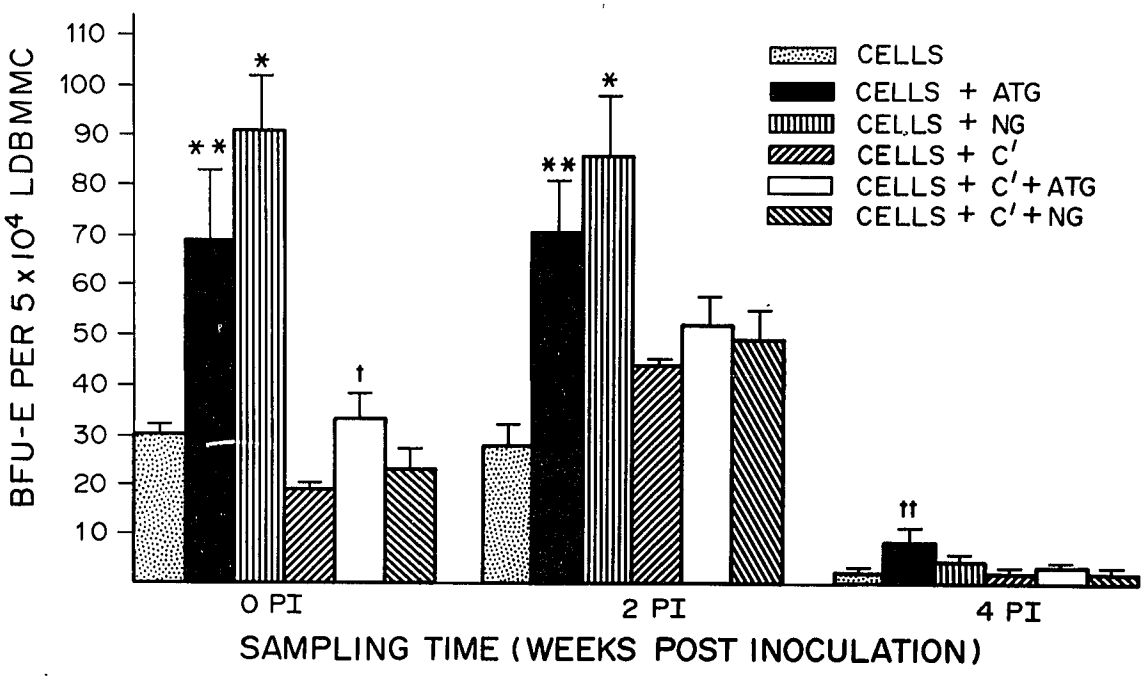

CELLS
CELLS + ATG
CELLS + NG
CELLS + C′
CELLS + C′ + ATG
CELLS + C′ + NG
CFU-E PER 5x10⁴ LDBMMC
500
400
300
200
100
0 PI
2 PI
4 PI
SAMPLING TIME (WEEKS POST INOCULATION)

Fig. 8. Effects of ATG or NG treatment in vitro on BFU-E (mean ± SEM) of LDBMMC from cats inoculated with FeLV (n=12) at 0, 2, and 4 weeks PI. For continuous exposure studies cells were incubated for 1 hour with media, ATG, or NG and added to the culture system without washing. For complement (C') studies cells were incubated for 30 minutes with media, ATG, or NG followed by addition of C' for 30 minutes. The cells were then washed, resuspended, and added to the culture system.

* = $p<.005$; ** = $p<.01$; $^{+}$ = $p<.05$; $^{++}$ = $p<.025$.


BFU-E PER 5 x 10⁴ LDBMMC
110
100
90
80
70
60
50
40
30
20
10
CELLS
CELLS + ATG
CELLS + NG
CELLS + C′
CELLS + C′ + ATG
CELLS + C′ + NG
**
*
**
*
†
††
0 PI
2 PI
4 PI
SAMPLING TIME (WEEKS POST INOCULATION)

REFERENCES

1. Sarma PS, Log T (1973) Subgroup classification of feline leukemia and sarcoma viruses by viral interference and neutralization tests. Virology 54:160-169.

2. Rojko JL, Olsen RG (1984) The immunobiology of the feline leukemia virus. Vet Immunol Immunopath 6:107-165.

3. Hoover EA, Kociba GJ, Hardy WD, Yohn DS (1974) Erythroid hypoplasia in cats inoculated with feline leukemia virus. J Natl Cancer Inst 53(5):1261-1265.

4. Onions D, Sherett O, Testa N, Frassoni F, Toth S (1982) Selective effect of feline leukaemia virus on early erythroid precursors. Nature 296:156-158.

5. Jarrett O, Golder MC, Toth S, Onions DE, Stewart MF (1984) Interaction between feline leukaemia virus subgroups in the pathogenesis of erythroid hypoplasia. Int J Cancer 34:283-288.

6. Gasper PW, Hoover EA (1983) Feline leukemia virus strain specific abatement of erythroid burst forming units (BFU-E). Leuk Res Internat 1:71-72.

7. Champling R, Ho W, Gale RP (1983) Antithymocyte globulin treatment in patients with aplastic anemia. N Engl J Med 308:113-118.

8. Miller WJ, Branda RF, Flynn PJ et al (1983) Antithymocyte globulin treatment of severe aplastic anemia. Brit J Haematol 55:17-25.

9. Camitta B, O'Reilly RJ, Sensenbrenner L et al (1983) Antithoracic duct lymphocyte globulin therapy of severe aplastic anemia. Blood 62:883-888.

10. Testa NG, Onions D, Jarrett O, et al (1983) Haemopoietic colony formation (BFU-E, GM-CFC) during the development of pure red cell hypoplasia induced in the cat by feline leukaemia virus. Leuk Res 7:103-116.

11. Hagberg H, Nilsson P, Nisell J (1980) Treatment of pure red cell anemia with antilymphocytic globulin. Scand J Haematol 24:360-364.

12. Marmont A, Peschle C, Sanguineti M et al (1975) Response of three patients to cyclophosphamide and antilymphocyte globulin and demonstration of two types of serum IgG inhibitors to erythropoiesis. Blood 45:247.

13. Torok-Storb B, Doney K, Sale G et al (1985) Subsets of patients with aplastic anemia identified by flow microfluorometry. N Engl J Med 312:1015-1022.

14. Bacigalupo A, Podesta M, Frassoni F et al (1985) In vitro tests in severe aplastic anaemia (SAA): A prospective study in 46 patients treated with immunosuppression. Brit J Haematol 59:611-616.

15. Mangan KF, Shadduck RK (1984) Successful treatment of chronic refractory pure red cell aplasia with antithymocyte globulin: correlation with in vitro erythroid culture studies. Am J Hematol 17:417-426.

16. Mangan KF, O'Allessandro L, Mullaney MT (1986) Action of antithymocyte globulin on normal human erythroid progenitor cell proliferation in vitro: Erythropoietic growth-enhancing factors are released from marrow accessory cells. J Lab Clin Med 107: 353-365.

17. Gascon P, Zoumbos NC, Scala G et al (1985) Lymphokine abnormalities in aplastic anemia: Implications for the mechanism of action of antithymocyte globulin. Blood 65:407-413.

18. Riedel N, Hoover EA, Gasper PW et al (1986) Molecular analysis and pathogenesis of the feline aplastic anemia retrovirus, feline leukemia virus C-sarma. J Virol 60:242-250.

19. Abkowitz JL, Oft RL, Nakamura JM et al (1985) Feline glucose-6-phosphate dehydrogenase cellular mosaicism. Application to the study of pure red cell aplasia. J Clin Invest 75:133.

20. Boyce JT, Hoover EA, Kociba GJ, Olsen RG (1981) Feline leukemia virus-induced erythroid aplasia: In vitro hemopoietic culture studies. Exp Hematol 9:990.

21. Atrah HI, Crawford RJ, Babra GS (1985) Modulation of suppressor T-cells for the treatment of aplastic anaemia. Lancet ii:339-340.

22. Templeton JG, Cocker JE, Crawford PJ (1985) Fc -receptor blocking antibodies in hyperimmune and normal pooled gammaglobulin. Lancet i:1337.

23. Hunter RF, Mold NG, Mitchell RG et al (1985) Differentiation of normal marrow and HL60 cells induced by antithymocyte globulin. Proc Natl Acad Sci USA 82:4823-4827.

24. Harada M, Gale RP (1982) Evaluation of antithymocyte globulin for human bone marrow transplantation. Transplantation 33:625-630.

25. Netzel B, Rodt H, Hoffmann-Fezer G et al (1978) The effect of crude and differently absorbed anti-human T-cell globulin on granulocytic and erythropoietic colony formation. Exp Hematol 6:410-420.

26. Abkowitz JL, Pwell JS, Nakamura JM et al (1986) Pure red cell aplasia: Response to therapy with antithymocyte globulin. AM J Hematol 23(4):363.

27. Vegh P, Erdos E, Janossy T et al (1978) Correlation of the in vivo and in vitro activities of antithymocyte sera (ATS) with the immunizing antigen dose. Transplantation 25:324-327.

28. Hoover EA, Mathes LE, Rojko JL et al (1978) Modification of the immunofluorescence assay for feline leukemia virus group-specific antigen. Am J Vet Res 39:1877-1880.

29. Heyworth MF (1981) Effects of antilymphocytic globulin in human subjects. Immunology 43:793-802.

30. Zoumbos NC, Gascon P, Djell JY et al (1985) Circulating activated suppressor T lymphocytes in aplastic anemia. N Engl J Med 312: 257-265.

31. Perryman LE, Hoover EA, Yohn DS (1972) Immunologic reactivity of the cat: Immunosuppression in experimental feline leukemia. J Natl Cancer Inst 49:1357-1365.

32. Cockerell GL, Hoover EA, Krakowka S (1976) Lymphocyte mitogen reactivity and enumeration of circulating B- and T-cells during feline leukemia virus infection in the cat. J Natl Cancer Inst 57:1095-1099.

33. Hoover EA, Kociba GJ, Olsen RG (1981) Experimental erythroid aplasia in cats induced by feline leukemia virus. Proceedings of the Conference on Aplastic Anemia: A stem cell disease, p 306. National Institutes of Health Publication No. 81-1008, June.

34. Kociba GJ, Lange RD, Dunn CDR, Hoover EA (1983) Serum erythropoietin changes in cats with feline leukemia virus-induced erythroid aplasia. Vet Pathol 20:548-552.

35. Weiser MG, Kociba GJ (1983) Erythrocyte macrocytosis in feline leukemia virus associated anemia. Vet Pathol 20:687-697.

36. Mookerjee BK, Azzolina L, Poultar L (1974) Interaction of antithymocyte serum with hematopoietic stem cells. J Immunol 112: 822-829.

37. Nagasawa T, Abe T, Hanada T (1982) Inhibitory effects of T-cells on in vitro granulopoiesis and immunoglobulin production in patients with aplastic anemia. Scand J Haematol 28:389-398.

38. Bagby GC, Rigas VD, Bennett RM et al (1981) Interaction of lactoferrin, monocytes, and T lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. J Clin Invest 68:56-73.

39. Greco B, Bielory L, Stephan D et al (1983) Antithymocyte globulin reacts with many normal human cell types. Blood 62:1047-1054.

40. Thomas JM, Williams AJ, Thomas FT et al (1978) T-cell-specific activity in rabbit anti-human thymocyte globulin. Transplantation 25:97-102.

41. Abkowitz JL, Ott RL, Holly RD, adamson JW (1987) Lymphocytes and antibody in retrovirus-induced feline pure red cell aplasia. J Natl Cancer Inst 78:135-139.

CHAPTER II

EFFECTS OF INCREASING CYCLIC AMP OR CALCIUM ON FELINE ERYTHROID PROGENITORS *IN VITRO*: NORMAL CELLS ARE STIMULATED WHILE CELLS FROM FeLV POSITIVE CATS ARE SUPPRESSED

INTRODUCTION

Feline leukemia virus (FeLV) is an exogenous type C retrovirus that causes a variety of neoplastic and degenerative diseases in cats, including erythroid aplasia (EA) (1). The Kawakami-Theilen strain of FeLV (FeLV-KT) consistently induces EA characterized by rapid and total depletion of erythroid colony-forming units (CFU-E) and erythroid burst-forming units (BFU-E), with moderate enhancement of granulocyte-macrophage colony-forming units (CFU-GM) in the bone marrow (2-5). Studies also have shown that the FeLV envelope protein, p15E, inhibits CFU-E and BFU-E but not CFU-GM from uninfected bone marrow cells *in vitro* (6).

The pathogenesis of EA is unknown. Recent experiments have failed to demonstrate serum or lymphocyte-mediated inhibition of erythroid progenitors (7,8). However, the possibility of viral alteration of progenitor responsiveness to humoral regulators or the inadequate production of such regulators by marrow accessory cells

resulting in "blocked" erythropoiesis has not been excluded as the mechanism of EA. Altered maturation of erythropoietic cells is a feature of several other retrovirus-induced syndromes including murine Friend leukemia, the erythroid dyscrasia associated with a variant of the murine Rauscher leukemia virus, and avian erythroblastosis (9-11). Studies of FeLV-mediated immunosuppression have demonstrated that FeLV and p15E alter T-lymphocyte function by impairing lymphokine secretion and interfering with the response to lymphokines (12, 13). Inhibition of concanavalin A stimulation of peripheral blood lymphocytes (PBL) from viremic cats or normal PBL exposed to FeLV or p15E was reversed by in vitro treatment with agents which increase intracellular cyclic 3',5' adenosine monophosphate (cAMP) (14,15). cAMP generally is regarded as a negative regulator of cell proliferation. However, in some studies, cAMP or calcium have been shown to enhance cell growth and differentiation of many cell types (16,17), including normal erythroid progenitors and precursors (18-21) and differentiation of Friend virus-infected murine erythroleukemia cells (22). Increased intracellular cAMP or calcium may mediate the action of growth promoting agents by acting as regulatory signals (23).

The objectives of these studies were to determine the effects of pharmacologic reagents known to increase intracellular cAMP and calcium on growth of CFU-E and BFU-E of normal cats and to determine whether the suppression of erythroid progenitors from cats with FeLV-induced EA or normal progenitors suppressed by p15E of FeLV could be reversed.

MATERIALS AND METHODS

Animals. All cats were obtained at 6 to 8 weeks of age from a specific pathogen-free (SPF) breeding colony maintained by the Department of Veterinary Pathobiology, The Ohio State University. This colony is uniformly free of infection and immunity to FeLV. The cats were tranquilized with intramuscular ketamine hydrochloride prior to bone marrow or blood collection. Infected cats were pre-treated one day prior to inoculation with methyl prednisolone (5 mg/kg) to enhance their susceptibility to productive FeLV infection.

Viral inoculum and p15E preparation. The FeLV inoculum was prepared from equal volumes of serum and bone marrow cell lysate pooled from cats with anemia induced by *in vivo* passage of FeLV-KT as described (5). The FeLV infectivity titer of the inoculum was assessed by the S+/L- focus induction assay as described previously (5). Cats were infected by intravenous injection of 0.4 ml inoculum containing 1×10^5 FFU FeLV-KT.

Methods for concentration (24) and purification (25) of p15E have been described previously. The preparation used did not contain detectable endotoxin as assayed by the *Limulus* amebocyte assay (M.A. Bioproducts, Walkersville, MD).

Culture of pre- and post-infection low density bone marrow mononuclear cells with modifiers of intracellular concentrations of cAMP and calcium. Four 6 to 8 week old kittens were inoculated with FeLV-KT. Blood was collected before inoculation and weekly post-inoculation from each cat to perform complete hemograms (Coulter S plus IV, Coulter Electronics Inc., Hialeah, FL) and reticulocyte

counts. Leukocytes on blood films were screened for the presence of FeLV using an indirect immunofluorescence test for FeLV p27 as modified by Hoover et al. (26). Bone marrow was sampled prior to inoculation and 2, 4, and 6 weeks post-inoculation from either the proximal humerus or the proximal femur. Samples were aspirated into a syringe containing 2 ml of 0.75% sodium citrate in phosphate buffered saline and placed immediately on ice. Low density bone marrow mononuclear cells (LDBMMC) were separated and cultured for CFU-E, BFU-E, and CFU-GM as described below. Cell separation was performed by diluting whole marrow cell suspensions to 20 ml with MEM- in 16 x 95 mm polystyrene tubes and underlayering them with an equal volume of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) adjusted to a specific gravity of 1.071. LDBMMC were collected from the interface after centrifugation for 30 minutes at 400g, washed twice in Hank's Balanced Salt Solution (GIBCO), and resuspended in MEM- .

Erythroid progenitors in LDBMMC were cultured with various modifiers of intracellular cyclic AMP or calcium. Except where indicated, stock solutions of the reagents were made prior to the experiment and stored in aliquots at -70°C. The reagents were added at 10 µl per 1 ml media directly to the culture wells to reach the specified concentration and remained in the wells throughout the culture period. Reagents used were: L-isoproterenol (Sigma, St. Louis, MO), a β_1 β_2 agonist (27) dissolved and diluted in MEM- media (Gibco, Grand Island, NY), and used at 10^{-6}M, 10^{-7}M, 10^{-8}M, and 10^{-9}M; D,L,-propranolol (Sigma), a β_1 βB_2 antagonist (28), dissolved

and diluted in MEM-α media, and used at 10^{-6}M alone and in combination with L-isoproterenol; dibutyryl cyclic AMP (db-cAMP) (Sigma), a more lipid-soluble cAMP analog, freshly dissolved and diluted in MEM- media for each experiment and used at 10^{-4}M, 10^{-5}M, 10^{-6}M, and 10^{-7}M; sodium butyrate (Sigma), included as a control for the dibutyryl group of db-cAMP, dissolved and diluted in MEM-α, and used at 10^{-4}M, 10^{-5}M, 10^{-6}M, and 10^{-7}M; forskolin (Calbiochem, La Jolla, CA), an activator of adenylate cyclase (29), dissolved in 95% ethanol to 10^{-2}M, diluted in MEM-α media, and used at 10^{-4}M, 10-$^{-5}$M, 10^{-6}M, and 10^{-7}M; RO-20-1724 (4-[2-butoxy-4-methoxybenzyl]-2-imidazolidine) (a generous gift from Hoffman-LaRoche Inc., Nutley, NJ), a phosphodiesterase inhibitor (30), dissolved in 95% ethanol to 10^{-1}M, diluted in MEM-α media, and used at 10^{-3}M, 10^{-4}M, 10^{-5}M, and 10^{-6}M; A23187 (Sigma), a calcium ionophore (31), dissolved and diluted in 95% ethanol, and used at 10^{-6}M, 10^{-7}M, 10^{-8}M, and 10^{-9}M in the presence of 2.14 mM calcium within the culture system; and indomethacin (Sigma), a cyclooxygenase and phosphodiesterase inhibitor (32), dissolved in 95% ethanol to 10^{-6}M, diluted in MEM-α media, and used at 10^{-6}M, 10^{-7}M, 10^{-8}M, and 10^{-9}M. Preliminary studies showed that erythroid progenitor numbers were unaffected by the addition of 95% ethanol to the culture system (10 µl/ml media).

Cultures of normal LDBMMC exposed to p15E and treated with modifiers of intracellular concentrations of cAMP and calcium. LDBMMC were harvested from one normal cat, suspended in MEM-α at 5 x 10^5 cells/ml, and incubated with p15E (7.5 µg/5 x 10^5 cells) in MEM-α or MEM-α alone (control) for one hour at 37°C as previously

described (6). The cells then were plated in culture dishes in the presence of the various chemical reagents as described above.

Progenitor assays. The culture methods for colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) were similar to those described by Abkowitz et al. (33) for feline bone marrow. Briefly, 7.5×10^4 LDBMMC in 0.15 ml MEM-α were added to 1.35 ml of medium containing 1.3% methylcellulose (Dow-Corning, Midland, MI) in Iscove's Modified DMEM (Hazleton Research Products), 1% deionized bovine serum albumin (Armour Pharmaceuticals, Tarrytown, NY), 10^{-4}M β-mercaptoethanol, 30% fetal bovine serum (Armour Pharmaceuticals), sheep plasma erythropoietin (Step III, Connaught Laboratories, Swiftwater, PA) at one unit per milliliter, and 5% pokeweed mitogen-conditioned medium from bone marrow mononuclear cells. The assays were performed in 35 mm cluster dishes (Costar, Cambridge, MA) with 5×10^4 cells in 1 milliliter of medium per well. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 10% O_2 in nitrogen-enriched air. Erythroid colonies were counted with an inverted microscope on day 4 of culture as red colonies containing 8-50 cells (CFU-E) and on day 9 as red colonies composed of more than 200 cells, usually with closely associated subunits (BFU-E).

Colony-forming units-granulocyte/macrophage (CFU-GM) were quantified using a modification of our previously described method (5). Briefly, 2.5×10^5 LDBMMC in 0.1 ml MEM-α were added to 0.9 ml medium consisting of 0.5% methylcellulose in MEM-α, 0.4% bovine serum albumin, glutamine (2 mM), 55 U/ml penicillin, 55 µ/ml streptomycin,

33% horse serum (Gibco) heat-inactivated at 56°C for 30 minutes, and 11% pokeweed mitogen-conditioned medium from bone marrow mononuclear cells. The cells were plated in 96-well culture dishes (Costar) with 5×10^4 cells in 0.2 ml medium per well. The cultures were incubated at 37°C in humidified air with 5% carbon dioxide. Colonies containing 40 or more cells were counted on day 7.

Statistical analysis. All values from treated cultures were normalized as the percentage of untreated control colony numbers. The Student's t-test was used to determine significance when a single treatment was compared to control. One-way analysis of variance and the Newman-Keul's follow-up test were used to identify significant differences when two or more treatments were compared or when parameters were compared at multiple time points.

RESULTS

Hematologic findings. All cats were viremic by 2 weeks post-inoculation (PI) and remained viremic throughout the course of the experiment as determined by indirect immunofluorescence for p27 of FeLV in circulating leukocytes. Packed cell volumes (PCV) initially increased ($p<.01$) from 0.29 ± 0.007 ($\bar{X}$ ± SEM) pre-inoculation to 0.32 ± 0.003 ($\bar{X}$ ± SEM) at 2 weeks PI (Fig.1). However, significant anemia became evident by 4 weeks PI with a mean PCV of 0.25 ± 0.01 (SEM) ($p<.01$). Anemia was severe by 6 weeks PI with a mean PCV of 0.16 ± 0.003 (SEM) ($p<.01$). A peripheral erythroid regenerative response did not develop in the cats with reticulocytes noted only rarely post-inoculation (data not shown).

Effect of FeLV-KT on hematopoietic progenitors. CFU-E increased significantly ($p<.025$) from 194 ± 3 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) pre-inoculation to 225 ± 8 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) at 2 weeks PI (Fig. 2.2). By four weeks PI, however, no CFU-E were detectable from any of the cats. In contrast to CFU-E, BFU-E had declined 39% ($p<.05$) by 2 weeks PI from 71 ± 1 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) pre-inoculation to 44 ± 1 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) (Fig. 2.2). As with CFU-E, no BFU-E were detectable in cultures of LDBMMC from cats at 4 and 6 weeks PI.

The number of CFU-GM increased 128% ($p<.01$) from 86 ± 6 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) pre-inoculation to 196 ± 8 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) at 2 weeks PI (Fig. 2.2). Levels declined slightly by four weeks PI but remained significantly increased ($p<.05$).

Effects of modulators of cAMP or calcium on erythroid progenitors from normal and viremic cats. In vitro exposure to reagents known to increase intracellular cAMP or calcium levels enhanced both CFU-E and BFU-E from LDBMMC of normal cats. However, these treatments either had no effect or, in most cases, caused significant suppression of progenitor numbers at 2 weeks PI. At both 4 and 6 weeks PI, CFU-E and BFU-E were 0 per 5 x 10^4 LDBMMC for all cats (Fig. 2.2) and the in vitro treatments failed to stimulate colony formation (data not shown).

Isoproterenol treatment of normal LDBMMC stimulated CFU-E at 10^{6}M - 10^{-8}M with the maximal effect of 161% ± 9.6 of control ($\bar{X}$ ± SEM) evident at 10^{-7}M ($p<.005$) (Fig. 2.3). BFU-E were increased to 156% ± 8 of control ($\bar{X}$ ± SEM) at 10^{-7}M ($p<.005$) and to a lesser extent at 10^{-8}M (Fig. 2.4). In all cases simultaneous treatment with propranolol (10^{-6}M) blocked the enhancement observed in cultures with isoproterenol alone. At 2 weeks PI, however, isoproterenol, neither stimulated nor suppressed CFU-E or BFU-E from LDBMMC (Figs. 3 and 4).

Dibutyryl-cAMP increased CFU-E at 10^{-5}M - 10^{-7}M with the maximal effect at 10^{-5}M (mean = 130% ± 2 SEM) ($p<.01$) (Fig. 2.5). BFU-E were increased at 10^{-5}M to 10^{-7}M with maximal stimulation to 139% ± 5 of controls ($\bar{X}$ ± SEM) at 10^{-6}M ($p<.01$) (Fig. 2.6). At 10^{-4}M db-cAMP significantly suppressed ($p<.01$) both CFU-E and BFU-E to 62% ± 6.1 ($\bar{X}$ ± SEM) and 21% ± 5 ($\bar{X}$ ± SEM), respectively, of pre-inoculation controls. Sodium butyrate had no significant effect on pre-inoculation CFU-E or BFU-E. At 2 weeks PI, although db-cAMP suppressed CFU-E at 10^{-4}M similar to levels observed pre-inoculation, no potentiating

effect occurred at 10^{-5}M - 10^{-7}M (Fig. 2.5). BFU-E not only were suppressed at 10^{-4}M but also at 10^{-5} - 10^{-7}M (Fig. 2.6). However, at the latter concentrations the effect could not be distinguished from the suppression caused by sodium butyrate.

Forskolin modestly enhanced both CFU-E and BFU-E of normal cats at 10^{-5}M and 10^{-6}M (Fig. 2.7). The peak response occurred at 10^{-6}M reaching 117% ± 7 ($\bar{X}$ ± SEM) ($p<.05$) and 127% ± 3 ($\bar{X}$ ± SEM) ($p<.01$) for CFU-E and BFU-E, respectively. At 10^{-4}M, CFU-E were suppressed to 54% ± 4 ($\bar{X}$ ± SEM) while BFU-E remained unaffected. In contrast, the erythroid progenitors of viremic cats were suppressed by forskolin at 10^{-5}M with maximum inhibition of CFU-E and BFU-E occurring at 10^{-4}M to 38% ± 5 ($\bar{X}$ ± SEM) and 42% ± 6 ($\bar{X}$ ± SEM), respectively ($p<.005$).

RO-20-1724 stimulated CFU-E of normal cats at 10^{-5}M and 10^{-6}M with the maximal effect of 160% ± 5 ($\bar{X}$ ± SEM) evident at 10^{-6}M ($p<.005$) (Fig. 2.8). BFU-E were increased significantly at 10^{-5}M to 137% ± 4 ($\bar{X}$ ± SEM) ($p<.01$). RO-20-1724 inhibited both CFU-E and BFU-E at a concentration of 10^{-3}M to 43% ± 9 ($\bar{X}$ ± SEM) and 10% ± 7 ($\bar{X}$ ± SEM), respectively ($p<.005$). Similar to the effects with forskolin, erythroid progenitors at 2 weeks PI were suppressed severely by RO-20-1724. BFU-E of viremic cats were inhibited at all concentrations reaching only 8% ± 5 ($\bar{X}$ ± SEM) at 10^{-3}M ($p<.005$). CFU-E of viremic cats were inhibited at 10^{-3}M - 10^{-5}M reaching only 17% ± 7 ($\bar{X}$ ± SEM) of control numbers at 10^{-3}M ($p<.005$).

A23187 enhanced CFU-E growth of normal cats at all concentrations producing peak stimulation of 144% ± 7 ($\bar{X}$ ± SEM) at 10^{-8}M

($p<.005$) (Fig. 2.9). BFU-E numbers increased at concentrations of 10^{-7}M - 10^{-9}M with the maximal effect of 155% ± 12 ($\bar{X}$ ± SEM) occurring also at 10^{-8}M ($p<.025$). In sharp contrast, both CFU-E and BFU-E of viremic cats were inhibited at 2 weeks PI. CFU-E suppression ranged from 75% ± 4 ($\bar{X}$ ± SEM) at 10^{-9}M ($p<.005$) to 41% ± 3 ($\bar{X}$ ± SEM) at 10^{-6}M. BFU-E also were inhibited between 10^{-6} - 10^{-8}M; the maximum suppression occurring at 10^{-7}M and reaching only 64% ± 6 ($\bar{X}$ ± SEM) of control numbers.

Indomethacin had no effect on numbers of erythroid progenitors pre-inoculation and no effect on CFU-E cultured at 2 weeks PI (Fig. 2.10). BFU-E of viremic cats, however, were decreased significantly at all concentrations reaching only 59% ± 6 ($\bar{X}$ ± SEM) at a concentration of 10^{-8}M.

Effects of in vitro treatments on erythroid progenitors suppressed by p15E. Addition of p15E to normal LDBMMC predictably inhibited CFU-E and BFU-E numbers to 18.4% and 13.2% of controls, respectively ($p<.005$) (Table 2.1). None of the treatments enhanced erythroid progenitor growth but several further suppressed colony numbers. CFU-E exposed to p15E were inhibited to approximately 50% of the p15E control by all concentrations of db-cAMP and A23187. They were totally inhibited by forskolin at 10^{-4}M and RO-20-1724 at 10^{-3}M but other concentrations of these two reagents appeared to have no effect on growth. BFU-E exposed to p15E were suppressed significantly by db-cAMP at all concentrations but similar inhibition was evident by sodium butyrate at comparable levels. Forskolin, RO-20-1724, and A23187 at all concentrations all inhibited BFU-E that had been

exposed to p15E. Isoproterenol and indomethacin had no effect on p15E suppressed progenitors.

DISCUSSION

In these studies, chemicals known to increase intracellular cAMP and calcium, and/or to reverse suppression of T-cell function by FeLV were assessed for their ability to enhance growth of erythroid progenitors from normal LDBMMC, normal LDBMMC treated with the FeLV envelope protein, p15E, and LDBMMC from cats with EA. The results indicate that both CFU-E and BFU-E are increased when cAMP or calcium levels are increased in cells from normal cats. However, treatment of bone marrow cells from viremic cats or of progenitors which were suppressed by exposure to p15E not only failed to enhance progenitor numbers and reverse suppression but actually caused further inhibition. These divergent effects are consistent with depletion of the progenitor subpopulation of progenitors which normally are responsive to agents which increase cAMP or calcium.

Previous studies have demonstrated that exposure of bone marrow cells to agents which increase intracellular cAMP or calcium will increase numbers of CFU-E (18-22) but few studies have addressed their effects on BFU-E. Isoproterenol, a β-agonist; db-cAMP, a cAMP analog; forskolin, an adenylate cyclase stimulator; RO-20-1724, a phosphodiesterase inhibitor; and A23187, a calcium ionophore; all stimulated both CFU-E and BFU-E growth from normal LDBMMC when added to the culture system at appropriate concentrations. Propranolol blocked the effects of isoproterenol and confirmed that its activity was β receptor specific. Sodium butyrate was used as a control for the dibutyryl moiety of db-cAMP and failed to have any effect on

normal cells. Indomethacin, in a previous study, had no effect on progenitor numbers (36). The ability of cAMP to enhance cell growth is partly related to the cell-cycle kinetics of the cell population being examined. All cells experience one or more surges of cAMP during active stages of the cycle ($S-G_2+M$) and in general are inhibited from progressing to a subsequent stage if levels do not decrease (16). However, slowly cycling or quiescent cells in the G_0/G_1 phase of the cycle, e.g. spleen colony-forming units (CFU-S) can be stimulated to proliferate by cAMP (16). Studies evaluating the cell cycle status of hemopoietic progenitors have revealed that 80% of CFU-S, BFU-E, and CFU-GM are normally in G_0/G_1, whereas this percentage may be less than 40% in a regenerating marrow (34). The enhancement of erythroid progenitor numbers from normal LDBMMC by increased cAMP most likely was due to mobilization of CFU-E and BFU-E from G_0/G_1 with concommitant suppression of those in $S-G_2+M$. As long as there is a greater percentage of cells slowly cycling, the net result of cAMP is stimulation. The mechanisms by which calcium stimulates cell growth is complex but part of its varied effects include elevated cAMP (17).

Increased levels of cAMP, however, were not able to reverse the total suppression of CFU-E and BFU-E at 4 and 6 weeks PI nor the partial suppression of CFU-E and BFU-E caused by p15E. These results are in contrast to the effects of indomethacin and forskolin on FeLV-induced suppression of lymphocyte Con A receptor mobility or lymphocyte mitogenic responses to Con A (14,15,37). In these studies, <u>in vitro</u> treatment of LDBMMC at 2 weeks PI as well as of normal LDBMMC

exposed to p15E resulted in additional suppression of both CFU-E and BFU-E by most reagents.

One possible explanation for the dramatic shift from stimulation to inhibition of erythroid progenitors may have been the number of BFU-E and CFU-E in the S-G_2+M phases of the cell cycle. Although cats at 2 weeks PI were not anemic, they had decreased BFU-E in conjunction with increased CFU-E and CFU-GM. This pattern has been seen previously with EA and attributed to rapid destruction of circulating erythrocytes with an extremely brief period of regeneration (35). The inhibition caused by enhanced cAMP, therefore, could be related to a reduced subpopulation of progenitors in G_0/G_1 and inhibitory effects on a larger population in S-G_2+M. This mechanism has been suggested as the reason for cAMP inhibition of Friend erythroleukemia cells (38). Furthermore, it is supported by cell cycle data from a previous study of FeLV-induced EA which showed that there was a significant increase of BFU-E in DNA synthesis by 3 weeks PI (41). Several additional results also are readily explained by this mechanism, including the suppression by sodium butyrate of BFU-E of FeLV-positive cats and p15E-treated BFU-E and the inhibition by indomethacin of FeLV-positive BFU-E. Indomethacin has been previously shown to increase cAMP in FeLV-positive lymphocytes while having no effect on normal cells (14,15). Sodium butyrate also has been reported to stimulate intracellular cAMP levels (39). A reason for the inability of isoproterenol to augment progenitor suppression, however, remains inexplicable although the expression of β receptors

in actively cycling progenitors could be different from those in G_0/G_1 phase.

These studies indicate that there is either a viral-induced block of pre-BFU-E progenitors or direct viral inhibition of BFU-E and CFU-E growth. The latter interpretation is supported by the failure of the treatments to reverse p15E-mediated suppression, implying that progenitors could still be present but are incapable of responding to growth regulation. However, CFU-E, BFU-E, and CFU-GM from viremic cats have been shown to display viral antigens and can be inhibited by antibody to FeLV gp70 plus complement suggesting lack of direct suppression of erythroid progenitors by FeLV. In view of previous studies demonstrating the reversal of FeLV-induced suppression of lymphocyte function, these results suggest that the ineffective function of lymphocytes as erythropoietic accessory cells is not likely to be a factor in the pathogenesis of FeLV-induced EA. Finally, the results indicate that cAMP and calcium are not only capable of stimulating CFU-E as described in previous studies but also dramatically enhancing growth of BFU-E.

SUMMARY

Pharmacologic modulators of cyclic 3',5'-adenosine monophosphate (cAMP) and calcium were added *in vitro* to cultures of low density bone marrow mononuclear cells (LDBMMC) of normal cats and LDBMMC of cats with retrovirus-induced erythroid aplasia. Treatment of cells from normal cats with the following reagents increased the number of erythroid progenitors (CFU-E and BFU-E) as compared to control growth: isoproterenol, a β-agonist, increased CFU-E 161% and BFU-E 156% at 10^{-7}M; dibutyryl cAMP, a cAMP analog, increased CFU-E 130% at 10^{-5}M and BFU-E 139% at 10^{-7}M; forskolin, an adenylate cyclase stimulator, increased CFU-E 117% and BFU-E 127% at 10^{-6}M; RO-20-1724, a phosphodiesterase inhibitor, increased CFU-E 160% and BFU-E 137% at 10^{-5}M; and A23187, a calcium ionophore, increased CFU-E 144% and BFU-E 155% at 10^{-8}M. However, treatment of LDBMMC collected at 2 weeks post-inoculation from viremic cats not only failed to enhance CFU-E and BFU-E but caused significant suppression of their growth. Additionally, the suppression of erythroid progenitors by exposure to the FeLV protein, p15E, was augmented when reagents were added at levels that caused enhancement of CFU-E and BFU-E numbers cultured from normal LDBMMC. Treatment of bone marrow cells from cats with no detectable erythroid progenitors failed to reverse FeLV-induced erythroid aplasia. These studies 1) demonstrate that agents which increase levels of cAMP or calcium enhance CFU-E and BFU-E from normal LDBMMC; 2) indicate that agents which increase levels of cAMP or calcium fail to reverse FeLV suppression of

erythropoiesis in contrast to the abrogation of FeLV-mediated suppression of lymphocyte blastogenesis as previously reported; 3) suggest that feline erythroid aplasia is related to a non-reversible block of pre-BFU-E progenitors or direct inhibition of BFU-E and CFU-E growth; 4) suggest that lymphocyte dysfunction as erythropoietic accessory cells is not a critical part of the pathogenesis of FeLV-induced erythroid aplasia.

Table 2.1

Effect *in vitro* of cAMP modulating agents, sodium butyrate, A23187, and indomethacin on colony formation from erythroid progenitors suppressed by p15E.

Treatments[a]	Colony Nos.[b]	
	CFU-E	BFU-E
Control medium	158 ± 9	38 ± 4
p15E	29 ± 5.8[c]	5 ± .3[c]
Isoproterenol (10^{-6}M)	22 ± 12	4 ± .5
Isoproterenol (10^{-7}M)	27 ± 1	4 ± 1.5
Isoproterenol (10^{-8}M)	22 ± 10	4 ± .5
Isoproterenol (10^{-9}M)	23 ± 7	3 ± .5
db-cAMP (10^{-4}M)	9 ± .5[d]	0[e]
db-cAMP (10^{-5}M)	11 ± 1.4[d]	0[e]
db-cAMP (10^{-6}M)	14 ± 1.5[d]	2 ± .5[d]
db-cAMP (10^{-7}M)	15 ± 1.4[d]	1 ± .5[d]
Forskolin (10^{-4}M)	0[e]	0[e]
Forskolin (10^{-5}M)	23 ± 3	1 ± .5[d]
Forskolin (10^{-6}M)	23 ± 7	1 ± .5[d]
Forskolin (10^{-7}M)	23 ± 3	0[e]
RO-20-1724 (10^{-3}M)	0[e]	0[e]
RO-20-1724 (10^{-4}M)	25 ± 1	2 ± .5[d]
RO-20-1724 (10^{-5}M)	25 ± 1.5	2 ± .5[d]
RO-20-1724 (10^{-6}M)	23 ± 9	0[e]

Table 2.1 (Continued)

Treatments[a]	Colony Nos.[b]	
	CFU-E	BFU-E
Sodium butyrate (10^{-4}M)	25 ± 5	0[e]
Sodium butyrate (10^{-5}M)	30 ± 10	3 ± 3
Sodium butyrate (10^{-6}M)	27 ± 1	1 ± .5[d]
Sodium butyrate (10^{-7}M)	26 ± 14	0[e]
A23187 (10^{-6}M)	9 ± 1[d]	0[e]
A23187 (10^{-7}M)	13 ± 2[d]	1 ± .5[d]
A23187 (10^{-8}M)	10 ± 2[d]	4 ± 4
A23187 (10^{-9}M)	11 ± 1[d]	1 ± .5[d]
Indomethacin (10^{-6}M)	26 ± 6	3 ± 1
Indomethacin (10^{-7}M)	29 ± 3.5	3 ± 1
Indomethacin (10^{-8}M)	25 ± 5	4 ± 3.5
Indomethacin (10^{-9}M)	23 ± 9	4 ± 3.5

[a] LDBMMC (5×10^5/ml) were preincubated with p15E (15 μg/10^6 cells) in α-MEM for 1 hour at 37°C and then cultured (5×10^4 cells/week) for CFU-E and BFU-E in the continual presence of various agents at different concentrations.

[b] Mean ± SEM, n=2.

[c] Significant from untreated control at $p<.005$.

[d] Significant from p15E treatment alone at $p<.05$.

[e] Significant from p15E treatment alone at $p<.005$.

Fig. 2.1 Changes in PCV (mean ± SEM) after inoculation with FeLV-KT (n=4). All cats became positive at 2 weeks PI and remained viremic through the course of the experiment.

+ $p<.01$

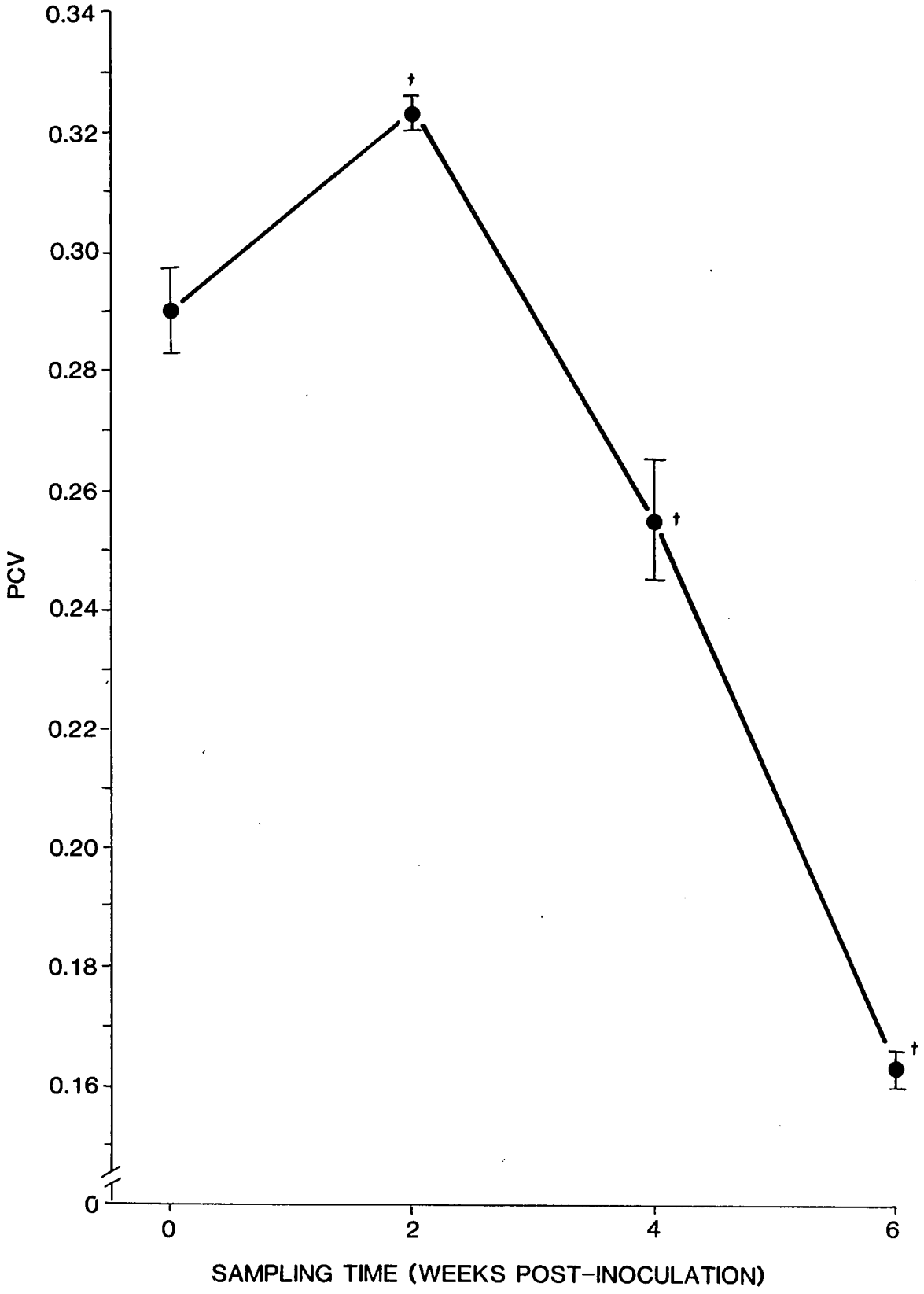

0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0
PCV
0
2
4
6
SAMPLING TIME (WEEKS POST-INOCULATION)

Fig. 2.2 Changes in CFU-E, BFU-E, and CFU-GM ($\bar{X}$ ± SEM) after inoculation with FeLV-KT (n=4).

* $p<.05$

** $p<.025$

\+ $p<.01$

++ $p<.005$

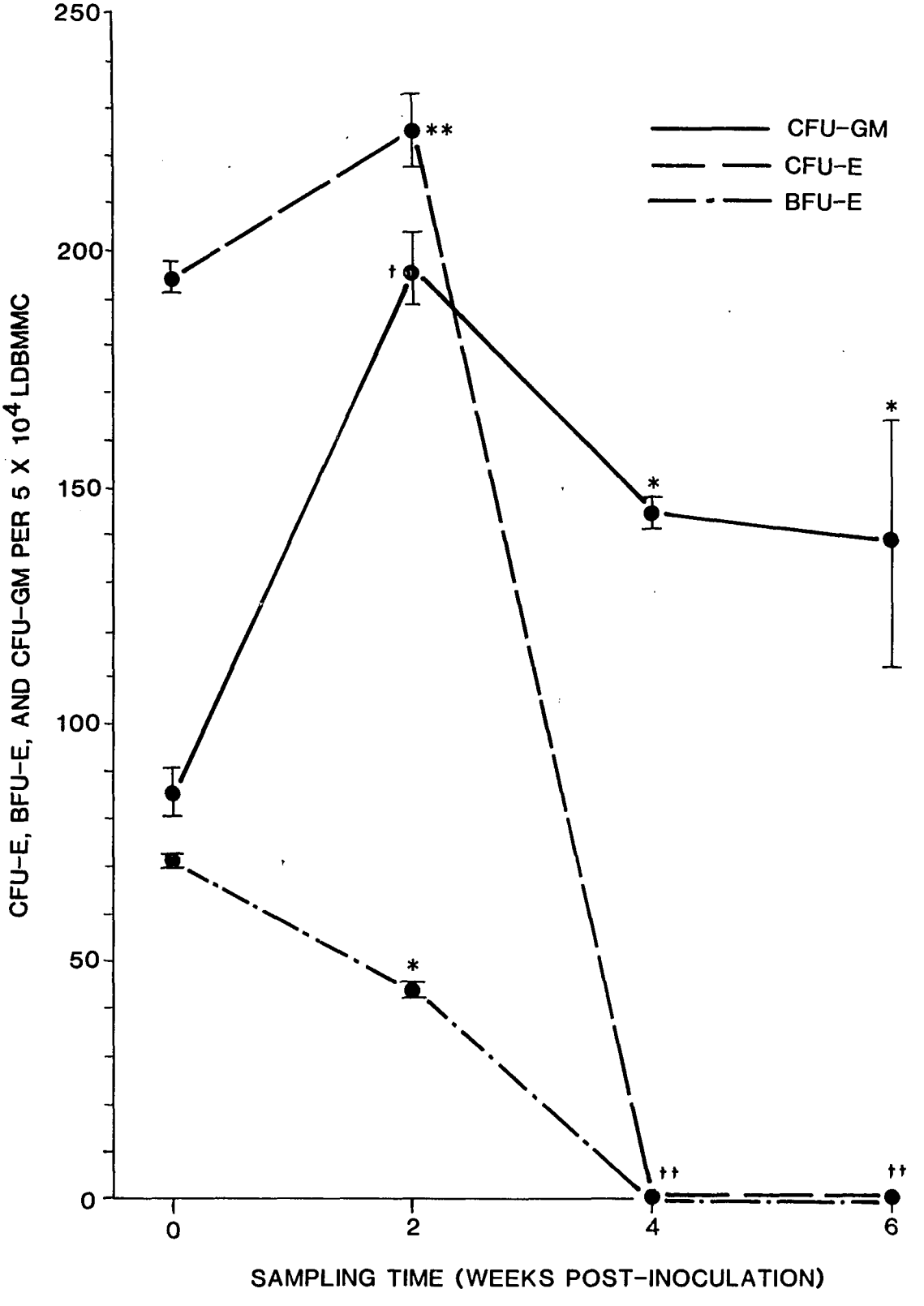

250
200
150
100
50
0
CFU-E, BFU-E, AND CFU-GM PER 5 X 10⁴ LDBMMC
CFU-GM
CFU-E
BFU-E
**
†
*
*
*
††
††
0
2
4
6
SAMPLING TIME (WEEKS POST-INOCULATION)

Fig. 2.3 Effect of various concentrations (M) of isoproterenol with and without propranolol on CFU-E ($\bar{X}$ ± SEM, % control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

* $p<.05$

\+ $p<.01$

++ $p<.005$

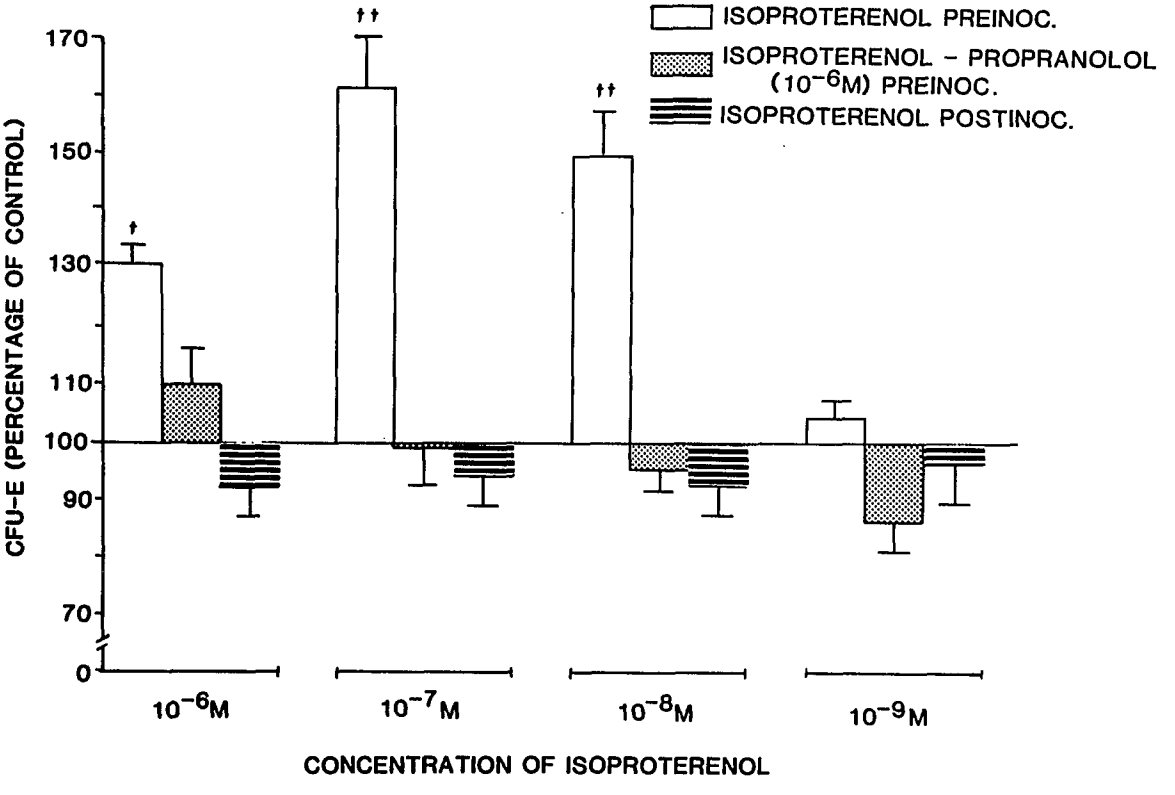

ISOPROTERENOL PREINOC.
ISOPROTERENOL - PROPRANOLOL (10⁻⁶M) PREINOC.
ISOPROTERENOL POSTINOC.
CFU-E (PERCENTAGE OF CONTROL)
170
150
130
110
100
90
70
0
†
††
††
10⁻⁶M
10⁻⁷M
10⁻⁸M
10⁻⁹M
CONCENTRATION OF ISOPROTERENOL

Fig. 2.4 Effect of various concentrations (M) of isoproterenol with and without propranolol on BFU-E ($\bar{X}$ ± SEM, % control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

** $p<.025$

++ $p<.005$

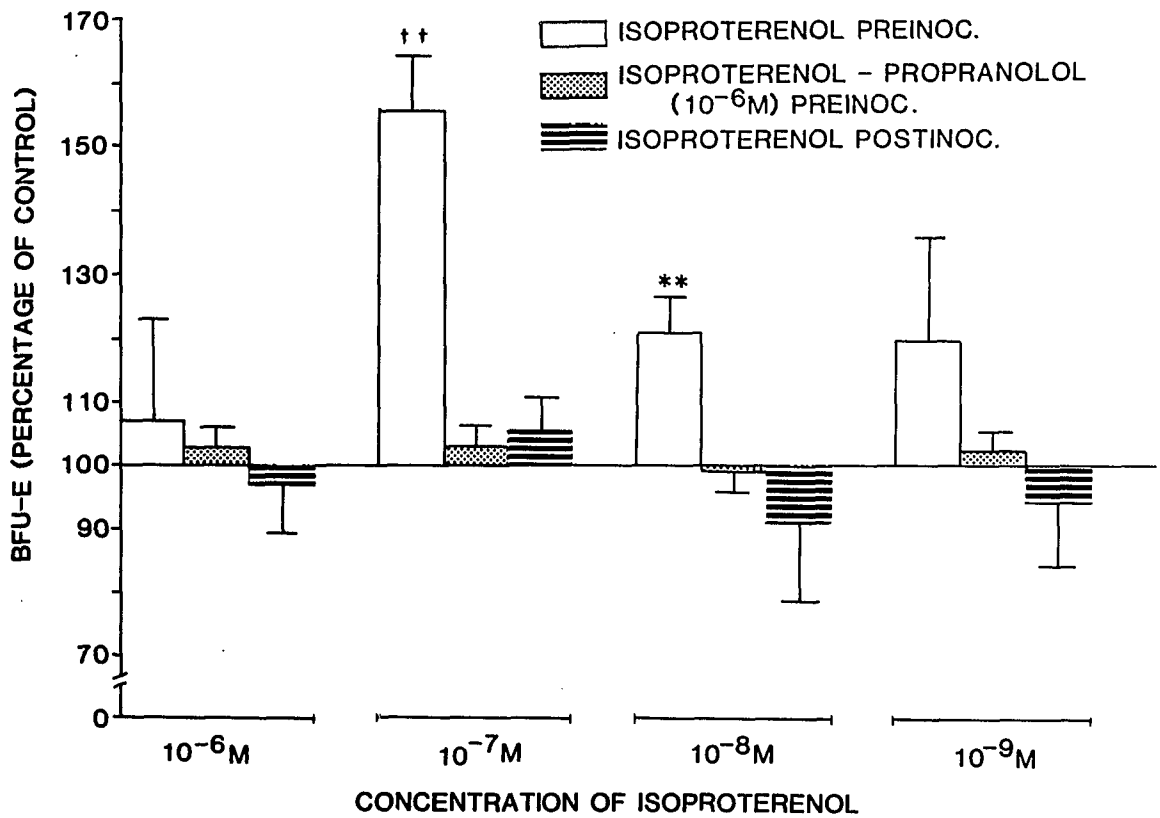

ISOPROTERENOL PREINOC.
ISOPROTERENOL - PROPRANOLOL (10^{-6}M) PREINOC.
ISOPROTERENOL POSTINOC.
BFU-E (PERCENTAGE OF CONTROL)
170
150
130
110
100
90
70
0
††
**
10^{-6}M
10^{-7}M
10^{-8}M
10^{-9}M
CONCENTRATION OF ISOPROTERENOL

Fig. 2.5 Effects of various concentrations (M) of dibutyryl cAMP and sodium butyrate on CFU-E ($\bar{X}$ ± SEM, % of control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

* $p<.05$

\+ $p<.01$

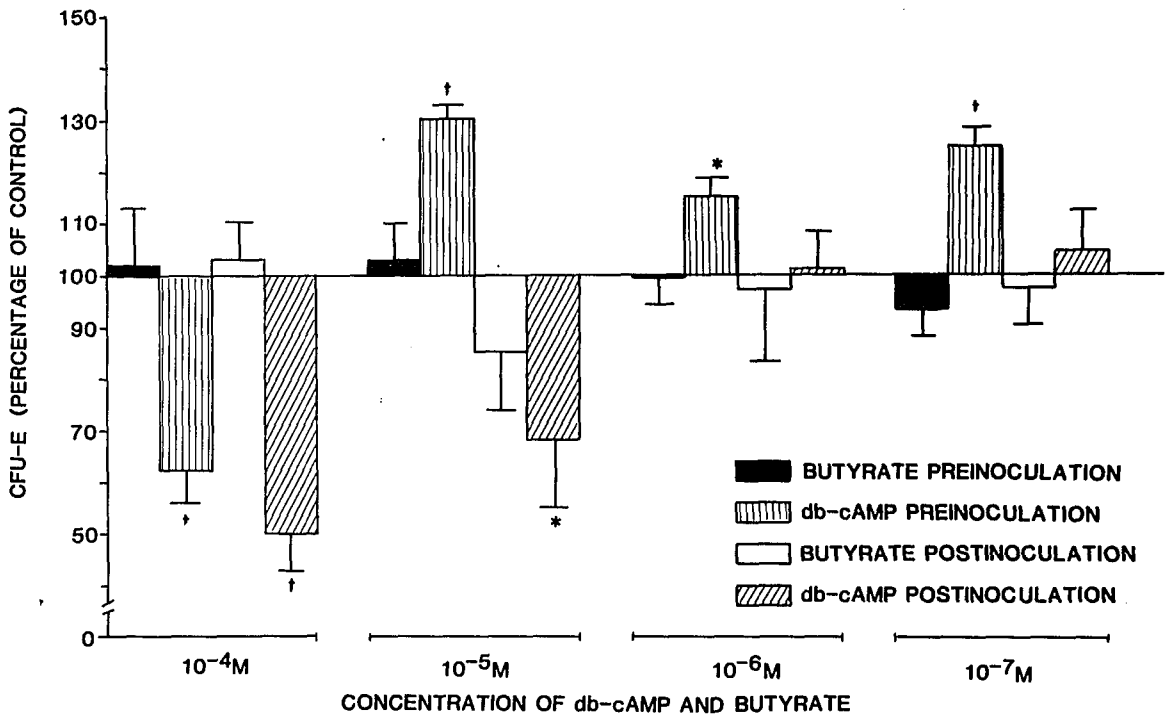

150
130
110
100
90
70
50
0
CFU-E (PERCENTAGE OF CONTROL)
BUTYRATE PREINOCULATION
db-cAMP PREINOCULATION
BUTYRATE POSTINOCULATION
db-cAMP POSTINOCULATION
10⁻⁴M
10⁻⁵M
10⁻⁶M
10⁻⁷M
CONCENTRATION OF db-cAMP AND BUTYRATE

Fig. 2.6 Effects of various concentrations (M) of dibutyryl cAMP and sodium butyrate on BFU-E ($\bar{X}$ ± SEM, % control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

* $p<.05$

\+ $p<.01$

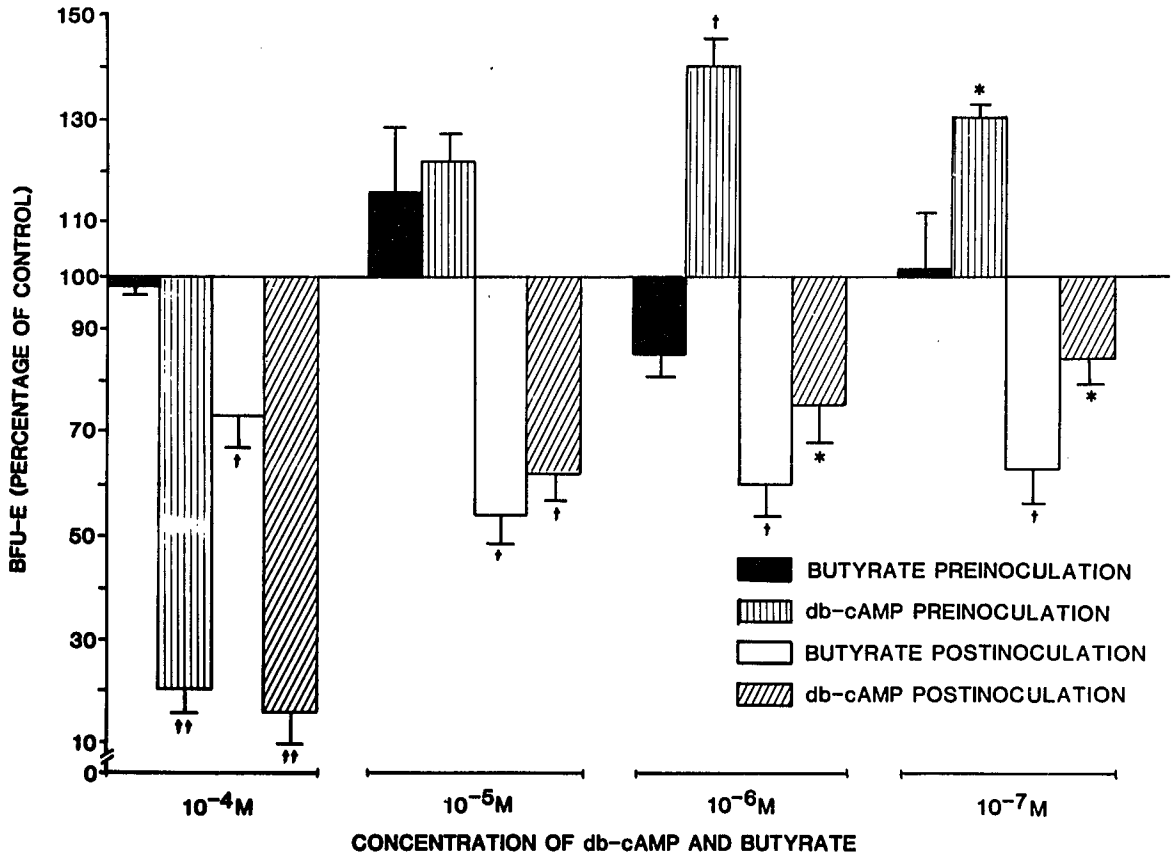

BFU-E (PERCENTAGE OF CONTROL)
150
130
110
100
90
70
50
30
10
0
BUTYRATE PREINOCULATION
db-cAMP PREINOCULATION
BUTYRATE POSTINOCULATION
db-cAMP POSTINOCULATION
10-4M
10-5M
10-6M
10-7M
CONCENTRATION OF db-cAMP AND BUTYRATE

Fig. 2.7 Effect of various concentrations (M) of forskolin on CFU-E and BFU-E ($\bar{X}$ ± SEM, % control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

* $p<.05$

** $p<.025$

\+ $p<.01$

++ $p<.005$

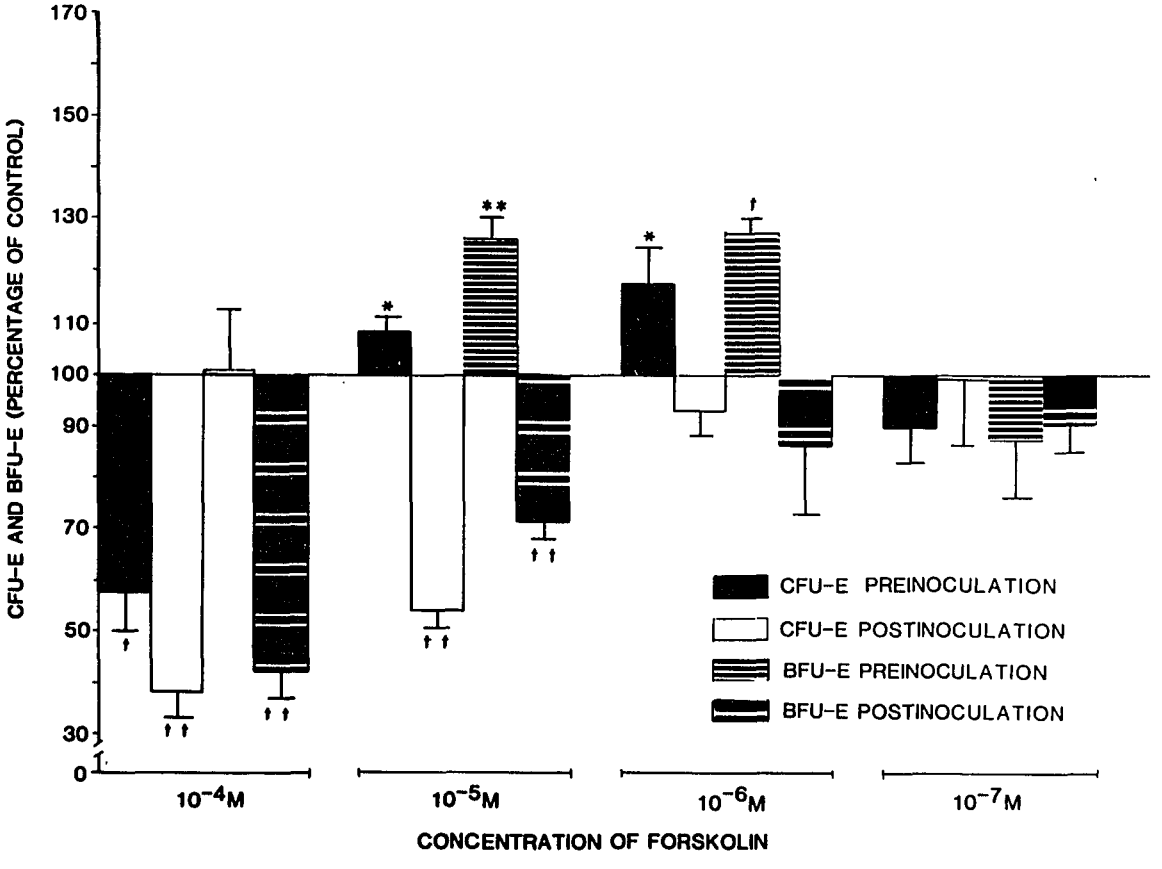

170
150
130
110
100
90
70
50
30
0
CFU-E AND BFU-E (PERCENTAGE OF CONTROL)
CFU-E PREINOCULATION
CFU-E POSTINOCULATION
BFU-E PREINOCULATION
BFU-E POSTINOCULATION
10⁻⁴M
10⁻⁵M
10⁻⁶M
10⁻⁷M
CONCENTRATION OF FORSKOLIN

Fig. 2.8 Effect of various concentrations (M) of RO-20-1724 on CFU-E and BFU-E ($\bar{X}$ ± SEM, % control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

* p<.05

** p<.025

\+ p<.01

++ p<.005

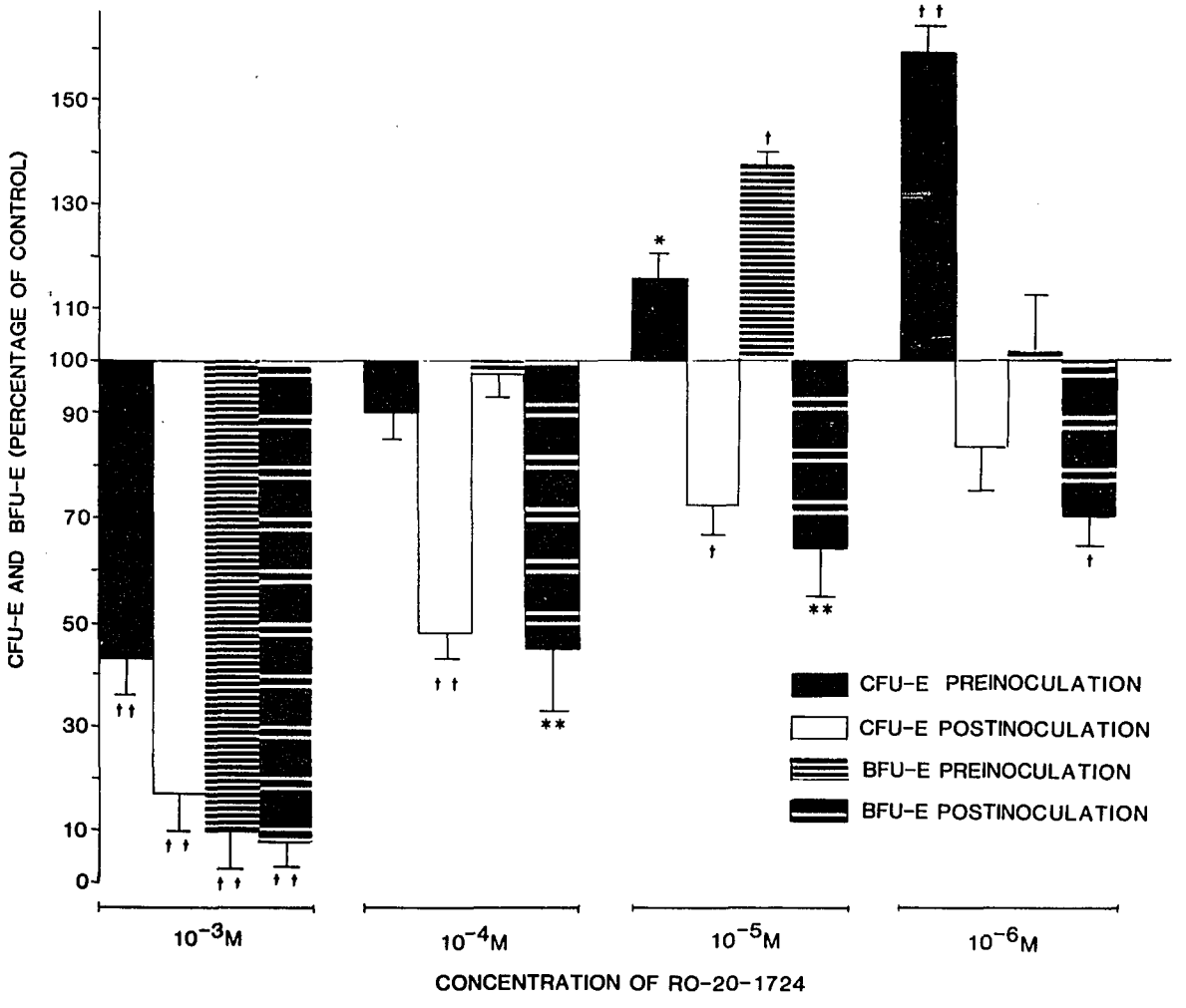

CFU-E AND BFU-E (PERCENTAGE OF CONTROL)
150
130
110
100
90
70
50
30
10
0
CFU-E PREINOCULATION
CFU-E POSTINOCULATION
BFU-E PREINOCULATION
BFU-E POSTINOCULATION
10-3M
10-4M
10-5M
10-6M
CONCENTRATION OF RO-20-1724

Fig. 2.9 Effect of various concentrations (M) of A23187 on CFU-E and BFU-E ($\bar{X}$ ± SEM, % control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

* $p<.05$

** $p<.025$

++ $p<.005$

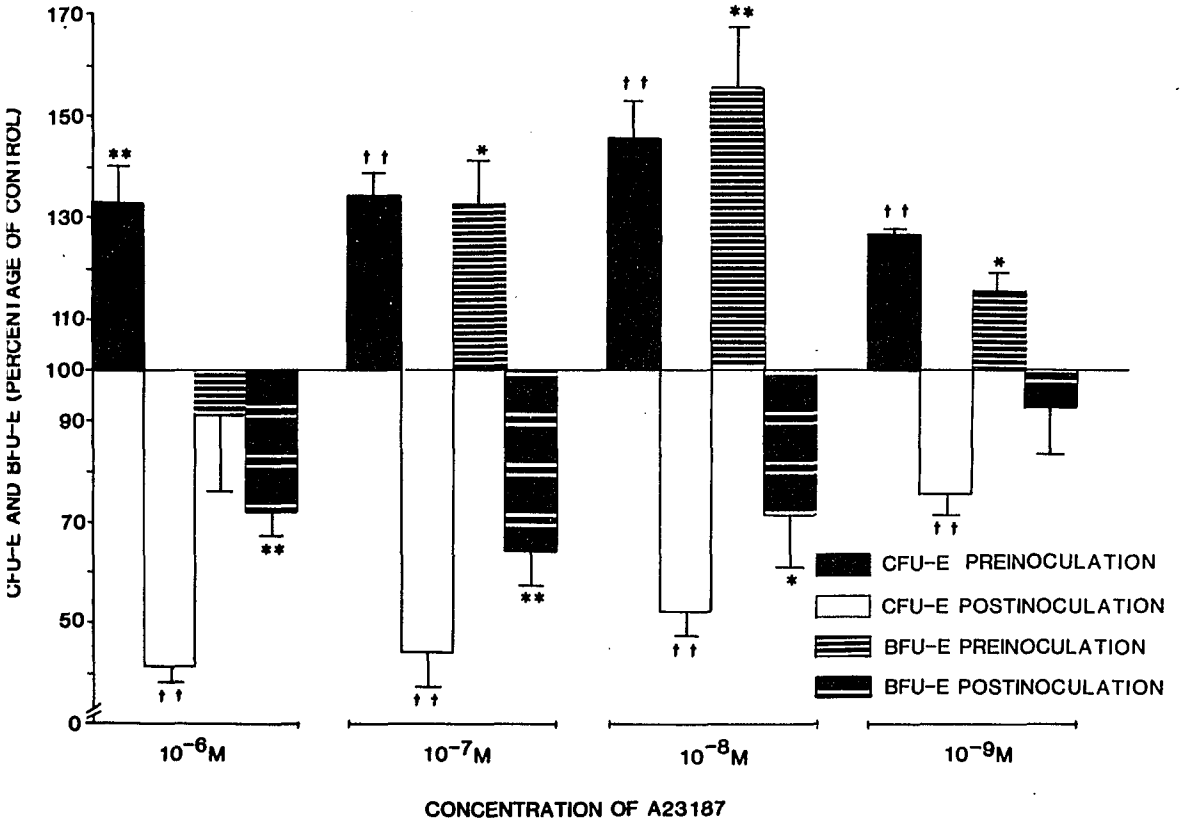
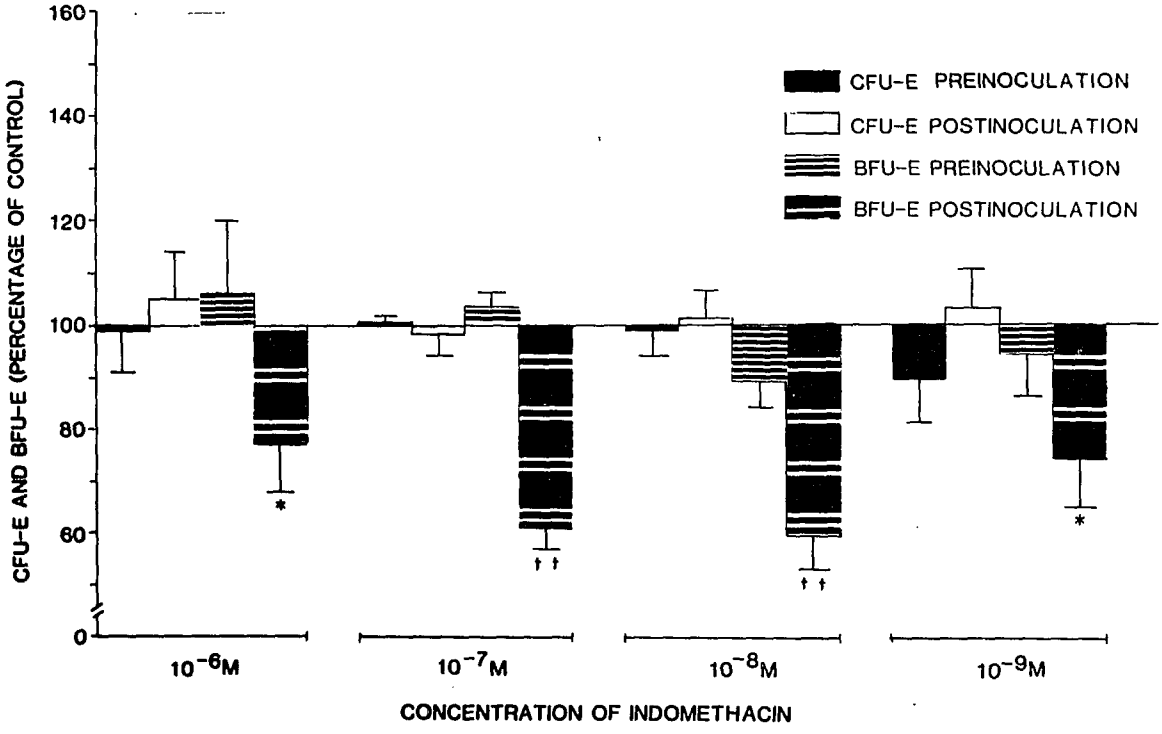

CFU-E AND BFU-E (PERCENTAGE OF CONTROL)
170
150
130
110
100
90
70
50
0
CFU-E PREINOCULATION
CFU-E POSTINOCULATION
BFU-E PREINOCULATION
BFU-E POSTINOCULATION
10⁻⁶M
10⁻⁷M
10⁻⁸M
10⁻⁹M
CONCENTRATION OF A23187

Fig. 2.10 Effect of various concentrations (M) of indomethacin on CFU-E and BFU-E ($\bar{X}$ ± SEM, % control growth) before inoculation and at 2 weeks after inoculation with FeLV (n=4).

* $p<.05$

++ $p<.005$


160
140
120
100
80
60
0
CFU-E AND BFU-E (PERCENTAGE OF CONTROL)
CFU-E PREINOCULATION
CFU-E POSTINOCULATION
BFU-E PREINOCULATION
BFU-E POSTINOCULATION
*
††
*
10^{-6}M
10^{-7}M
10^{-8}M
10^{-9}M
CONCENTRATION OF INDOMETHACIN

REFERENCES

1. Rojko JL, Olsen RG (1984) The immunobiology of the feline leukemia virus. Vet Immunol Immunopathol 6:107-165.

2. Onions D, Jarrett O, Testa N, Frassoni F, Toth S (1982) Selective effect of feline leukaemia virus on early erythroid precursors. Nature 296:156-158.

3. Gasper PW, Hoover EA (1983) Feline leukemia virus strain specific abatement of erythroid burst-forming units (BFU-E). Leuk Rev Internatl 1:71-72.

4. Testa NG, Onions, Jarrett O et al (1983) Haemopoietic colony formation (BFU-E, GM-CFC) during the development of pure red cell hypoplasia induced in the cat by feline leukaemia virus. Leuk Res 7:103-116.

5. Boyce JT, Hoover EA, Kociba GJ, Olsen RG (1981) Feline leukemia virus-induced erythroid aplasia: In vitro hemopoietic culture studies. Expl Hematol 9:990.

6. Wellman ML, Kociba GJ, Lewis MG, Mathes LE, Olsen RG (1984) Inhibition of erythroid colony-forming cells by a M_r 15,000 protein of feline leukemia virus. Cancer Res 44:1527-1529.

7. Kociba GJ (1987) Lymphocyte coculture studies of retrovirus-induced feline erythroid aplasia. In preparation.

8. Abkowitz JL, Ott RL, Holly RD, Adamson JW (1987) Lymphocytes and antibody in retrovirus-induced feline pure red cell aplasia. J Natl Cancer Inst 78:135-139.

9. Tambourin PE, Wendling F, Jasmin C, Smadja-Joffe F (1979) The physiopathology of Friend leukemia. Leuk Res 3:117-129.

10. Lobue J (1984) Viral-associated alterations in hematopoiesis in the mouse. Blood Cells 10:211-222.

11. Graf T, Beug H, Roussel M, Saule S, Stehelin D, Hayman MJ (1980) Avian leukemia viruses and hematopoietic cell differentiation. Brit J Cancer 41:659-661.

12. Orosz CG, Zinn NE, Olsen RG, Mathes LE (1985) Retrovirus-mediated immunosuppression. I. FeLV-UV and specific FeLV proteins alter T lymphocyte behavior by inducing hyporesponsiveness to lymphokines. J Immunol 134:3396-3403.

13. Orosz CG, Zinn NE, Olsen RG, Mathes LE (1985) Retrovirus-mediated immunosuppression. II. FeLV-UV alters in vitro murine T lymphocyte behavior by reversibly impairing lymphokine secretion. J Immunol 135:584-590.

14. Lewis MG Determination of a mechanism of feline retroviral suppression of the feline immune system. PhD Dissertation, Ohio State University, 1983, pp 30-49.

15. Lewis MG (1985) Reversal of feline retroviral suppression by indomethacin. Leuk Res 9:1451-1456.

16. Boynton AL, Whitfield JF (1983) The role of cyclic AMP in cell proliferation: A critical assessment of the evidence. Adv Cyc Nucl Res 15:193-294.

17. Whitfield JF, Boynton AL, MacManus JP, Rixon RH, Sikorska M, Tsang B, Walter PR, Swierenga S (1980) The roles of calcium and cyclic AMP in cell proliferation. Ann NY Acad Sci 339:216.

18. Brown JE, Adamson JW (1977) Modulation of in vitro erythropoiesis: The influence of -adrenergic agonists on erythroid colony formation. J Clin Invest 60:70-77.

19. Brown JE, Adamson JW (1977) Studies of the influence of cyclic nucleotides on in vitro haemoglobin synthesis. Brit J Haemat 35:193-208,

20. Gallicchio VS, Chen MG, Murphy MG (1982) Modulation of murine in vitro erythroid and granulopoietic colony formation by ouabain, digoxin, and theophylline. Expt Hematol 10:682-697.

21. Misiti J, Spivak JL (1979) Erythropoiesis in vitro. Role of calcium. J Clin Invest 64:1573-1579.

22. Bridges K, Levenson R, Housman D, Cantley L (1981) Calcium regulates commitment of murine erythroleukemia cells to terminal differentiation. J Cell Biol 90:542-545.

23. Rozengurt E (1986) Early signals in the mitogenic response. Science 234:161-166.

24. Mathes LE, Yohn DS, Olsen RG (1977) Purification of infectious feline leukemia virus from large volumes of tissue culture fluids. J Clin Microbiol 5:372-374.

25. Mathes LE, Olsen RG, Hebebrand LC, Hoover EA, Schaller JP (1978) Abrogation of lymphocyte blastogenesis by a feline leukemia virus protein. Nature (Lond) 274:687-689.

26. Hoover EA, Mathes LE, Rojko JL et al (1978) Modification of the immunofluorescence assay for feline leukemia virus group-specific antigen. Am J Vet Res 39:1877-1880.

27. Lands AM, Arnold A, McAuliff JP, Luduena FP, Brolon TG (1967) Differentiation of receptor systems activated by sympathomimetic amines. Nature (Lond) 214:597-598.

28. Nickerson M, Collier B (1975) Drugs inhibiting adrenergic nerves and structures innervated by them. In The Pharmacological Basis of Therapeutics. LS Goodman, A Gilman, editors. MacMillan Publ Co Inc., NY, pp 533-546.

29. Seamon KB, Daly JW (1981) Forskolin: A unique diterpene activator of cyclic AMP-generating systems. J Cyc Nucl Res 7:201-224.

30. Sheppard H, Wiggan A (1971) Different sensitivities of the phosphodiesterases (adenosine-3',5'-cyclic phosphate 3',phosphohydrolase) of DDG cerebral cortex and erythrocytes to inhibition by synthetic agents and cold. Biochem Pharmacol 20:2128-2130.

31. Reed PW, Laroy HA (1972) A23187: A divalent cation ionophore. J Biol Chem 247:6970.

32. Kantor HS, Hampton TN (1979) Indomethacin at submicromolar concentrations inhibits cyclic AMP-dependent protein kinase. Nature 276:817-820.

33. Abkowitz JL, Oft RL, Nakamura JM et al (1985) Feline glucose-6-phosphate dehydrogenase cellular mosaicism. Application to the study of pure red cell aplasia. J Clin Invest 75:133-140.

34. Cillo C, Sekaly RP, Magli MC, Odartchenko N (1985) Early hemopoietic progenitor cells: Direct measurement of cell cycle status. Ann NY Acad Sci 459:150-161.

35. Zack PM, Kociba GJ (1987) Antithymocyte globulin treatment of retrovirus-induced feline erythroid aplasia: in vivo and in vitro studies. Submitted for publication.

36. Snyder DS, Desforges JF (1986) Lipoxygenase metabolites of arachidonic acid modulate hematopoiesis. Blood 67:1675-1679.

37. Lewis MG, Fertel RH, Olsen RG (1985) The reversal of feline retroviral-induced suppression of lymphocyte Con A receptor mobility by indomethacin and PGE_2. Leuk Res 9:1105-1109.

38. Beckman B, Mirand E, Fisher JW (1980) Effects of beta adrenergic agents and prostaglandin E_1 on erythroid colony (CFU-E) growth and cyclic AMP formation in Friend erythroleukemic cells. J Cell Physiol 105:355-361.

39. Prasad KN (1980) Butyric acid: A small fatty acid with diverse biological functions. Life Sci 37:1351-1358.

40. Riedel N, Hoover EA, Gasper PW et al (1986) Molecular analysis and pathogenesis of the feline aplastic anemia retrovirus. feline leukemia virus C-sarma. J Virol 60:242-250.

41. Abkowitz JL, Adamson JW (1985) Kinetics of marrow failure in feline leukemia virus (FeLV-C)-induced pure red cell aplasia (PRCA). Blood 66(Suppl 1):118A.

CHAPTER III

ROLE OF INTERFERON (IFN) IN FELINE RETROVIRUS-INDUCED ERYTHROID APLASIA: LACK OF DETECTABLE SERUM OR BONE MARROW IFN *IN VIVO* AND SUPPRESSION BY IFN OF HEMATOPOIETIC PROGENITOR CELLS *IN VITRO*

INTRODUCTION

Interferons (IFN) are small proteins and glycoproteins produced by nucleated cells in response to viruses as well as a wide variety of other stimuli. IFN is capable of a diverse series of functions both *in vivo* and *in vitro*, including the inhibition of viral replication, the regulation of cellular proliferation or differentiation, the expression of cell surface antigens, and immunoregulatory effects on both cellular and humoral immune responses (1).

In vitro studies have been performed examining the effects on hematopoiesis of α-IFN produced by leukocytes, β-IFN produced by fibroblasts, and γ-IFN produced by stimulated T lymphocytes. All three types effectively suppress the proliferation of normal hematopoietic progenitors including pluripotent colony-forming units (CFU-GEMM) (2,3), erythroid progenitors (CFU-E and BFU-E) (2,4,9), and granulocyte/macrophage colony-forming units (CFU-GM) (2,7,8). This

suppression has been correlated directly with IFN concentrations and can be blocked by anti-IFN antibodies (7,10).

The inhibitory effects in vitro have prompted evaluation of IFN as a mediator of hematopoietic suppression in vivo. α-IFN or γ-IFN has been detected in the blood of some patients with aplastic anemia (AA) (7,11). In these patients, γ-IFN has been shown to be produced by OKT8(+) Tac(+) lymphocytes (10) and may play a central role in T-cell-mediated suppression of hematopoiesis (10). The possibility that a viral etiologic agent may be responsible for some cases of AA has been suggested. γ-IFN is produced during chronic viral infection (12), circulating α-IFN is present in acquired immunodeficiency syndrome (AIDS) patients (13), and viruses can induce stimulation of suppressor lymphocytes (14).

Feline leukemia virus (FeLV) is a horizontally transmitted retrovirus that is the major cause of spontaneous neoplastic and non-neoplastic disease in outbred cats, including erythroid aplasia (EA) (15). The Kawakami-Theilen strain of FeLV (FeLV-KT) consistently induces erythroid aplasia characterized by rapid and total depletion of CFU-E and BFU-E, with moderate enhancement of the number of CFU-GM in the bone marrow and severe immunosuppression (16-19). The pathogenesis of retroviral-induced EA is unknown. The combination of retroviral infection, immune dysfunction, and erythroid aplasia in cats infected with FeLV-KT suggests the possibility of interferon-mediated dysregulation of hematopoiesis. The objectives of these studies were to characterize the sensitivity of various feline hematopoietic progenitors to feline α/β-IFN and γ-IFN in vitro and

determine if IFN can be detected in the blood or the bone marrow in association with feline retrovirus-induced erythroid aplasia.

MATERIALS AND METHODS

Animals. All cats were obtained at 6 to 8 weeks of age from a specific pathogen-free breeding colony maintained by the Department of Veterinary Pathobiology, The Ohio State University. This colony is uniformly free of infection and immunity to FeLV. The cats were tranquilized with intramuscular ketamine hydrochloride prior to bone marrow or blood collection. Infected cats were pretreated one day prior to inoculation with methyl prednisolone (5 mg/kg) to enhance their susceptibility to productive FeLV infection.

Viral inoculum. The FeLV inoculum was prepared from equal volumes of serum and BMMC lysate pooled from cats with anemia induced by *in vivo* passage of FeLV-KT as described (19). The FeLV infectivity titer of the inoculum was assessed by the S+/L- focus induction assay as described previously (19). Cats were infected by intravenous or intraperitoneal injection of 0.4 ml inoculum containing 1×10^5 FFU FeLV-KT.

Determination of interferon (IFN) levels in the serum and bone marrow plasma of cats with EA. Six 6 to 8 week old kittens were inoculated with FeLV-KT. Blood was collected prior to inoculation and biweekly post-inoculation (PI) from each cat to perform complete hemograms (Coulter S plus IV, Coulter Electronics Inc., Hialeah, FL) and reticulocyte counts, and to evaluate circulating leukocytes for the presence of FeLV using an indirect immunofluorescence test for FeLV p27 as modified by Hoover *et al.* (24). Blood samples for IFN assays were collected at weekly intervals from each cat starting

at the onset of anemia (4 weeks PI). The samples were placed immediately on ice and serum was collected within 6 hours of collection. Bone marrow was sampled pre-inoculation and 4, 8, and 10 weeks PI from either the proximal humerus or the proximal femur. Samples were aspirated into a syringe previously flushed with heparin (500 U/ml) and placed immediately on ice. Bone marrow plasma was obtained after centrifugation of the samples at 400 x g for 10 minutes and was assayed for IFN as described below. Bone marrow cells were then resuspended in MEM- medium (Gibco, Grand Island, NY). Low density bone marrow mononuclear cells (LDBMMC) were separated and cultured for CFU-E, BFU-E, and CFU-GM as described below. All serum and bone marrow plasma samples were frozen at -70°C prior to determination of IFN levels.

Additionally, serum samples from 16 cats of a separate EA study were assayed for interferon. These cats all became viremic and anemic and had severely reduced numbers of erythroid progenitors between 4 and 6 weeks PI. Serum samples were collected at 0, 4, 5, 6, 8, 10, and 12 weeks PI, placed immediately on ice, and frozen at -70°C. Subsequently, they were thawed at room temperature and assayed for interferon as described below.

Low density bone marrow mononuclear cell separation (LDBMMC). Whole marrow cell suspensions were diluted to 20 ml with MEM-α in 16 x 95 mm polystyrene tubes and underlayered with an equal volume of Percoll (Pharmacia Fine CHemicals, Uppsala, Sweden) adjusted to a specific gravity of 1.071. LDBMMC were collected from the interface

after centrifugation for 30 minutes at 400g, washed twice in Hank's Balanced Salt Solution, and resuspended in MEM-*α*.

Progenitor assays. The culture methods for colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) were similar to those described by Abkowitz et al. (20) for feline bone marrow. Briefly, 1.25×10^5 LDBMMC in 0.2 ml MEM-*α* were added to 0.9 ml of medium containing 1.3% methylcellulose (Dow-Corning, Midland, MI) in Iscove's Modified DMEM (Hazleton Research Products), 1% deionized bovine serum albumin (Armour Pharmaceuticals, Tarrytown, NY), 10^{-4}M β-mercaptoethanol, 30% fetal bovine serum (Armour Pharmaceuticals), TC erythropoietin (Amgen Biologicals, Thousand Oaks, CA) at two units per milliliter, and 5% pokeweed mitogen-conditioned medium from bone marrow mononuclear cells. The assays were performed in duplicate in 24-well cluster dishes (Costar, Cambridge, MA) with 5×10^4 cells in 0.4 ml of medium per well. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 10% O_2 in nitrogen-enriched air. Erythroid colonies were counted on day 4 of culture as red colonies containing 8-50 cells (CFU-E) and on day 9 as red colonies composed of more than 200 cells, usually with closely associated subunits (BFU-E).

Colony-forming units-granulocyte/macrophage (CFU-GM) were quantified using a modification of our previously described method (19). Briefly, 2.5×10^5 LDBMMC in 0.1 ml MEM-*α* were added to 0.9 ml medium consisting of 0.5% methylcellulose in MEM-*α*, 0.4% bovine serum albumin, glutamine (2 mM), 55 U/ml penicillin, 55 µg/ml streptomycin, 33% horse serum (Gibco) heat-inactivated at 56°C for 30 minutes, and

11% pokeweed mitogen-conditioned medium from bone marrow mononuclear cells. The cells were plated in 96-well culture dishes (Costar) with 5×10^4 cells in 0.2 ml medium per well. The cultures were incubated at 37°C in humidified air with 5% carbon dioxide, and 10% O_2 in nitrogen-enriched air. Colonies containing 40 or more cells were counted on day 7.

Colony-forming units-fibroblast (CFU-F) were quantified using a modification of our previously described method (21). Briefly, 2.6×10^5 LDBMMC in 0.1 ml MEM-α were added to 0.9 ml medium containing MEM-α supplemented with 25% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cultures were performed in 12-well culture dishes (Costar) with 2.5×10^5 cells in 1 ml medium per well. The plates were incubated 8 days at 37°C in a humidified atmosphere containing 5% CO_2. The medium was depleted on day 8 and the wells were stained with 1% crystal violet. Colonies containing more than 500 spindle shaped cells were counted with an inverted microscope.

Preparation of feline α/β-interferon (α/β-IFN) and feline γ-interferon (γ-IFN). α/β-IFN was prepared using standard procedures (22). Briefly, normal peripheral blood mononuclear cells (PBMC) were separated from whole blood using a Ficoll-Hypaque gradient (lymphocyte separation medium, Litton Bionetics Inc., Charleston, SC) with a specific gravity of 1.077. The cells collected from the interface were washed with MEM-α and suspended at 10^7 cells/ml in RPMI 1640 (Gibco) containing 10% heat-inactivated fetal bovine serum, 2% glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml).

Newcastle disease virus (NDV) strain B_1, type B_1 (generously provided by Dr. Fred Melchior, Sterwin Laboratories, Millsboro, DE) was added to the cell suspension at a concentration of 1.5 x 10^8 50% egg-infectious dose units per 10^7 PBMC. The cells were incubated for 1 hour at 37°C, diluted to 5 x 10^6 cells/ml, and incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2 with gentle agitation. After incubation, the cells were removed by centrifugation and the supernatant containing α/β-IFN was treated with HCl to pH 2.0. After 24 hours at 4°C the pH was adjusted to 7.4 using NaOH and filtered (0.45μ). IFN produced by this method was acid- and heat-stable (56°C, 1 hour) and had antiviral activity after 30 minutes incubation with FEA cell monolayers in the IFN assay described below.

γ-IFN was prepared according to procedures developed for murine and human γ-IFN (23). Briefly, a spleen was collected aseptically from an SPF cat, minced, and passaged through stainless steel mesh to obtain a single cell suspension. The splenic cells were washed three times with MEM-α and suspended to 5 x 10^6/ml in RPMI 1640 (Gibco) containing 10% heat-inactivated fetal bovine serum, 2% glutamine, penicillin (100 μ/ml), streptomycin (100 μg/ml), and 10^{-5}M β-mercaptoethanol. Staphylococcal enterotoxin A (SEA) (Toxin Technology Inc., Madison, WI) was added to a final concentration of 0.25 μg/ml and the cell suspension incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2 with gentle stirring. After incubation, the cells were removed by centrifugation and the supernatant filtered (0.45μ). IFN produced by this method was both acid

labile (pH 2) and heat labile (56°C, 1 hour) and required 4 to 6 hours of incubation with FEA cell monolayers to induce measurable antiviral activity.

Interferon assay. IFN was measured by its ability to inhibit the cytopathic effects (CPE) of vesicular stomatitis virus (VSV) on FEA cells (a feline embryonic lung cell line generously provided by Dr. Oswald Jarrett, Glasgow, Scotland). Briefly, two-fold serial dilutions of serum samples, filtered (0.45μ) bone marrow plasma samples, or standards prepared from IFN concentrates were added in duplicate to confluent monolayers of FEA cells in 96-well flat bottomed microtiter plates (Costar). After 18 hours of incubation at 37°C, the wells were decanted, washed once with 0.2 ml of medium, and challenged with 20 times the 50% tissue culture infectious dose of VSV in 0.2 ml of medium. The plates were read at 18-24 hours when the control virus wells showed 100% CPE. The wells were decanted, fixed with absolute methanol, and stained with 1% crystal violet. The interferon titer was calculated as the reciprocal of the dilution that yielded 50% CPE and was expressed in laboratory units/ml (U/ml). All assays included control wells for virus, cells, IFN negative sera, and α/β-IFN and γ-IFN internal reference standards. No international reference standards are available for feline IFNs.

Interferon suppression of bone marrow progenitors in vitro. Studies were performed to evaluate the sensitivity of CFU-E, BFU-E, CFU-F, and CFU-GM to the anti-proliferative effects of feline α/β-IFN and γ-IFN. Four separate experiments were conducted in duplicate using LDBMMC from two different cats and using three different α/β-IFN preparations with specific activities of 25,000 U/ml, 16,000 U/ml

and 12,500 U/ml and γ-IFN preparations with specific activities of 4000 U/ml and 2000 U/ml. Control media preparations for both α/β-IFN (NDV control) and γ-IFN (SEA control) were included with each experiment. LDBMMC were suspended in MEM-α at twice the concentration normally used in the progenitor assays. The cells then were preincubated with an equal volume of an IFN preparation appropriately diluted with MEM-α for 1 hour at room temperature. The cell/IFN mixtures were added to the progenitor culture systems as described above. All values were normalized as the percentage of control colony growth vs. U IFN per ml of assay media. Data points were calculated as the mean and standard error of the mean for 4 experiments.

Statistical analysis. The Student's t-test was used to determine significance when a single treatment was compared to control. One-way analysis of variance and the Newman-Keul's follow-up test were used to identify significant differences when treatments were compared to two controls or when parameters were compared at multiple time points.

RESULTS

Hematologic findings. All cats were viremic by 2 weeks post-inoculation (PI) and remained viremic throughout the course of the experiment as determined by indirect immunofluorescence for p27 of FeLV in circulating leukocytes. Packed cell volumes remained unchanged from 31 ± 1.4% ($\bar{X}$ ± SEM) pre-inoculation to 31 ± 8% at 2 weeks PI (Fig. 3.1.). However, significant anemia became evident by 4 weeks PI with a mean PCV of 26 ± 1.7% (SEM) ($p<.05$). Anemia became severe by 8 weeks PI with a mean PCV of 11 ± 1.4% (SEM) ($p<.01$). A regenerative response was absent in the blood of all cats with reticulocytes only noted rarely post-inoculation (data not shown).

Effect of FeLV-KT on hematopoietic progenitors. CFU-E numbers declined 83% ($p<.01$) from 113 ± 4 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) before inoculation to 19 ± 7 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) at 4 weeks PI (Fig. 3.2). Likewise, BFU-E declined 91% ($p<.01$) from 22 ± 2 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) prior to inoculation to 2 ± 1 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) at 4 weeks PI (Fig. 3.2). Both CFU-E and BFU-E continued to decrease until they were undetectable in cultures of LDBMMC from cats at 10 weeks PI.

The number of CFU-GM were increased 70% to 148 ± 10.3 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) by 4 weeks PI from 87 ± 6 per 5 x 10^4 LDBMMC ($\bar{X}$ ± SEM) before inoculation (Fig. 3.2). Although CFU-GM remained significantly increased ($p<.01$) at 8 weeks PI (135 ± 4, $\bar{X}$ ± SEM), they progressively declined to normal levels by 10 weeks PI.

Interferon levels in cats with EA. Assays for IFN from the six cats studied prospectively were conducted on serum before inoculation and biweekly from 4-10 weeks PI, and on bone marrow plasma pre-inoculation and at 4, 8, and 10 weeks PI. Additionally, serum samples from 16 other cats with EA were evaluated at 0, 4, 5, 6, 8, 10, and 12 weeks PI. Antiviral activity was not detected in any of the sera (<4 U/ml).

Effects of α/β-IFN and γ-IFN on the growth of hematopoietic progenitors. The effect of α/β-IFN on CFU-E and BFU-E was similar. Both CFU-E and BFU-E were suppressed to 50% of controls at 100 U/ml while exposure to 600 U/ml reduced CFU-E to 6.9 ± 3.5% ($\bar{X}$ ± SEM) and BFU-E to 7.4 ± 4.3% ($\bar{X}$ ± SEM) of control growth ($p<.005$) (Fig. 3.3, 3.4). Complete inhibition of proliferation of CFU-E and BFU-E occurred in the presence of 1200 U/ml α/β-IFN. γ-IFN inhibited BFU-E 15-20% more than comparable concentrations of α/β-IFN. A similar effect was evident with CFU-E at 200 U/ml and 400 U/ml (Figs. 3.3, 3.4) Additionally, BFU-E were more sensitive to inhibition by γ-IFN than CFU-E by approximately 10-15%. One hundred U/ml γ-IFN reduced BFU-E to 40 ± 6.9% ($\bar{X}$ ± SEM) ($p<.005$) of control growth while at this level CFU-E were reduced to 52.1 ± 3.2% ($\bar{X}$ ± SEM) ($p<.005$). Complete inhibition of proliferation of both erythroid progenitors was evident at a γ-IFN concentration of 400 U/ml. NDV or SEA media control preparations added to the CFU-E/BFU-E assay at full concentration did not inhibit progenitors.

Evaluation of the effects of α/β-IFN and γ-IFN on CFU-F growth revealed that these progenitors are extremely sensitive to γ-IFN

but have a suppression pattern similar to erythroid progenitors upon exposure to α/β-IFN. At 25 U/ml, γ-IFN caused suppression of CFU-F to 56.9 ± 5.6% ($\bar{X}$ ± SEM) of control growth ($p<.005$) (Fig. 3.5). At 100 U/ml, γ-IFN inhibited CFU-F to only 1.1 ± 1.1% ($\bar{X}$ ± SEM) of control growth ($p<.005$) and caused complete inhibition at 200 U/ml or above. In contrast, suppression of CFU-F by α/β-IFN was first detected at 100 U/ml, causing suppression to 52.2 1.6% ($\bar{X}$ ± SEM) of control growth ($p<.005$). Inhibition reached 6.2 ± 4% ($\bar{X}$ ± SEM) at 600 U/ml and was complete at 1200 U/ml. As in the erythroid progenitor assays, neither NDV nor SEA media control preparations at full concentration had any effect on CFU-F numbers as compared to untreated controls.

Suppression of CFU-GM growth by either α/β-IFN or γ-IFN required greater concentrations to cause inhibition. Addition of 100 U/ml of γ-IFN caused significant suppression ($p<.01$) of CFU-GM to 64.2 ± 2.4% ($\bar{X}$ ± SEM) of control growth, while 1000 U/ml of α/β-IFN or γ-IFN suppressed CFU-GM to 48.5 ± 4.9% ($\bar{X}$ ± SEM) and 40.4 ± 3.4% ($\bar{X}$ ± SEM) of control growth, respectively ($p<.01$). Inhibition to 23.4 ± 2.7% ($\bar{X}$ ± SEM) of control was achieved at 5000 U/ml α/β-IFN ($p<.01$). Compounding the interpretation of the suppression of these progenitors was the significant enhancement of CFU-GM growth by SEA control media at a concentration of 5% final volume (125.5 ± 7%, $\bar{X}$ ± SEM) and at 50% final volume (165.5 ± 24.9%, $\bar{X}$ ± SEM) and by NDV control media at 50% final volume (114.6 ± 2.4%, $\bar{X}$ ± SEM).

DISCUSSION

In these studies, interferon levels were measured in the serum and bone marrow plasma of cats with retrovirus-induced erythroid aplasia, while the anti-proliferative effects of feline α/β-IFN and γ-IFN were assessed in vitro using normal LDBMMC. The results showed that, in agreement with results from other species, feline hematopoietic progenitor growth was inhibited by IFN. Additionally, feline CFU-F were found to be particularly sensitive to γ-IFN. IFN was undetectable in the blood or bone marrow plasma of cats with EA, demonstrating that this syndrome is not likely to be mediated by IFN and suggesting that it probably is not a result of T-cell suppression of erythroid progenitors.

The investigation of IFN levels in retrovirus-induced EA was undertaken for several reasons. FeLV infection produces many of the immunologic abnormalities associated with AIDS in which there are increased levels of acid-labile α-IFN (13). The association of circulating γ-IFN with T-suppressor/cytotoxic cells in a significant number of patients with AA has prompted speculation that at least some of these cases may be due to an undiscovered viral etiologic agent (10). Also, there have been reports of T-cell-mediated AA secondary to Epstein-Barr virus infections and viral hepatitis. In some cases these have been associated with IFN production (25-27). Feline EA provides a reproducible model of hematopoietic suppression with an established retroviral etiology. In these studies, FeLV-KT infection of cats produced severe nonregenerative anemia by four

weeks PI at which time PCV, reticulocyte numbers, and marrow CFU-E and BFU-E were decreased significantly. Sequential examination of sera from 21 cats throughout the course of anemia, however, failed to reveal the presence of circulating IFN. The lack of circulating γ-IFN may not be surprising as FeLV has been reported to suppress γ-IFN production by normal feline PBMC (28). More importantly, however, was the absence of IFN in bone marrow plasma measured at 4, 8, and 10 weeks PI. FeLV viremia is associated with infection of bone marrow cells and first occurs 7-21 days PI (29). Although IFN response to viral infections can be transient even at primary sites of viral replication, measurable IFN levels would be expected if IFN plays a direct role in the pathogenesis of EA. In patients with AA IFN levels in bone marrow sera were several-fold higher than circulating IFN (7). IFN has also been detected in normal human bone marrow (7) but could not be measured in the bone marrow plasma of normal cats (data not shown).

In order to substantiate the ability of our IFN assay to measure IFN at levels capable of causing hematopoietic suppression, in vitro studies were performed to characterize the dose-response relationship of feline α/β-IFN and γ-IFN with various progenitors in short-term clonogenic assays. The results demonstrate that CFU-E, BFU-E, CFU-GM, and CFU-F all are sensitive to the anti-proliferative effects of both species of IFN. In general, the erythroid progenitors were inhibited as reported in previous studies (2,4-9). Fifty percent inhibition by either type of IFN occurred at approximately 100 U/ml. However, γ-IFN caused complete suppression of both CFU-E and BFU-E at

400 U/ml while comparable suppression with α/β-IFN required 1200 U/ml. CFU-GM were less sensitive than erythroid progenitors to growth inhibition by either type of IFN requiring 1000 U/ml to achieve greater than 50% suppression. However, high concentrations of the SEA or NDV control preparations were able to stimulate CFU-GM and might increase the amount of IFN necessary for suppression. Colony stimulating factor as well as γ-IFN is induced by SEA and has been reported to overcome the anti-proliferative effects of γ-IFN (31). The effects of α/β-IFN versus γ-IFN on CFU-F formation were dramatically different. While α/β-IFN reduced CFU-F numbers to 50% control growth at 100 U/ml and 100% at 1200 U/ml, γ-IFN achieved this level of inhibition with only 25 U/ml and caused complete suppression by 100 U/ml. Previous studies have not addressed the effects of IFN on CFU-F per se although crude human γ-IFN was approximately 20-fold more potent per unit of antiviral activity than human α-IFN when tested for anti-proliferative effects on normal human fibroblasts (32).

The lack of circulating or bone marrow IFN in cats with EA provides significant implications for the pathogenesis of this syndrome. First, the absence of IFN, at least in the bone marrow, may contribute to the inability of some cats to recover from FeLV infection. Secondly, IFN does not appear to mediate EA, an aplastic syndrome with an established retroviral etiology. Finally, although the correlation has not always been substantiated, the lack of IFN suggests that suppressor T-cells do not mediate EA, a conclusion

supported by recent lymphocyte co-culture studies and by the failure of EA to respond to ATG treatment (33,34).

SUMMARY

The role of interferon (IFN) in the pathogenesis of retrovirus-induced erythroid aplasia (EA) was investigated in cats inoculated with the Kawakami-Theilen strain of feline leukemia virus (FeLV-KT). Serum samples were collected weekly or biweekly in 21 cats starting at 4 weeks post-inoculation (PI) when packed cell volumes, reticulocyte counts, and erythroid progenitor numbers (CFU-E, BFU-E) were initially decreased. Additionally, in 6 cats, bone marrow plasma levels were measured at 4, 8, and 10 weeks PI. Our results demonstrated that, in cats with EA, IFN was not present in the peripheral circulation or in the bone marrow which is the origin of cell-associated viremia in FeLV infection. Further in vitro studies were performed to measure the response of feline hematopoietic progenitors to feline α/β-IFN and γ-IFN based upon antiviral activity. α/β-IFN at 100 U/ml caused 50% inhibition and at 1200 U/ml caused 100% inhibition of growth of CFU-E, BFU-E and fibroblast-colony forming units (CFU-F). γ-IFN at 100 U/ml caused 50% suppression of CFU-E and BFU-E, 40% suppression of granulocyte/macrophage colony-forming units (CFU-GM) and complete inhibition of CFU-F. CFU-GM could not be suppressed completely with either 1000 U/ml -IFN or 5000 U/ml / -IFN.

These data indicate that 1) feline hematopoietic progenitors are sensitive to growth inhibition by α/β-IFN or γ-IFN similar to other species, 2) IFN does not appear to be part of the pathogenesis of EA, and 3) EA probably is not mediated by T-suppressor cells.

Fig. 3.1 Changes in PCV (mean ± SEM) vs. time after inoculation with FeLV-KT (n=6). All cats became positive by 2 weeks PI and remained viremic through the course of the experiment.

* $p<.05$

+ $p<.01$

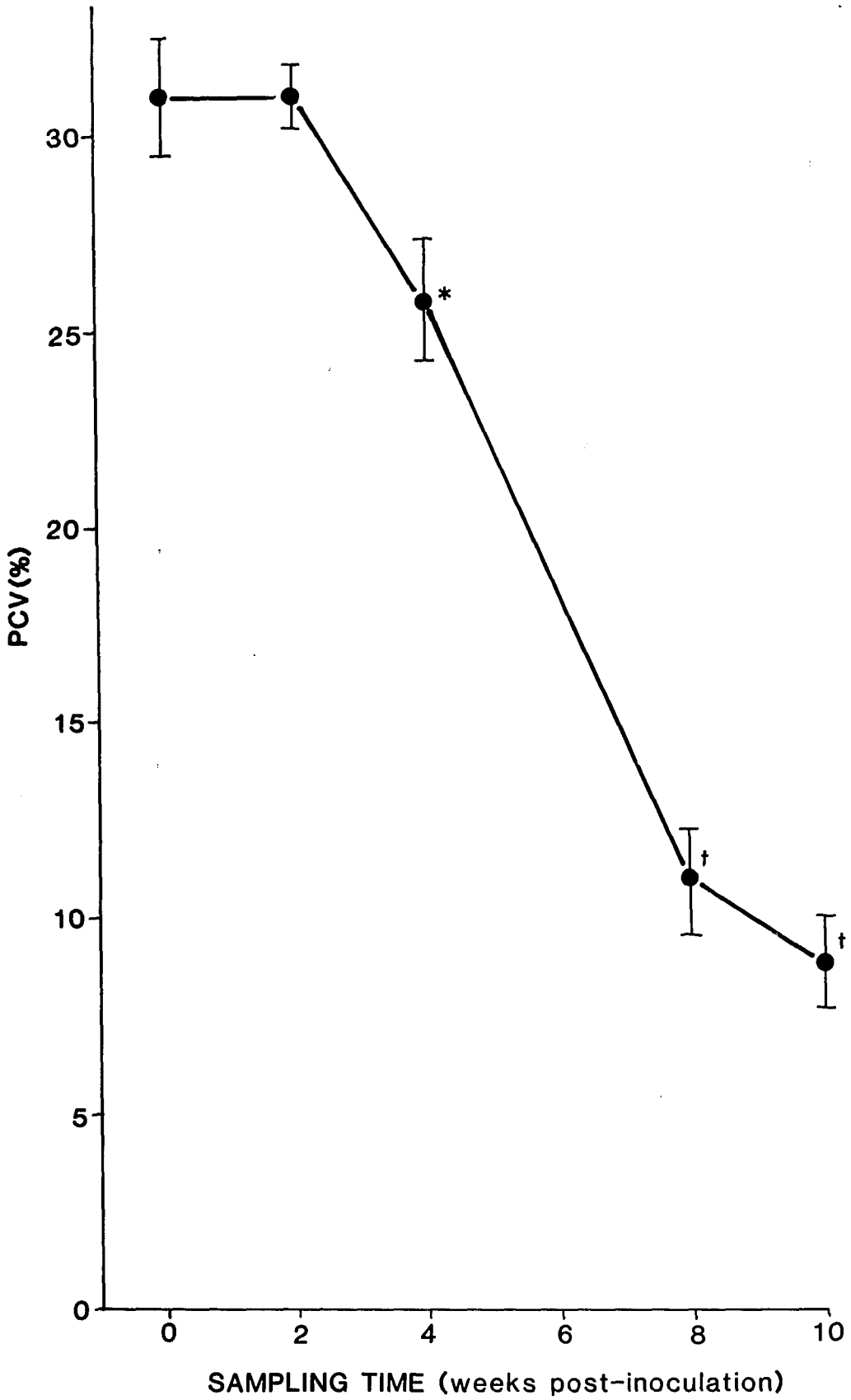

30
25
20
15
10
5
0
PCV(%)
*
t
t
0
2
4
6
8
10
SAMPLING TIME (weeks post-inoculation)

Fig. 3.2 Changes in numbers of CFU-E, BFU-E, and CFU-Gm ($\bar{X} \pm SEM$) after inoculation with FeLV-KT (n=6).

\+ $p<.01$

++ $p<.005$

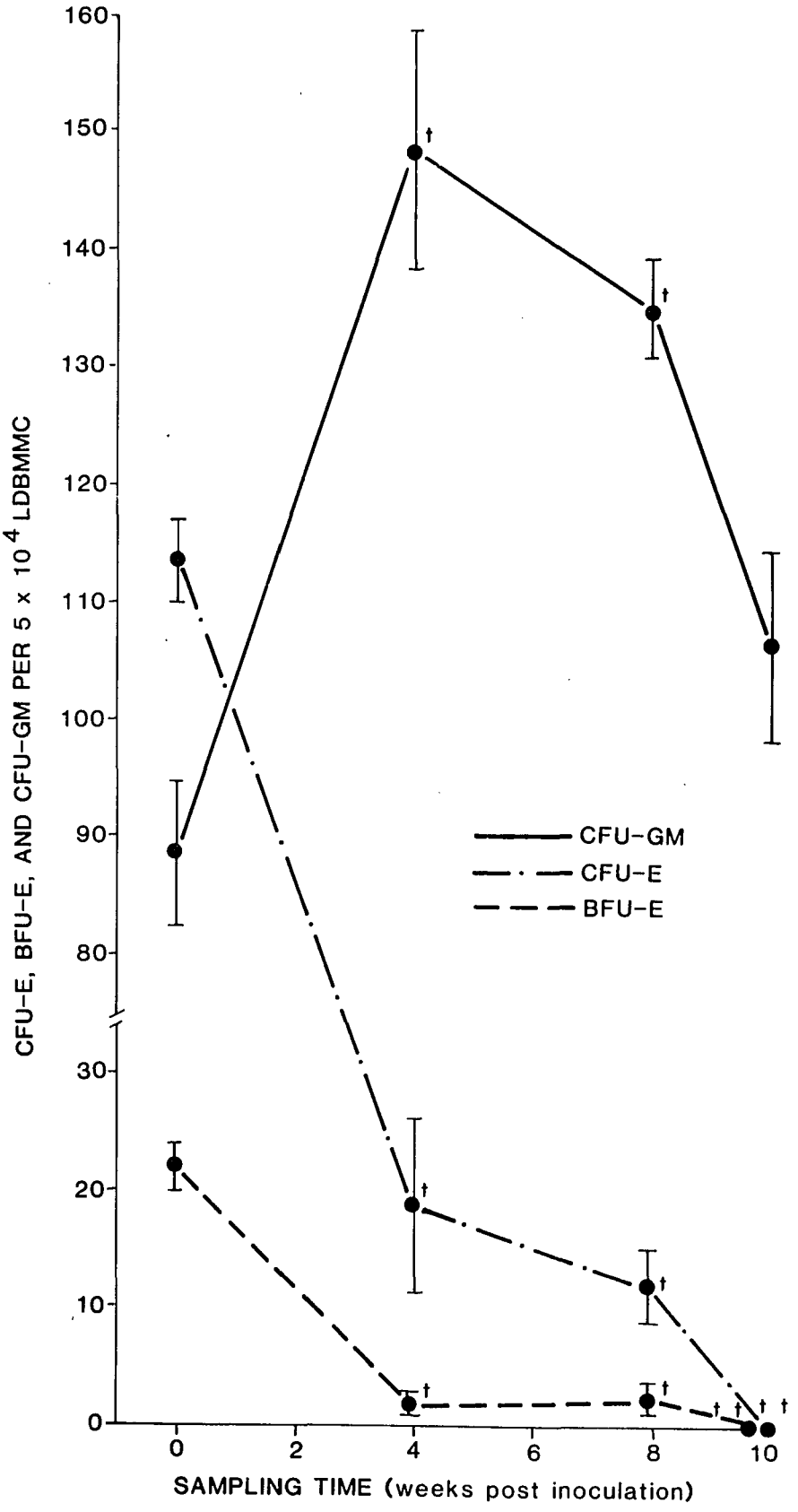

160
150
140
130
120
110
100
90
80
30
20
10
0
0
2
4
6
8
10
CFU-E, BFU-E, AND CFU-GM PER 5 x 10 4 LDBMMC
SAMPLING TIME (weeks post inoculation)
CFU-GM
CFU-E
BFU-E

Fig. 3.3 Effect of various concentrations (U/ml) of α/β-IFN or γ-IFN incorporated within the culture system on CFU-E ($\bar{X}$ ± SEM, % of control growth). Control colony numbers were 114 ± 8 ($\bar{X}$ ± SEM) for 4 experiments. NDV and SEA control media had no significant effect on growth.

** $p<.025$

++ $p<.005$

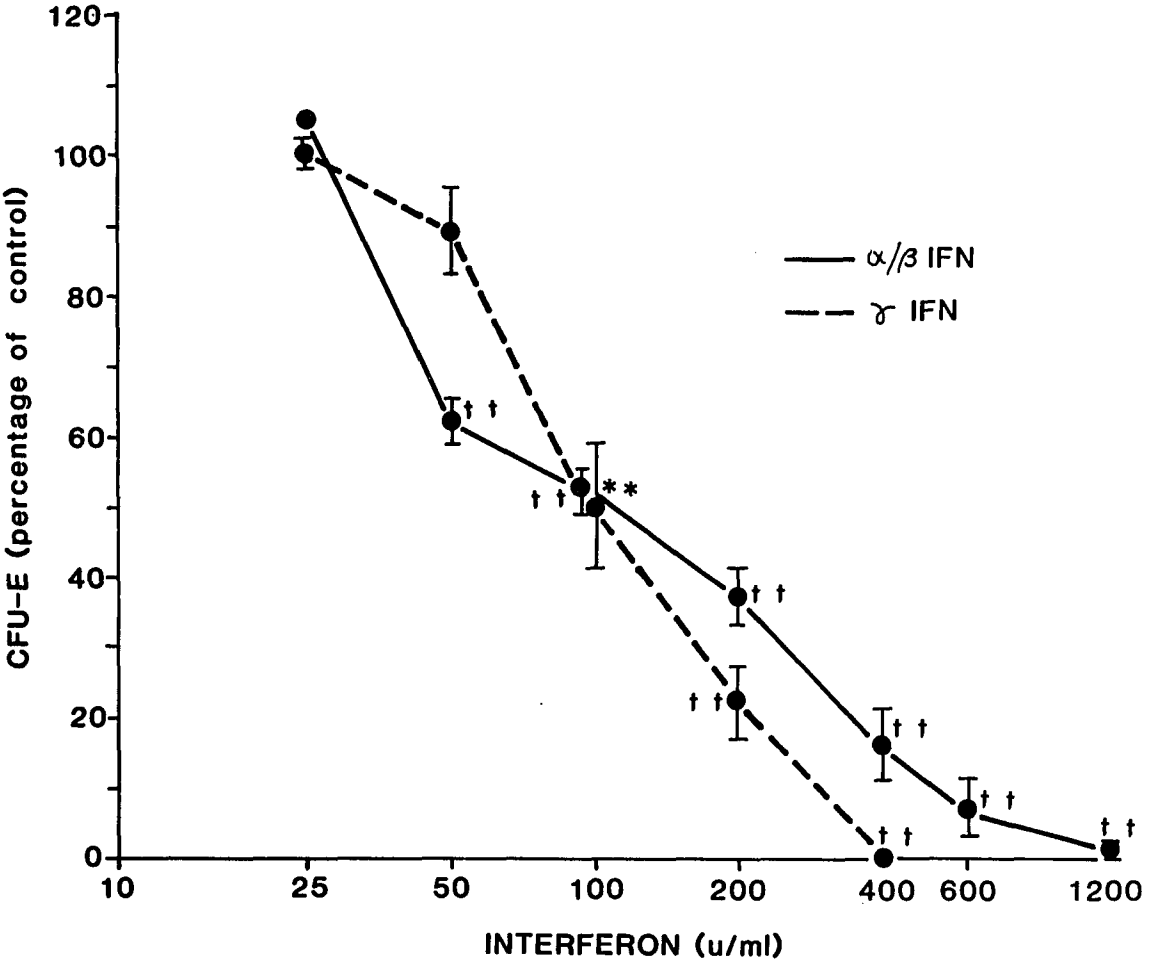

120
100
80
60
40
20
0
CFU-E (percentage of control)
10
25
50
100
200
400
600
1200
INTERFERON (u/ml)
α/β IFN
γ IFN
**

Fig. 3.4 Effect of various concentrations (U/ml) of α/β-IFN or γ-IFN incorporated within the culture system on BFU-E ($\bar{X}$ ± SEM, % control growth). Control colony numbers were 32 ± 5 ($\bar{X}$ ± SEM) for 4 experiments. NDV and SEA control media had no significant effect on growth.

** $p<.025$

++ $p<.005$

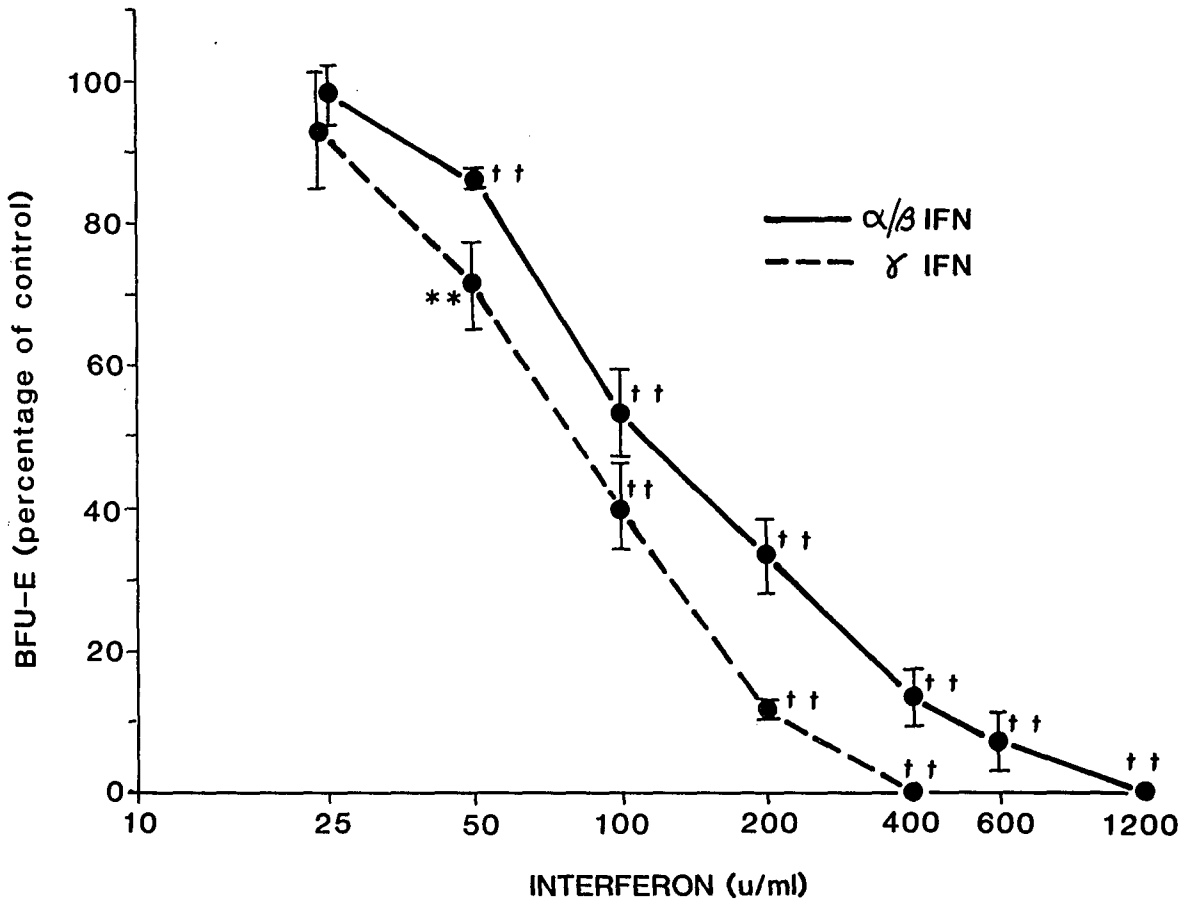

100
80
60
40
20
0
BFU-E (percentage of control)
10
25
50
100
200
400
600
1200
INTERFERON (u/ml)
α/β IFN
γ IFN
**

Fig. 3.5 Effect of various concentrations (U/ml) of α/β-IFN or γ-IFN incorporated within the culture system on CFU-F ($\bar{X}$ ± SEM, % control growth). Control colony numbers were 21 ± 2 ($\bar{X}$ ± SEM) for 4 experiments. NDV and SEA control media had no significant effect on growth.

++ $p<.005$

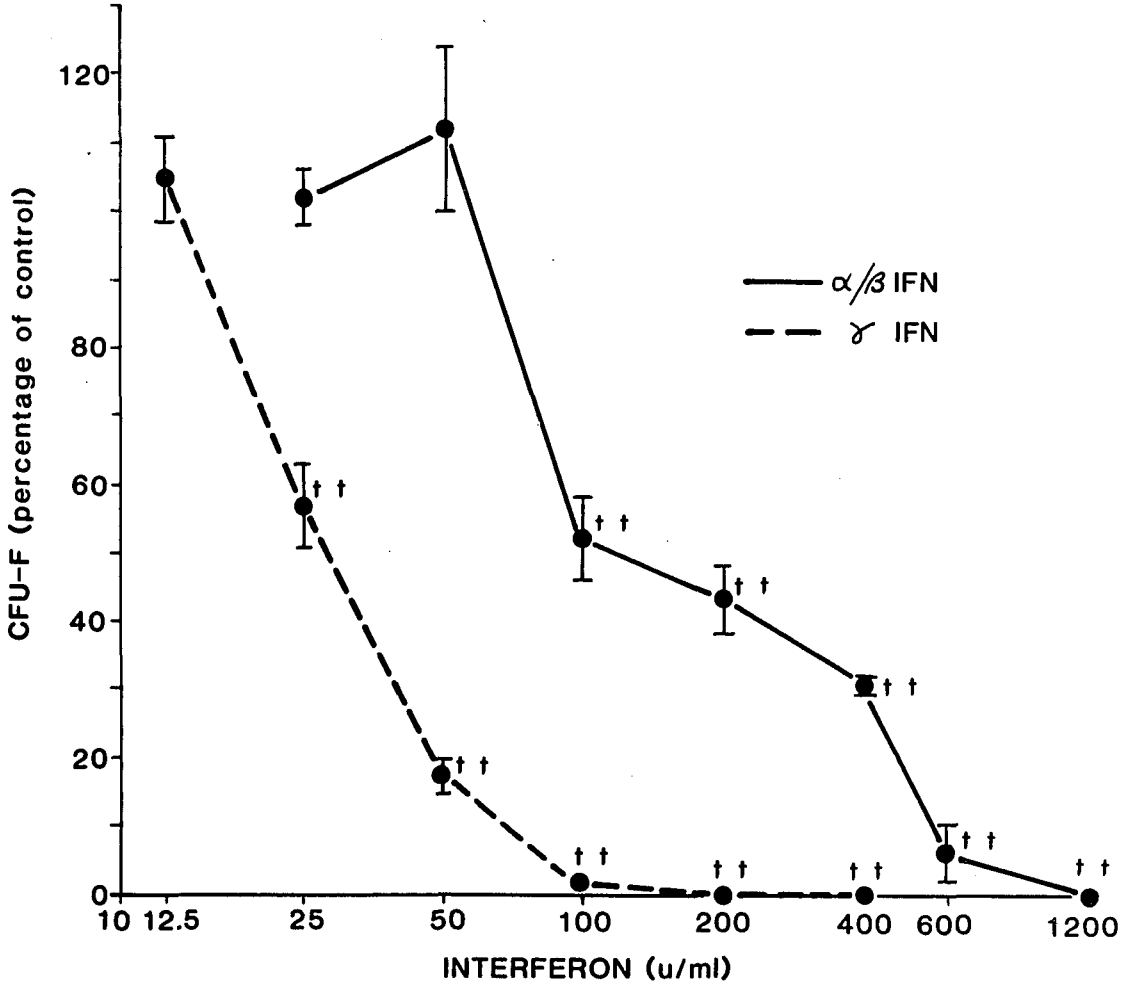
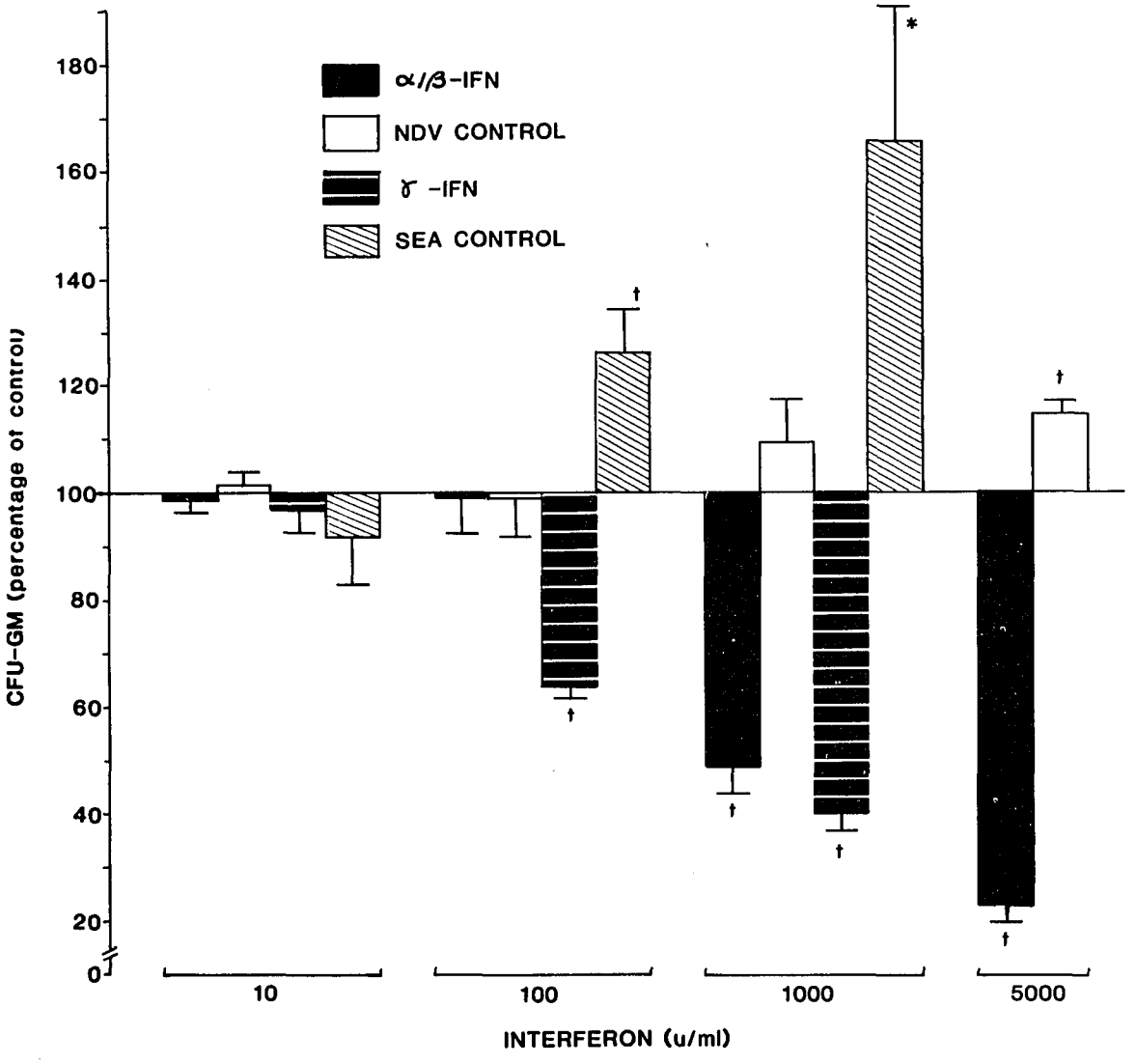

120
80
60
40
20
0
CFU-F (percentage of control)
α/β IFN
γ IFN
10 12.5 25 50 100 200 400 600 1200
INTERFERON (u/ml)

Fig. 3.6 Effect on CFU-GM ($\bar{X}$ ± SEM, % control growth) of various concentrations (U/ml) of α/β-IFN or γ-IFN compared to NDV-treated and SEA-treated control media incorporated within the culture system. Control colony numbers were 55 ± 4 ($\bar{X}$ ± SEM) for 4 experiments.

* $p<.05$

+ $p<.01$


α/β-IFN
NDV CONTROL
γ -IFN
SEA CONTROL
CFU-GM (percentage of control)
180
160
140
120
100
80
60
40
20
0
10
100
1000
5000
INTERFERON (u/ml)
*
†

REFERENCES

1. Preble OT, Friedman RM (1983) Biology of Disease: Interferon-induced alterations in cells: Relevance to viral and nonviral diseases. Lab Invest 49(1):4-18.

2. Broxmeyer HE, Lu L, Puatzer E, Feit C, Juliano L, Rubin BY (1983) Comparative analysis of the influences of human gamma, alpha, and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. J Immunol 131:1300-1305.

3. Neumann HA, Fauser AA (1982) Effect of interferon on pluripotent hemopoietic progenitors (CFU-GEMM) derived from human bone marrow. Exp Hematol 10:587-590.

4. Ortega JA, Ma A, Shore NA, Dukes PP, Merigan TC (1979) Suppressive effect of interferon on erythroid cell proliferation. Exp Hematol 7:145-150.

5. Mamus SW, Beck-Schroeder S, Zanjani ED (1985) Suppression of normal human erythropoiesis by gamma interferon in vitro. J Clin Invest 75:1496-1503.

6. Zoumbos NC, Djell JY, Young NS (1984) Interferon is the suppressor of hematopoiesis generated by stimulated lymphocytes in vitro. J Immunol 33:769-774.

7. Zoumbos NC, Gascon P, Djell JY, Young NS (1985) Interferon is a mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo. Proc Natl Acad Sci 82:188-192.

8. Mazur EM, Richtsmeier WJ, South K (1986) Alpha-interferon: Differential suppression of colony growth from human erythroid, myeloid, and megakaryocytic hematopoietic progenitor cells. J Interferon Res 6:199-206.

9. Mamus SW, Oken MM, Zanjani ED (1986) Suppression of normal human erythropoiesis by human recombinant DNA-produced alpha-*a*-interferon *in vitro*. Exp Hematol 14:1015-22.

10. Zoumbos NC, Gascon P, Djell JY, Trost SR, Young NS (1985) Circulating activated suppressor T lymphocytes in aplastic anemia. N Engl J Med 312:257-265.

11. Mangan KF, Zidar B, Shadduck RK, Zeigler Z, Winkelstein A (1985) Interferon-induced aplasia: Evidence for T-cell-mediated suppression of hematopoiesis and recovery after treatment with horse antihuman thymocyte globulin. Am J Hematol 19:401-413.

12. Cunningham AL, Merigan TC (1984) Leu-3+ T cells product γ-interferon in patients with recurrent Herpes labialis. J Immunol 132:197-202.

13. Preble OT, Rook AH, Quinnan GU *et al* (1984) Role of interferon in AIDS. Ann NY Acad Sci 437:65-75.

14. Tosato G, Magrath I, Koski I, Dooley N, Blaese M (1979) Activation of suppressor T cells during Epstein-Barr virus-induced infectious mononucleosis. N Engl J Med 301:1133-1137.

15. Rojko JL, Olsen RG (1984) The immunobiology of the feline leukemia virus. Vet Immunol Immunopathol 6:107-165.

16. Onions D, Sharrett O, Testa N, Frassoni F, Toth S (1982) Selective effect of feline leukaemia virus on early erythroid precursors. Nature 296:156-158.

17. Gasper PW, Hoover EA (1983) Feline leukemia virus strain specific abatement of erythroid burst-forming units (BFU-E). Leuk Rev Internat 1:71-72.

18. Testa NG, Onions D, Jarrett O, et al (1983) Haemopoietic colony formation (BFU-E, GM-CFC) during the development of pure red cell hypoplasia induced in the cat by feline leukaemia virus. Leuk Res 7:103-116.

19. Boyce JT, Hoover EA, Kociba GJ, Olsen RG (1981) Feline leukemia virus-induced erythroid aplasia: In vitro hemopoietic culture studies. Exp Hematol 9:990-1001.

20. Abkowitz JL, Oft RL Nakamura JM et al (1985) Feline glucose-6-phosphate dehydrogenase cellular mosaicism. Application to the study of pure red cell aplasia. J Clin Invest 75:133-140.

21. Wellman ML, Kociba GJ (1987) Characterization of normal feline bone marrow fibroblast colony forming units. Am J Vet Res, In press.

22. Carter WA, Johnson FH (1981) Induction and production of interferon with porcine, bovine, and equine leukocytes. Meth Enz 78:48-54.

23. Johnson HM, Dianzani F, Georgiades JA (1981) Large-scale induction and production of human and mouse immune interferons. Meth Enz 78:158-162.

24. Hoover EA, Mathes LE, Rojko JL et al (1978) Modification of the immunofluorescence assay for feline leukemia virus group-specific antigen. Am J Vet Res 39:1877-1880.

25. Socinski MA, Ershler WB, Tosato G (1984) Pure red blood cell aplasia associated with chronic Epstein-Barr virus infection: Evidence for T cell-mediated suppression of erythroid colony-forming units. J Lab Clin Med 104:995-1006.

26. Young N, Mortimer P (1984) Viruses and bone marrow failure. Blood 63:729-737.

27. Herrmann F, Griffin JD, Meuer SG, et al (1986) Establishment of an interleukin 2-dependent T cell line derived from a patient with severe aplastic anemia, which inhibits in vitro hematopoiesis. J Immunol 136:1629-1634.

28. Engelman RW, Fulton RW, Good RA, Day NK (1985) Suppression of gamma interferon production by inactivated feline leukemia virus. Science 227:1368-1370.

29. Olsen RG, Mathes LE, Tarr MJ, Blakeslee JR (1986) Oncogenic viruses of domestic animals. Vet Clin N Am:Sm Anim Pr 16:1129-1143.

30. Hosoi T, Ozawa K, Urabe A, Takaku F (1985) Effects of recombinant interferons on the clonogenic growth of leukemic cells and normal hemopoietic progenitors. Int J Cell Cloning 3:304-312.

31. Klimpel GR, Fleischmann WR, Klimpel KO (1982) Gamma interferon (IFN-γ) and IFN α/β suppress murine myeloid colony formation (CFU-C): Magnitude of suppression is dependent upon level of colony-stimulating factor (CSF). J Immunol 129:76-129.

32. Rubin BY, Gupta SL (1980) Differential efficacies of human Type I and Type II interferons as antiviral and antiproliferative agents. Proc Natl Acad Sci USA 77:5928-5931.

33. Abkowitz JL, Ott RL, Holly RD, Adamson JW (1987) Lymphocytes and antibody in retrovirus-induced feline pure red cell aplasia. J Natl Cancer Inst 78:135-130.

34. Zack, PM, Kociba GJ (1987) Antithymocyte globulin treatment of retrovirus-induced feline erythroid aplasia: In vivo and in vitro studies. Submitted for publication.

CHAPTER IV

THE INTERFERON RESPONSE IN CATS INFECTED WITH FELINE LEUKEMIA VIRUS: HIGH PREVIREMIC LEVELS OF GAMMA INTERFERON SIGNAL REGRESSIVE INFECTION

INTRODUCTION

Feline leukemia virus (FeLV) is one of the most important viral pathogens of the domestic cat. It is an exogenous type C retrovirus capable of inducing several cytoproliferative and cytosuppressive syndromes of the hemic-lymphatic system (1). Depending upon age, immune status, and/or dosage of virus, cats may develop progressive infection characterized by persistent viremia or regressive infection characterized by transient viremia and latency. Although FeLV can cause leukemia, lymphoma, and myeloproliferative disease, FeLV infection more frequently results in severe immunosuppression with associated thymic atrophy (3), paracortical lymphoid depletion (4), failure to reject skin allografts (6), depression of peripheral blood lymphocyte blastogenic response to mitogens (2), and reduction in lymphocyte-membrane concanavalin A-receptor mobility (capping) (7). Studies of the pathogenesis of FeLV replication have revealed that bone marrow cells are infected between 7-21 days post-inoculation (PI) followed by viremia of marrow origin between 14-28 days PI (8).

The role of interferon (IFN) in the response of cats to FeLV infection has received limited attention. Previous reports have revealed that FeLV suppresses the induction of γ-IFN from peripheral blood lymphocytes *in vitro* (9); that exogenous administration or endogenous stimulation of IFN may result in partial to complete remission of viremia (10,20); and that FeLV replication *in vitro* can be inhibited by human leukocyte IFN (11). Other retroviruses are capable of inducing IFN *in vivo* (12). Production of IFN in mice after infection with various murine retroviruses has been reported (13-16). Furthermore, treatment of MuLV-infected mice with antibody against IFN often has resulted in reduction of the latent period, higher virus titers, earlier appearance of tumors and larger tumor size (16-18). Additionally, in man, the acquired immunodeficiency syndrome (AIDS) includes the production of an acid-labile γ-IFN (19).

The objective of these studies was to evaluate the interferon response to FeLV *in vivo* both in the circulation and locally in the bone marrow, particularly focusing on activity occurring prior to viremia. Additionally, the production of α/β-IFN and γ-IFN from bone marrow cells and γ-IFN from peripheral blood lymphocytes was evaluated at sequential time intervals and compared to the onset of immunosuppression. The results indicate that production of circulating γ-like-IFN is a consistent feature of early infection with FeLV and shows that there is a significant difference in the level of production between persistently viremic and transiently viremic cats.

MATERIALS AND METHODS

Animals. All cats were obtained either from the specific pathogen-free (SPF) breeding colony maintained by the Department of Veterinary Pathobiology, The Ohio State University, or from Liberty Labs (Liberty Corners, NJ). All cats were uniformly free of infection and immunity to FeLV. The cats were tranquilized with intramuscular ketamine hydrochloride prior to bone marrow or blood collection. Except where indicated, infected cats were pre-treated with methyl prednisolone (5 mg/kg) to enhance their susceptibility to productive FeLV infection.

Viral inoculum. The FeLV inoculum was prepared from equal volumes of serum and bone marrow cell lysate pooled from cats with persistent viremia induced by *in vivo* passage of FeLV-KT as described (5). The FeLV infectivity titer of the inoculum was assessed by the S+/L- focus induction assay as described previously (5). Eight cats were infected by intravenous injection of 1.0 ml inoculum containing 2×10^5 FFU FeLV-KT, and 32 cats by intraperitoneal injection of 0.5 ml inoculum containing 1×10^5 FFU FeLV-KT. FeLV antigen concentration of the inoculum was determined for the FeLV structural core protein, p27, by enzyme-linked immunosorbent assay (ELISA).

Determination of interferon (IFN) and FeLV p27 levels in the sera and bone marrow plasma of persistently viremic and transiently viremic cats. Thirty-two 18-week-old cats were pre-treated with methyl prednisolone and inoculated with 1×10^5 FFU FeLV-KT intraperitoneally. Blood was collected before inoculation and weekly

post-inoculation (PI), placed immediately on ice, allowed to clot at 4°C, and centrifuged at 400 x g for 10 minutes at 4°C in order to obtain serum. All serum samples were stored at -70°C until assayed for IFN or p27. Blood films were made weekly PI to evaluate circulating leukocytes for the presence of FeLV using an indirect immunofluorescence test (IFA) for FeLV p27 as modified by Hoover et al. (26). Cats were designated as transiently viremic if they ceased to be positive for circulating p27 via ELISA and intracellular p27 via IFA.

In a second experiment, six cats were pre-treated with methyl prednisolone, five of which were inoculated with 2×10^5 FFU FeLV-KT intravenously. Serum was separated at 4°C from blood samples collected prior to inoculation and at 1-12, 14, 17, 21, 28, and 35 days PI for IFN and p27 assays. Blood was collected prior to inoculation and weekly PI to evaluate circulating leukocytes for the presence of FeLV using the IFA procedure and to perform complete hemograms (Coulter S plus IV, Coulter Electronics Inc., Hialeah, FL). Bone marrow samples were aspirated into syringes previously flushed with heparin (500 U/ml), and blood samples were collected in heparin (20 U/ml) at 3, 7, 10, 14, 21, 28, and 35 days PI. Samples were maintained at 4°C. Bone marrow plasma was obtained after centrifugation of the samples at 400 x g for 15 minutes at 4°C and was assayed for IFN and p27 as described below. Bone marrow cells then were resuspended in MEM-α medium (GIBCO, Grand Island, NY). Bone marrow mononuclear cells (BMMC) and peripheral blood mononuclear cells (PBMC) were separated as follows. Cell suspensions were diluted to

20 ml with 10 ml MEM-α and 10 ml Hank's Balanced Salt Solution (GIBCO) in 50 ml conical centrifuge tubes and underlayered with 10 ml of Ficoll-Hypaque (lymphocyte separation medium, Litton Bionetics Inc., Charleston, SC) with a specific gravity of 1.077. BMMC and PBMC were collected from the interface after centrifugation for 30 minutes at 400 x g, washed once with Hank's Balanced Salt Solution and resuspended in MEM-α. BMMC were used in α/β-IFN and γ-IFN induction assays and in suspensions to assay constitutive IFN production (described below). PBMC were used in γ-IFN induction assays and in lymphoblast transformation assays.

Two cats were inoculated with 2 x 10^5 FFU FeLV-KT intravenously without pre-treatment with methyl prednisolone to induce transient viremia. Blood, serum and bone marrow plasma samples were collected at 4°C on 3, 7, 14, 21, 28, and 35 days PI to perform IFN and p27 assays. IFA tests for intracellular p27 in circulating leukocytes were performed weekly.

Interferon assay. IFN was measured by its ability to inhibit the cytopathic effects (CPE) of vesicular stomatitis virus (VSV) on FEA cells (a feline embryonic lung cell line generously provided by Dr. Oswald Jarrett, Glasgow, Scotland). Briefly, two-fold serial dilutions of serum samples, filtered (0.45μ) bone marrow plasma samples, or induced IFN standards were added in duplicate to confluent monolayers of FEA cells in 96-well flat-bottomed culture plates (Costar). After 18 hours of incubation at 37°C, the wells were decanted, washed once with 0.2 ml of media, and challenged with 20 times the 50% tissue culture infectious dose of VSV in 0.2 ml of

medium. The plates were read at 18-24 hours when the virus control wells showed 100% CPE. The wells were decanted, fixed with absolute methanol, and stained with 1% crystal violet. The interferon titer was calculated as the reciprocal of the dilution that yielded 50% CPE and was expressed in laboratory units/ml (U/ml). All assays included control wells for virus, cells, IFN negative sera, and α/β-IFN and γ-IFN internal reference standards. No international reference standards are available for feline IFNs. Positive samples were further characterized as α/β-IFN or γ-IFN by separately retesting samples after treatment with heat (56°C, 1 hour) and acid (pH 2 at 4°C for 24 hours) (22). Antiviral activity sensitive to both heat and acid was classified as γ-IFN.

FeLV p27 assay. p27 was measured using a modification of the FeLV enzyme-linked immunosorbent assay (ELISA) test (TechAmerica Diagnostics, Elwood, KS). Briefly, 50 µl of each sample was incubated in wells precoated with monoclonal antibody to FeLV p27. Monoclonal antibody to FeLV p27 and conjugated to horseradish peroxidase was added. After 10 minutes incubation the wells were decanted and washed five times with distilled water. Chromogenic substrate was added and incubated in the wells for 10 minutes. The well contents were then transferred to a 96-well plate and absorbance read at 405 using an eight channel photometer (Titertek Multiscan, Flow Laboratories, McLean, VA). Plates included blanks consisting of phosphate-buffered saline with 1% bovine serum albumin. A standard curve was established for each run using p27 controls of 600, 300, 150, and 75 ng/ml.

Induction of α/β-IFN from BMMC. BMMC were standardized to 10^7 cells/ml in RPMI 1640 (GIBCO), 10% heat-inactivated fetal bovine serum, 2% glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Newcastle disease virus (NDV) strain B_1, type B_1, (generously provided by Dr. Fred Melchior, Sterwin Laboratories, Millsboro, DE) was added to 0.5 ml to 1.0 ml of the cell suspension at a concentration of 1.5×10^8 50% egg-infectious dose units per 10^7 BMMC. The cell-virus suspensions were placed in 24-well flat-bottomed culture plates (Costar) and incubated for 24 hours at 37°C in 5% CO_2 with gentle stirring. After incubation, the cells were removed by centrifugation and the supernatant adjusted to pH 2 using HCl. After 24 hours at 4°C, the pH was adjusted to 7.4 using NaOH and the supernatant was filtered (0.45µ).

Induction of γ-IFN from BMMC and PBMC. BMMC and PBMC both were standardized to 5×10^6 cells/ml in RPMI 1640 (GIBCO), containing 10% heat-inactivated fetal bovine serum, 2% glutamine, 10^{-5}M β-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 µg/ml). Staphylococcal enterotoxin A (SEA) (Toxin Technology Inc., Madison, WI) was added to 1.0 ml of the cell suspension at a final concentration of 0.25 µg/ml. The SEA-cell suspensions were placed in 24-well flat-bottomed culture plates and incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2 with gentle stirring. After incubation, the cells were removed by centrifugation.

Measurement of IFN from BMMC suspensions. Constitutive production of IFN from BMMC was measured in suspension of BMMC (5×10^6 cells/ml) in RPMI 1640 containing 10% heat-inactivated fetal bovine

serum, 2% glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). The suspensions (1.0 ml) were placed in 24-well flat-bottomed culture plates and incubated for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2 with gentle stirring. After incubation, the cells were removed by centrifugation.

Lymphoblast transformation assay. The lymphoblast transformation test (LBT) was a modification of one which has been previously described (2). Briefly, PBMC were added at a concentration of 1 x 10^5 cells/well to a 96-well plate. Concanavalin A (10 µg/well) was added to bring the total volume per well to 200 µl. Five percent fetal bovine serum (Sterile Systems, Salt Lake City, UT) was added and the suspension was incubated four days. Eighteen hours before harvest the cultures were pulsed with [^{3}H]-thymidine (New England Nuclear, Boston, MA). The plates were harvested on glass wool using a Mash unit (Brandel, Gaithersburg, MD) and assessed for thymidine uptake using a liquid scintillation counter (Packard Instruments, Downers Grove, IL). A stimulation index was calculated for each test by dividing counts per minute (CPM) for stimulated cells by CPM control.

Statistical analysis. Either the Student's t-test or the Mann-Whitney-U test was used to determine significance when comparing transiently viremic and persistently viremic groups at a single time point. The paired t-test was used to compare sequential PI values with pre-inoculation values from the same group or parameter.

RESULTS

Interferon levels in the serum and bone marrow of FeLV-KT-infected cats. All cats inoculated with FeLV-KT produced measurable circulating levels of interferon during the first 14 days of infection. The interferon always was heat- and acid-labile consistent with the properties of γ-IFN as described for other species (Table 4.1). The maximum concentration of interferon averaged 72 ± 20 U/ml ($\bar{X}$ ± SEM) with a median of 16 U/ml. This large variance can be attributed in part to a significant difference between the concentration of IFN produced by transiently viremic cats versus persistently viremic cats. At 1 week PI, even before viremia was detected, serum concentrations of IFN were much higher in cats that eventually became nonviremic (143 ± 57 U/ml, $\bar{X}$ ± SEM) than those that became persistently viremic (12 ± 1 U/ml, $\bar{X}$ ± SEM) (Table 4.2). This significant difference remained through 2 weeks PI but was not evident by 3 weeks PI, at which time only occasional transiently viremic cats had measurable levels of IFN. Inoculation of a second group of cats with twice the usual dose of FELV-KT (2×10^5 FFU) doubled the IFN concentrations measured at 1 week PI. Persistently viremic cats averaged 28 ± 23 U/ml ($\bar{X}$ ± SEM) while transiently viremic cats averaged 343 ± 111 U/ml ($\bar{X}$ ± SEM) (Fig. 4.1). Evaluation of this smaller group of cats at more closely spaced sampling times revealed that peak IFN concentrations occurred between 4 and 6 days PI. Additionally, IFN was detected 1 day sooner in transiently viremic cats (2 days PI vs. 3 days PI) and remained detectable

longer, being present sporadically at 21 and 28 days PI. Examination of IFN levels in bone marrow plasma (Fig. 4.2) also showed that transiently viremic cats produced higher levels of IFN for a longer period of time than persistently viremic cats. Peak IFN levels in the bone marrow plasma occurred between days 3 and 7 PI similar to the peripheral blood. Interestingly, comparison of the concentrations of IFN in the blood vs. bone marrow revealed little difference in persistently viremic cats, e.g. 24 ± 12 U/ml ($\bar{X}$ ± SEM) in the bone marrow vs. 28 ± 23 U/ml ($\bar{X}$ ± SEM) in the serum at 7 days PI. However, this comparison revealed significant differences in transiently viremic cats. At 30 days PI, serum IFN was 213 ± 43 U/ml ($\bar{X}$ ± SEM) while bone marrow plasma IFN was 117 ± 46 U/ml ($\bar{X}$ ± SEM) and at 7 days PI serum IFN was 343 ± 111 U/ml ($\bar{X}$ ± SEM) while bone marrow plasma IFN was 64 ± 0 U/ml ($\bar{X}$ ± SEM).

FeLV p27 levels in the serum and bone marrow. Cats inoculated with FeLV-KT developed measurable levels of p27 in both the serum (Table 4.2, Fig. 4.3) and bone marrow plasma (Fig. 4.4) between 7 and 14 days PI. Peak levels were attained at 14 days PI and, as expected, were lower in transiently viremic cats, becoming undetectable by 4-6 weeks PI. Infection with the higher dose inoculum resulted in a comparably higher peak level (300± 29 ng/ml, $\bar{X}$ ± SEM) at 14 days PI in persistently viremic cats vs. infection with the lower dosage (52 ± 29 ng/ml, $\bar{X}$ ± SEM). In both groups of persistently viremic cats, serum p27 levels became lower by 4-5 weeks PI, e.g. 258 ± 32 ng/ml ($\bar{X}$ ± SEM) at 3 weeks PI vs. 140 ± 19 ng/ml ($\bar{X}$ ± SEM) at 4 weeks PI (Table 4.2). This reduction in p27 was reversed in

persistently viremic cats so that by 8 weeks PI, p27 concentrations averaged 483 ± 37 ng/ml ($\bar{X}$ ± SEM). Peak levels of p27 in the marrow were lower than serum concentrations for both transiently and persistently viremic cats: e.g. at 14 days in persistently viremic cats, p27 was 399 ± 29 ng/ml ($\bar{X}$ ± SEM) in the serum but only 118 ± 11 ng/ml ($\bar{X}$ ± SEM) in the bone marrow. Comparison of IFN and p27 for all cats showed that p27 concentrations began to rise as IFN levels were starting to fall with a small overlap (approximately 1 day) in the persistently viremic cats (Figs. 4.1 and 4.3).

Numbers and function of circulating lymphocytes. Lymphocyte counts were performed weekly in 4 persistently viremic cats and 3 transiently viremic cats as well as 1 normal control cat. Lymphocytes decreased in all cats, including the control, by 1 week PI, apparently in part due to pre-treatment with methyl prednisolone (Fig. 4.5). In transiently viremic cats and the control cat lymphocytes returned to normal by 3 weeks PI. Persistently viremic cats, however, maintained lymphopenia, e.g., 0.900 ± 147 x 10^9 cells/L ($\bar{X}$ ± SEM) at 3 weeks PI versus 3.825 ± 1.064 x 10^9 cells/L ($\bar{X}$ ± SEM) prior to inoculation.

Lymphoblast transformation assays were performed at 3, 7, 10, 14, 21, and 35 days PI in 4 persistently viremic cats as well as in 1 transiently viremic cat and 1 normal control cat. In the normal and persistently viremic cats there was a modest but not significant reduction in stimulation indices at 3 days PI followed by slight increases at 7 and 10 days, respectively (Fig. 4.6). Lymphocyte

function became significantly suppressed by 21 days PI in persistently viremic cats: 128.4 ± 22 SI ($\bar{X}$ ± SEM) at -1 day PI versus 36 ± 10 SI ($\bar{X}$ ± SEM) at 21 days PI. The control cat's SIs fluctuated normally from 7 days PI to 35 days PI. The single transiently viremic cat tested showed a dramatic increase in its SI from 118.0 on day -1 PI to 336.1 on day 3 PI. At 7 days PI, its SI had dropped to 52.5 and but then returned to normal by 10 days PI. Interestingly, the sharp rise in this cat's SI at 21 days PI to 335 was concurrent with an increase in serum γ-IFN and a reduction in serum p27.

Constitutive and stimulated production of IFN from BMMC and PBMC in vitro. BMMC and PBMC suspension cultures were established from 4 persistently viremic cats, as well as 1 transiently viremic and 1 normal control cat at 0, 3, 7, 14, 28, and 35 days PI. The BMMC suspensions were cultured for 72 hours with and without the α/β-IFN inducer, NDV, or the γ-IFN inducer, SEA, and the PBMC suspensions were cultured with the γ-IFN inducer, SEA (Table 4.3). Although not detectable in bone marrow plasma, culture of normal BMMC revealed endogenous production of α/β-IFN averaging approximately 25 U/ml of supernatant. This level was reduced significantly at 3 days PI in the control (8 U/ml) and persistently viremic cats (2 ± 2 U/ml, $\bar{X}$ ± SEM), possibly due to pre-treatment with methyl prednisolone. By 7 days PI, the α/β-IFN level returned to within normal limits in the persistently viremic cats (16 ± 8, $\bar{X}$ ± SEM), but from 14 days onward it fell continually and was 5 ± 5 U/ml ($\bar{X}$ ± SEM) at 35 days PI. The IFN level from cells of the control cat remained stable from 7 through 35 days PI. The transiently viremic cat showed a dramatic

increase in α/β-IFN starting at 3 days PI (128 U/ml), which peaked at 7 days PI (256 U/ml), and ultimately dropped to 8 U/ml by 28 days PI. γ-IFN was not detected in suspensions of BMMC of normal cats but was produced significantly at 3 days PI by both the persistently viremic (10 ± 3 U/ml, $\bar{X}$ ± SEM) and the transiently viremic (32 U/ml) cats. γ-IFN was produced only sporadically by persistently viremic cats at 7 and 14 days PI, after which it was unmeasurable in the culture supernatants. The transiently viremic cat produced comparably higher IFN levels which peaked at 7 days PI (64 U/ml).

NDV induced α/β-IFN production by BMMC of all cats through 7 days PI and from the control cat through 35 days PI. BMMC of persistently viremic cats produced lower IFN levels in response to NDV in vitro at 14 days PI (1579 ± 887 U/ml, $\bar{X}$ ± SEM) vs. pre-inoculation (5622 ± 1560 U/ml, $\bar{X}$ ± SEM). However, production of α/β-IFN in vitro was significantly increased at both 28 and 35 days PI, reaching 2596 ± 3131 U/ml ($\bar{X}$ ± SEM) for persistently viremic cats and 57,500 U/ml for the transiently viremic cat at 35 days PI.

SEA induced γ-IFN production by both BMMC and PBMC, although consistently higher levels of IFN were produced from BMMC. No changes were noted with cells of persistently viremic cats until 14 days PI when γ-IFN concentrations were reduced to 4 ± 2 U/ml ($\bar{X}$ ± SEM) in PBMC suspensions and at 28 days PI when BMMC suspension levels were only 14 ± 9 U/ml ($\bar{X}$ ± SEM). γ-IFN could not be induced from PBMC of persistently viremic cats at 28 and 35 days PI. γ-IFN from the PBMC suspension of the transiently viremic cat at 3 days PI (256 U/ml) was increased dramatically over the pre-inoculation value

(16 U/ml). This production level fell to 48 U/ml by 7 days and was 16 U/ml at 35 days PI. The BMMC suspension from the transiently viremic cat at pre-inoculation produced a high level of IFN in response to SEA (512 U/ml) vs. the control (64 U/ml) or persistently viremic cats (32 ± 8 U/ml, $\bar{X}$ ± SEM). This high level was maintained throughout the course of the experiment, peaking at 768 U/ml at 7 days PI.

DISCUSSION

In these studies, the IFN response of cats to infection with FeLV was examined. γ-IFN was found to be produced consistently prior to viremia in both the serum and bone marrow plasma of all cats but in significantly higher concentrations in cats ultimately developing transient viremia and regressive infections. In vitro cultures of BMMC and PBMC revealed that γ-IFN production by BMMC or PBMC exposed to SEA is reduced or inhibited in persistently viremic cats concurrent with immunosuppression. The results suggest that IFN may play an important role in recovery from FeLV infection.

The interferon system represents an integral part of the body's nonspecific immune system and is the earliest appearing of the known host defenses. Three types of IFN appear to exist in all animal species tested, including α-IFN, produced predominantly by leukocytes, β-IFN by fibroblasts, and γ-IFN by lymphocytes (21). In addition to their ability to inhibit viral replication, IFNs possess the capacity to affect a wide range of biological functions including cell proliferation and differentiation, expression of cell surface antigens, cytoskeleton and plasma membrane characteristics, as well as immunoregulatory effects on both humoral and cell-mediated immune responses (22).

The IFN response of cats to FeLV was different from expectations based on other virus-host systems in which IFN production overlaps extensively with viremia and consists of α/β-IFN (23,24). α/β-IFN was not detectable in either bone marrow plasma or serum at any time

before or after onset of viremia. Suspension cultures of normal BMMC, however, did contain α/β-IFN after 72 hours incubation consistent with the normal low-level endogenous production of IFN described in man (25). The levels of α/β-IFN produced by the suspensions of BMMC, however, did not increase in cats becoming persistently viremic but actually decreased. The transiently viremic cat showed high levels of α/β-IFN in suspensions of BMMC extending through 14 days PI that suggest it may serve a protective role locally. No α/β-IFN was detected in supernatants from suspensions of PBMC (data not shown). When NDV was used to evaluate the ability of BMMC to produce α/β-IFN, a progressive five-fold enhancement over pre-inoculation levels developed with BMMC of in persistently viremic cats and an almost 10-fold enhancement with cells of the transiently viremic cat.

Instead of releasing α/β-IFN within the circulation, FeLV-infected cats produced γ-IFN. All cats tested were found to have measurable γ-IFN in both their serum and bone marrow plasma between 0-14 days PI. Results of the two experiments showed that the levels produced by cats at 7 days or less PI and prior to peak viremia predicted whether the cats would become persistently or transiently viremic. Furthermore, inoculation with twice the concentration of virus doubled the corresponding IFN levels on day 7 PI. FeLV p27 levels always were lower in cats that became transiently viremic and in most of these animals were not detectable by 4 weeks PI. Interestingly, both IFN and p27 levels in the bone marrow plasma were lower than corresponding levels in the serum at all times PI. This

is surprising in view of the numerous leukocytes with intracellular p27 evident in the circulation at 14 days PI which have been interpreted as evidence of marrow origin viremia. These results suggest that the marrow is not a major source of circulating free virus and actually may remove circulating virus via phagocytosis by neutrophils and macrophages.

In vitro studies with BMMC and PBMC suspensions revealed that γ-IFN was produced constitutively in FeLV-infected cats. As expected, cats with persistent viremia produced lower levels than the transiently viremic cat. Similarly, both BMMC and PBMC from persistently viremic cats produced lower levels of γ-IFN in response to SEA. The most interesting comparison is the levels of SEA-induced IFN from BMMC of persistently viremic cats versus the transiently viremic cat prior to inoculation. The cat that ultimately became transiently viremic was able to produce 16 times more IFN than the other cats including the control and maintained this ability over the course of the infection. This suggests that it may be possible to predict transiently viremic cats prior to inoculation and that a key cell in resistance occurs in the bone marrow but not the peripheral blood. The reduction of γ-IFN induction from BMMC and the total inability to produce γ-IFN from PBMC at 28 and 35 days PI in persistently viremic cats is consistent with the immunosuppression that occurs between 14 and 21 days PI (1). Examination of lymphocyte numbers and LBT assays in these cats confirms significant loss of function and circulating lymphocytes by 21 days PI. The combination of T-cell hyporesponsiveness to lectins and the inability to produce γ-IFN is similar to

findings in AIDS (27) and is not surprising since γ-IFN is a lymphokine product of T-lymphocytes.

The in vivo production of γ-IFN in response to a viral agent without prior sensitization is novel. In vitro induction of γ-IFN can be accomplished with mitogens, allogeneic cells, or antigens after previous sensitization (28). The typical cell-mediated immune response to viral infection was exemplified in studies of the acute lymphocytic choriomeningitis virus in mice. NK cells and α/β-IFN both reached maximum levels at day 3 and were followed by the generation of cytotoxic T-lymphocytes (CTL) on day 5. These CTL peaked on day 7 and then rapidly declined (29,30). Although circulating γ-IFN was not detected in this system, suspensions of splenic CTL from day 7 produced γ-IFN in culture (31). Conceivably, the CTL response to FeLV in cats is rapid and these activated cells circulate, producing γ-IFN. Lymphocytes of the OKT8+, TAC+, Fc-γR phenotype which are consistent with activated T-suppressor/cytotoxic cells have been reported to produce circulating γ-IFN in patients with aplastic anemia (32) and T_γ lymphoproliferative disease (33). Studies examining the susceptibility and resistance to FeLV infection demonstrated that viral proliferation was enhanced dramatically in FcγR depleted peripheral blood lymphocytes suggesting that naturally occurring FcγR cells limit the outgrowth of FeLV-infected cells or maintain them in a virus-nonproductive state (34). An alternative to specific antigen induction of γ-IFN is nonspecific induction similar to the response to mitogens and allogeneic cells. Direct stimulation of γ-IFN by a soluble protein produced by Mycoplasma arthriditis is believed to act

in this manner (21). Possibly FeLV at very low concentrations interacts with receptors on T-cells to induce γ-IFN synthesis, thus accounting for its rapid occurrence in the circulation prior to viremia.

Regardless of the cell type producing γ-IFN prior to viremia, the higher levels of γ-IFN in cats that develop transient viremia versus persistent viremia indicate that it is associated with a more effective immune response. γ-IFN is an effective antiviral agent, e.g. human T-lymphocyte lymphoma/leukemia virus expression cultured in vitro is inversely proportional to constitutive γ-IFN production (35). In addition to direct antiviral effects, γ-IFN has been shown to enhance CTL and NK cell numbers and activity, activate macrophages, stimulate histocompatibility antigens and β_2 microglobulin, increase the expression of interleukin-2 receptors, and drive the maturation of B-cells to active immunoglobulin synthesis (21,22,28). In this context, higher levels of γ-IFN may be a significant factor in the ultimate regression of FeLV viremia.

SUMMARY

The interferon (IFN) response after infection of cats with the Kawakami-Theilen strain of feline leukemia virus (FeLV-KT) was evaluated in the blood and bone marrow. Serum levels of γ-IFN ranging from 8-512 U/ml developed in all 39 cats tested during the first 14 days after inoculation (PI) and occurred prior to the onset of viremia. Higher levels of γ-IFN (143 ± 57 U/ml) and significantly lower levels of circulating viral antigen (163 ± 69 ng/ml) were produced in transiently viremic cats versus cats with persistent viremia 12 ± 2 U/ml IFN) (258 ± 32 ng/ml viral antigen). Inoculation of 7 cats with twice the FeLV-KT dose caused a two-fold increase in serum IFN concentrations of both transiently viremic (343 ± 111 U/ml) and persistently viremic (28 ± 23 U/ml) cats. γ-IFN was detectable in the bone marrow plasma in persistently viremic cats at levels comparable to the serum. However, serum concentrations in transiently viremic cats were much higher than bone marrow levels (343 ± 111 U/ml vs. 64 ± 0 U/ml). Cultures of bone marrow cells (BMMC) and peripheral blood mononuclear cells (PBMC) demonstrated that in persistently viremic cats: 1) constitutive α/β-IFN production from BMMC significantly decreased by 4 weeks PI while α/β-IFN production in response to Newcastle disease virus (NDV) in vitro increased five-fold; 2) constitutive γ-IFN was produced transiently between 3 and 14 days PI; 3) γ-IFN levels produced in response to staphylococcal enterotoxin A (SEA) in vitro significantly decreased from BMMC and was absent from PBMC by 28 days PI. The loss of γ-IFN inducibility

corresponded to T-lymphocyte dysfunction evident in lymphocyte blastogenic transformation assays. In contrast to the persistently viremic cats, the following changes were noted in a transiently viremic cat 1) increased constitutive α/β-IFN by BMMC from 32 U/ml prior to inoculation to 256 U/ml at 7 days PI, 2) a 10-fold increase in NDV-induced α/β-IFN from BMMC at 35 days PI, 3) higher constitutive γ-IFN production from BMMC, and 4) 15 times more SEA-induced IFN from BMMC at pre-inoculation and consistent production of SEA-induced γ-IFN at all times from BMMC and PBMC. These studies reveal that production of circulating γ-IFN even prior to viremia is a consistent feature of early FeLV infection. Furthermore, higher levels of γ-IFN are observed in cats ultimately recovering from viremia demonstrating the association of γ-IFN with a more effective immune response and suggesting that it may function to directly enhance the cell-mediated and/or humoral response to FeLV.

Table 4.1. Features of serum interferon in cats 0-14 days after inoculation with FeLV-KT.

Frequency of Detection	
No. cats tested:	32
No. with IFN:	32
Maximum Concentration of Interferon (U/ml)	
Mean ± SEM:	72 ± 20
Median:	16
Range:	8-512
Effects of Acid or Heat Treatment on Antiviral Activity	
Untreated:	100%
pH 2:	0%
56°C:	0%

Table 4.2. Levels of γ-interferon and FeLV p27 in the sera of persistently viremic versus transiently viremic cats.

Weeks PI		Serum IFN (U/ml)		Serum p27 (ng/ml)	
		Persistent Viremia[a]	Transient Viremia[b]	Persistent Viremia	Transient Viremia
1	$\bar{X}$ ± SEM	12 ± 2	143 ± 57[e]	<10 ± 0	<10 ± 2
	Median	16	48	<10	<10
	Range	<4-16	<4-400	0	<10-20
2	$\bar{X}$ ± SEM	3 ± 1	48 ± 24[d]	52 ± 29	21 ± 6
	Median	<4	24	20	20
	Range	<4-16	<4-256	<10-650	<10-60
3	$\bar{X}$ ± SEM	<4 ± 0	18 ± 12	258 ± 32	163 ± 69[c]
	Median	<4	<4	220	130
	Range	0	<4-128	90-530	<10-530
4	$\bar{X}$ ± SEM	<4 ± 0	<4 ± 1	140 ± 19	50 ± 35[f]
	Median	<4	<4	113	<10
	Range	0	<4-12	30-400	<10-320
8	$\bar{X}$ ± SEM	<4 ± 0	<4 ± 0	483 ± 37	<10 ± 0[f]
	Median	<4	<4	595	<10
	Range	0	0	50-600	0

[a] n = 22.
[b] n = 10.
[c] Significantly lower than persistently viremic cats at $p<.05$.
[d] Significantly higher than persistently viremic cats at $p<.025$.
[e] Significantly higher than persistently viremic cats at $p<.005$.
[f] Significantly lower than persistently viremic cats at $p<.005$.

Table 4.3. Comparative Production of α/β-IFN and γ-IFN from stimulated and unstimulated BMMC, and of γ-IFN from stimulated PBMC, before and after inoculation with FeLV.

	Interferon Concentration U/ml Days Post-Inoculation					
Viremia Status	0	3	7	14	28	35
A. α/β-IFN in BMMC suspensions						
Control	32	8	32	16	16	8
Persistent[a]	22 ± 4	2 ± 2[e]	16 ± 8	14 ± 6[b]	4 ± 2[b]	5 ± 5[b]
Transient	32	128	256	128	8	8
B. NDV induction of α/β-IFN from BMMC						
Control	8200	6150	6144	4100	8200	8200
Persistent	5622 ± 1560	3459 ± 1768	2544 ± 841	1579 ± 887[b]	18450 ± 6150[b]	25967 ± 3131[b]
Transient	6144	4100	5120	12296	24600	57500
C. γ-IFN in BMMC suspensions						
Control	<4	<4	<4	<4	<4	<4
Persistent	<4 ± 0	10 ± 3[c]	28 ± 23	15 ± 8	<4 ± 1	<4 ± 1
Transient	<4	32	64	32	<4	<4
D. SEA induction of γ-IFN from BMMC						
Control	64	67	160	96	96	96
Persistent	32 ± 8	39 ± 15	86 ± 38	16 ± 7	14 ± 9[b]	11 ± 6[b]
Transient	512	384	768	448	640	640
E. SEA induction of γ-IFN from PBMC						
Control	24	8	24	24	24	24
Persistent	30 ± 6	33 ± 14	58 ± 25	4 ± 2[c]	<4 ± 0[d]	<4 ± 0[d]
Transient	16	256	48	6	ND[f]	16

[a]Mean ± SEM, n=4.
[b]Significant difference from pre-inoculation at $p<.05$.
[c]Significant difference from pre-inoculation at $p<.025$.
[d]Significant difference from pre-inoculation at $p<.01$.
[e]Significant difference from pre-inoculation at $p<.005$.
[f]ND = not done.

Fig. 4.1 Changes in serum IFN ($\bar{X}$ ± SEM, U/ml) after inoculation with FeLV-KT in transiently viremic (n=3) and persistently viremic cats (n=4).

$^{**}p<.025$

$^{++}p<.005$

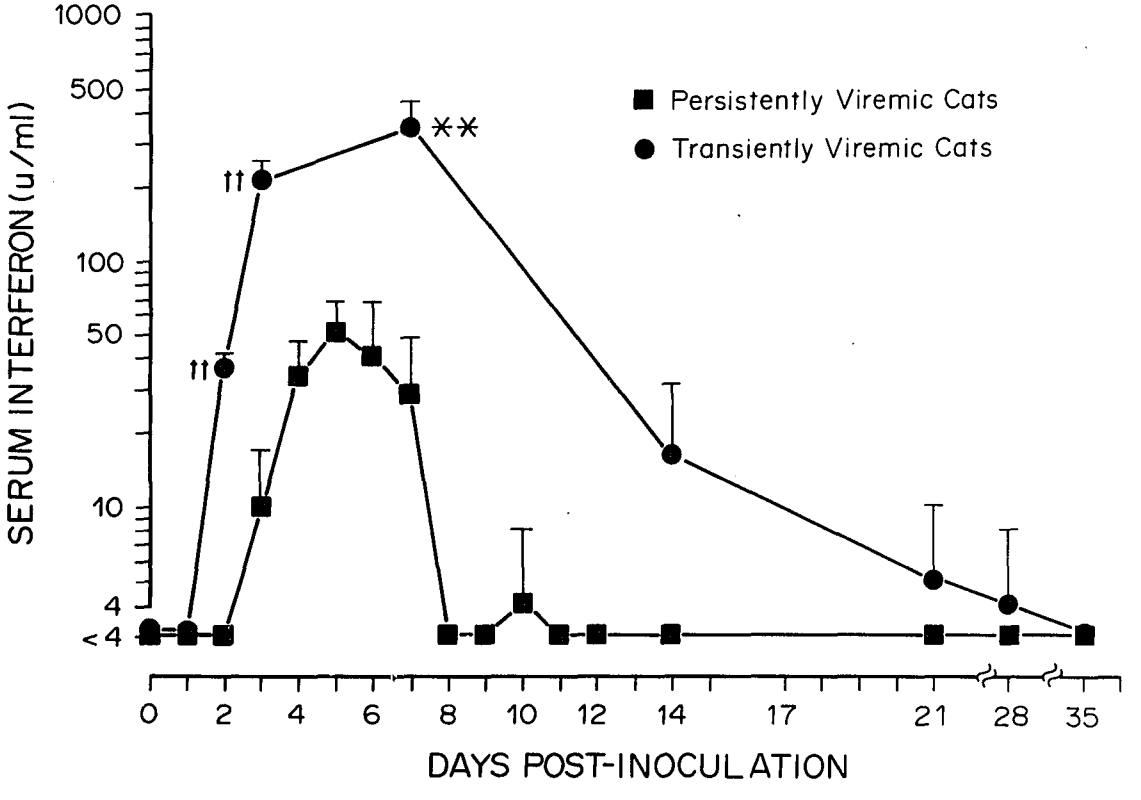

1000
500
100
50
10
4
<4
SERUM INTERFERON (u/ml)
Persistently Viremic Cats
Transiently Viremic Cats
††
††
**
0
2
4
6
8
10
12
14
17
21
28
35
DAYS POST-INOCULATION

Fig. 4.2 Changes in bone marrow plasma IFN ($\bar{X}$ ± SEM, U/ml) after inoculation with FeLV-KT in transiently viremic (n=3) and persistently viremic cats (n=4).

** $p<.025$

++ $p<.005$

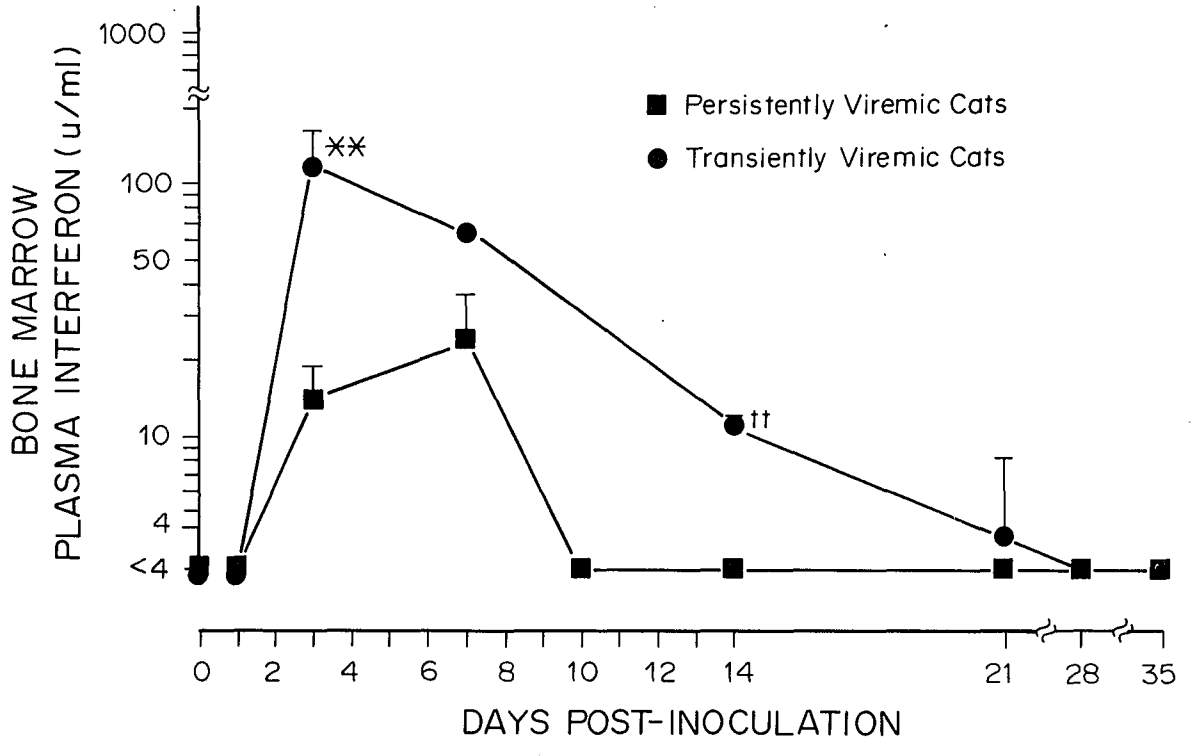

BONE MARROW
PLASMA INTERFERON (u/ml)
1000
100
50
10
4
<4
Persistently Viremic Cats
Transiently Viremic Cats
**
tt
0
2
4
6
8
10
12
14
21
28
35
DAYS POST-INOCULATION

Fig. 4.3 Changes in serum p27 ($\bar{X}$ ± SEM, ng/ml) after inoculation with FeLV-KT in transiently viremic cats (n=3) and persistently viremic cats (n=4).

** $p<.025$

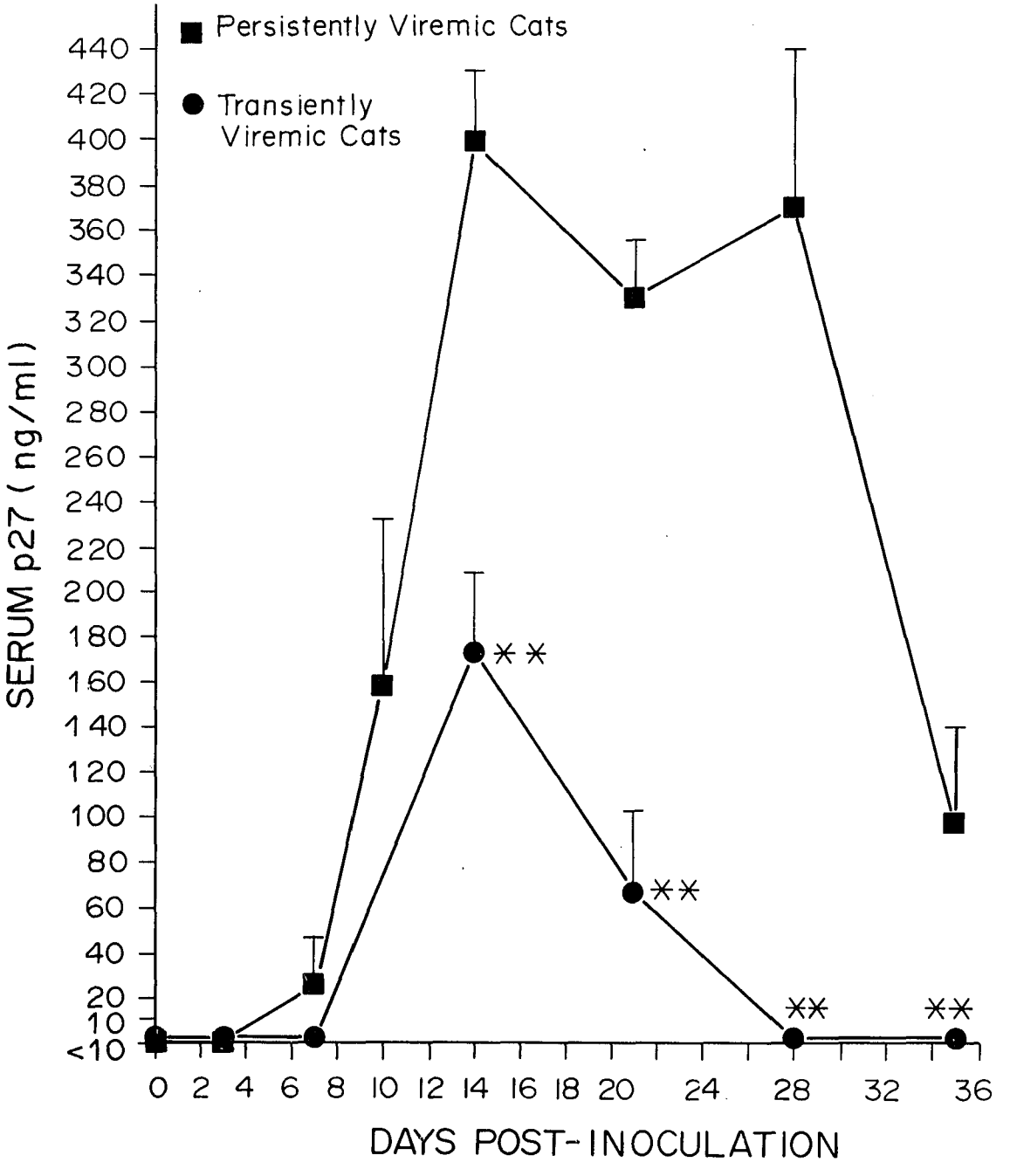

Persistently Viremic Cats
Transiently Viremic Cats
SERUM p27 (ng/ml)
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
10
<10
**
**
**
**
0 2 4 6 8 10 12 14 16 18 20 24 28 32 36
DAYS POST-INOCULATION

Fig. 4.4 Changes in bone marrow plasma p27 ($\bar{X}$ ± SEM, ng/ml) after inoculation with FeLV-KT in transiently viremic cats (n=3) and persistently viremic cats (n=4).

*$p<.05$

**$p<.025$

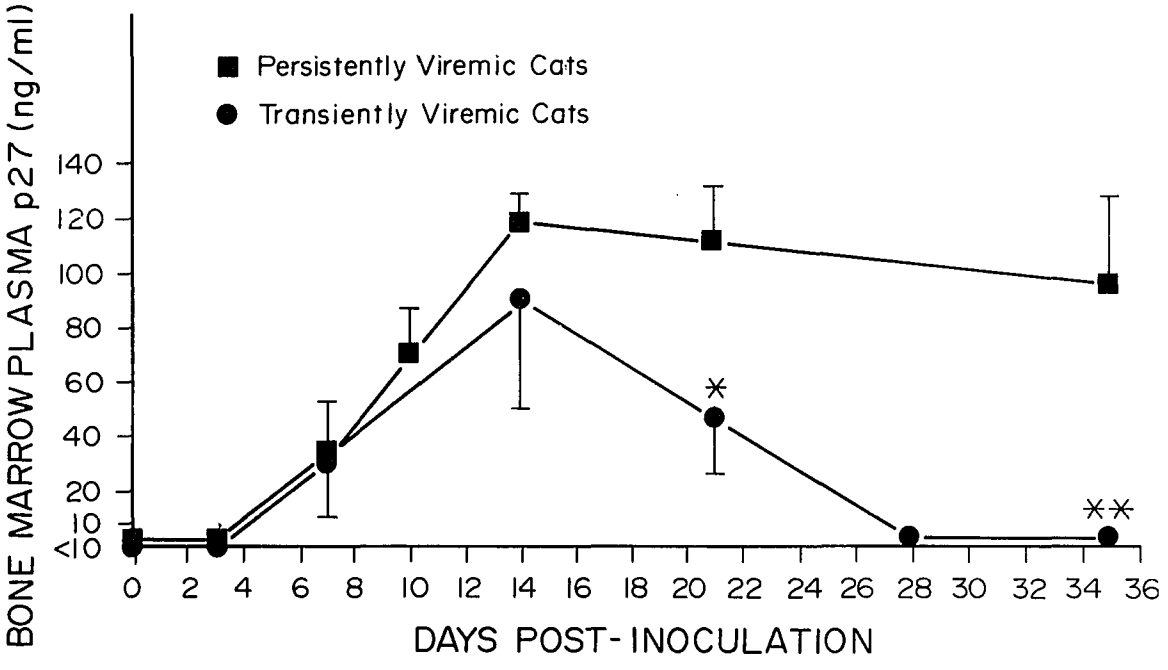

Persistently Viremic Cats
Transiently Viremic Cats
BONE MARROW PLASMA p27 (ng/ml)
≤10
20
40
60
80
100
120
140
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
DAYS POST-INOCULATION
*
**

Fig. 4.5 Changes in numbers of lymphocytes ($\bar{X}$ ± SEM) after inoculation with FeLV-KT in transiently viremic cats (n=3) and persistently viremic cats (n=4), and in an uninoculated normal cat.

*$p<.05$

+$p<.01$

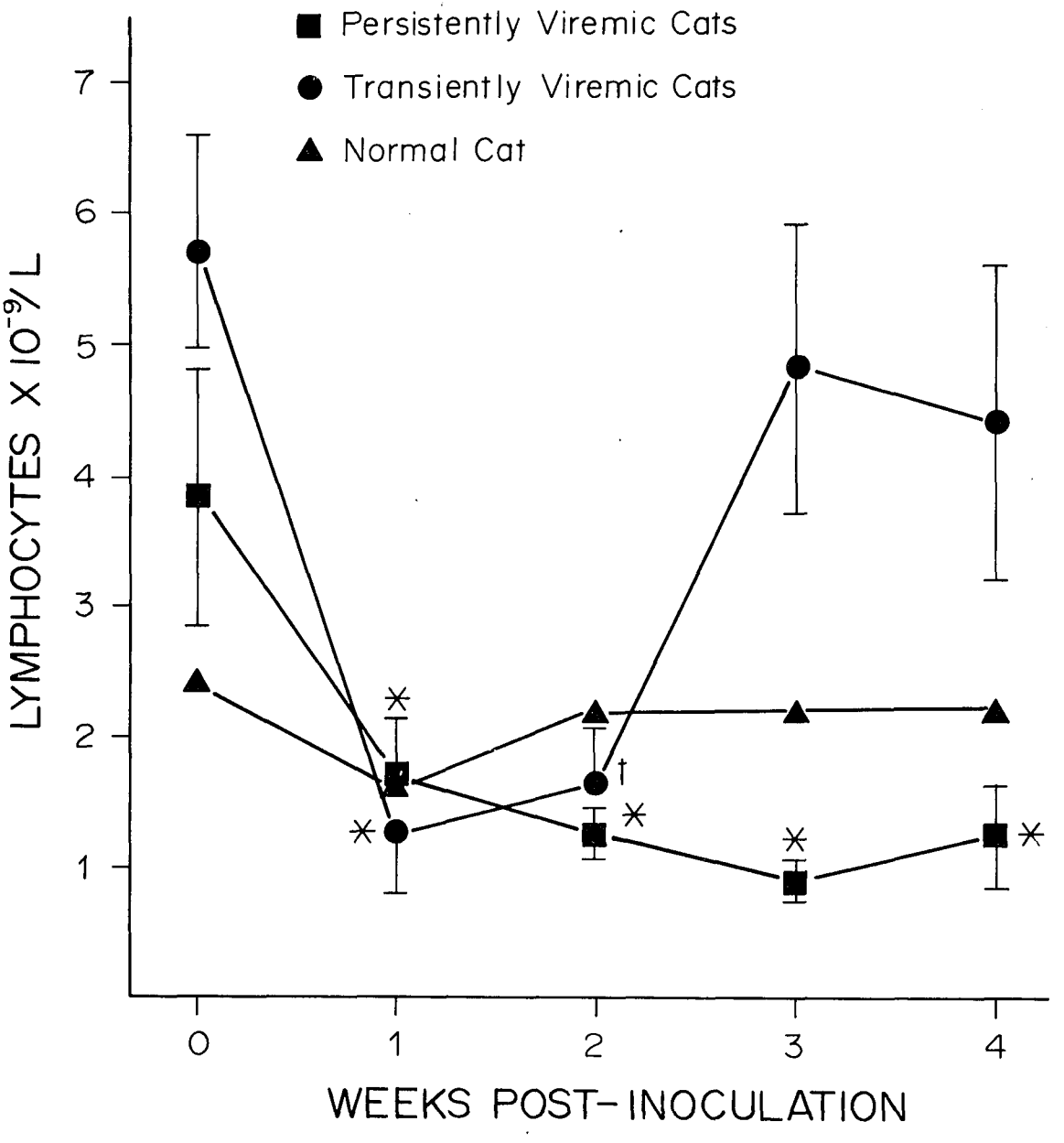
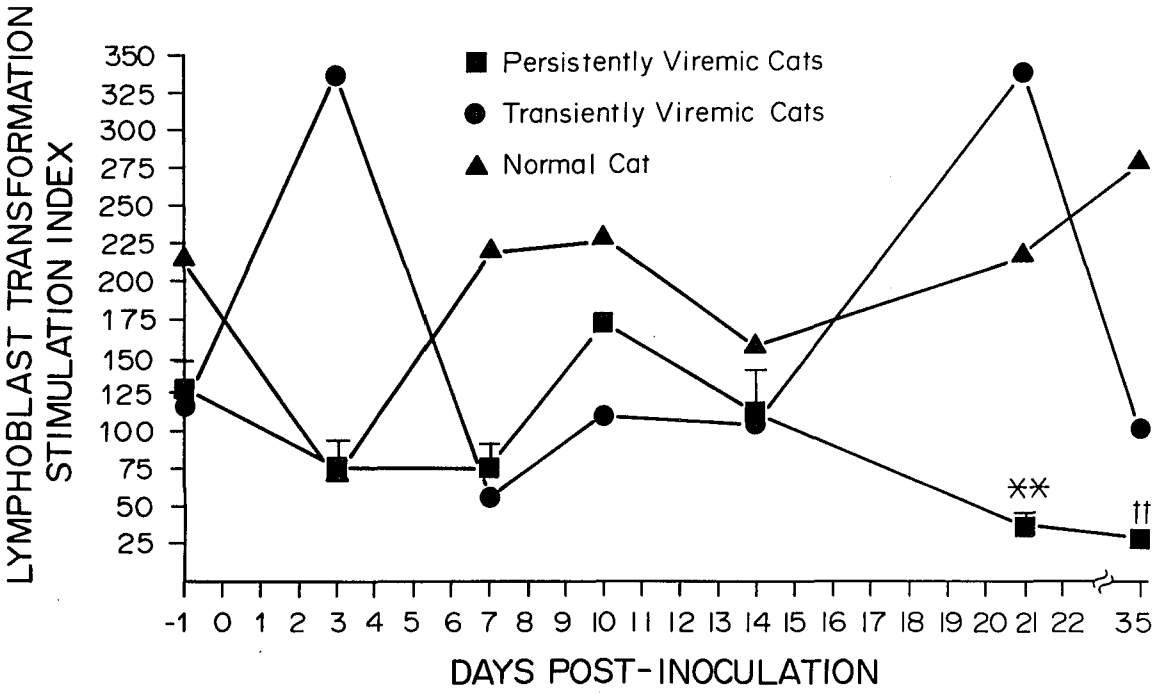

Persistently Viremic Cats
Transiently Viremic Cats
Normal Cat
LYMPHOCYTES X 10⁻⁹/L
7
6
5
4
3
2
1
0
1
2
3
4
WEEKS POST-INOCULATION
*
*
*
*
*
†

Fig 4.6 Changes in the lymphoblast transformation stimulation indices ($\bar{X}$ ± SEM) of PBMC of transiently viremic (n=1) and persistently viremic cats (n=4) after inoculation with FeLV-KT and in an uninoculated normal cat (n=1).

** $p<.025$

++ $p<.005$


LYMPHOBLAST TRANSFORMATION
STIMULATION INDEX
350
325
300
275
250
225
200
175
150
125
100
75
50
25
Persistently Viremic Cats
Transiently Viremic Cats
Normal Cat
**
††
-1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 35
DAYS POST-INOCULATION

REFERENCES

1. Rojko JL, Olsen RG (1984) The immunobiology of the feline leukemia virus. Vet Immunol Immunopathol 6:107-165.

2. Cockerell GL, Hoover EA, Krakowka S, Olsen RG, Yohn DS (1976) Lymphocyte mitogen reactivity and enumeration of circulating B- and T-cells during feline leukemia virus infection in cats. J Natl Cancer Inst 57:1095-1099.

3. Hoover EA, Perryman LE, Kociba GJ (1973) Early lesions in cats inoculated with feline leukemia virus. Cancer Res 33:145-152.

4. Anderson LJ, Jarrett WFH, Jarrett O, Laird HM (1971) Feline leukemia-virus infection of kittens: Mortality associated with atrophy of the thymus and lymphoid depletion. J Natl Cancer Inst 47:807-817.

5. Boyce JT, Hoover EA, Kociba GJ, Olsen RG (1981) Feline leukemia virus-induced erythroid aplasia: In vitro hemopoietic culture studies. Expl Hematol 9:990.

6. Perryman LE, Hoover EA, Yohn DS (1972) Immunological reactivity of the cat: Immunosuppression in experimental feline leukemia. J Natl Cancer Inst 49:1357-1365.

7. Dunlap JE, Nichols WS, Hebebrand LC, Mathes LE, Olsen RG (1979) Mobility of lymphocyte surface membrane concanavalin A receptor of normal and feline leukemia virus-infected viremic felines. Cancer Res 39:956-958.

8. Rojko JL, Hoover EA, Mathes LE, Schaller JP, Olsen RG (1979) Pathogenesis of experimental feline leukemia virus infection. J Natl Cancer Inst 63:759-778.

9. Engelman RW, Fulton RW, Good RA, Day NK (1985) Suppression of gamma interferon production by inactivated feline leukemia virus. Science 227:1368-1370.

10. Liu WT, Good RA, Trang LQ, et al (1984) Remission of leukemia and loss of feline leukemia virus in cats injected with Staphylococcus protein A associated with increased circulating interferon and complement-dependent cytotoxic antibody. Proc Natl Acad Sci 81:6471-6475.

11. Jameson P, Essex M (1983) Inhibition of feline leukemia virus replication by human leukocyte interferon. Antiviral Res 3:115-120.

12. Stewart WE. The Interferon System. Springer-Verlag, NY. 1979, p. 30.

13. De Maeyer E, De Maeyer-Guignaro J, Hall WT, et al (1972) Induction of interferon synthesis by mammary tumor virus preparations in mice. In Recherches Fundamentals sur les Tumeurs Mammaires, 119-126, ed. by J. Mouriquand. Edition S Inserm.

14. Glasgow LA, Friedman SB (1969) Interferon and host resistance to Rauscher virus-induced leukemia. J Virol 47:99-105.

15. Blank KJ, Murasko DM (1980) Induction of interferon in AKR mice by murine leukaemia viruses. Nature 283:494-495.

16. Blank KJ, Murasko DM (1981) Effect of anti-interferon serum on Friend virus infection and FV-1-mediated resistance. J Interferon Res 1:437-442.

17. Inglot AD, Oleszak E (1978) Application of antisera to interferon in studying oncogenic viruses. Arch Immunol Ther Exp 26: 529-536.

18. Gresser I, Tovey MG, Maury C, et al (1976) Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. J Exp Med 144:1316-1323.

19. Preble OT, Rook AH, Quinnan GV, et al (1984) Role of interferon in AIDS. Ann NY Acad Sci 437:65-75.

20. Tompkins MB, Cummins JM (1982) Response of feline leukemia virus-induced nonregenerative anemia to oral administration of an interferon-containing preparation. Feline Pract 12(3):6-15.

21. Kirchner H (1986) The interferon system as an integral part of the defense system against infections. Antiviral Res 6:1-17.

22. Preble OT, Friedman RM (1983) Biology of disease: Interferon-induced alterations in cells: Relevance to viral and nonviral diseases. Lab Invest 49:4-18.

23. Baron S, Buckler CE, McCloskey RV, et al (1966) Role of interferon during viremia. I. Production of circulating interferon. J Immunol 96:12-16.

24. Buchmeier MJ, Welse RM, Dukto FJ et al (1980) The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv Immunol 30:275-313.

25. Zoumbos NC, Gascon P, Djell JY, Young NS (1985) Interferon is a mediator of hematopoietic suppression in aplastic anemia *in vitro* and possibly *in vivo*. Proc Natl Acad Sci 82:188-192.

26. Hoover EA, Mathes LE, Rojko JL *et al* (1978) Modification of the immunofluorescence assay for feline leukemia virus group-specific antigen. Am J Vet Res 39:1877-1880.

27. Hart CC, Murray HW (1984) Cellular immunity and the acquired immune deficiency syndrome: Failure to secrete lymphokines and gamma interferon. Surv Synth Path Res 3:397-408.

28. Epstein LB (1984) The special significance of interferon-gamma. Interferon 2:185-220.

29. Blanden RV (1974) T-cell response to viral and bacterial infection. Transplant Rev 19:56-88.

30. Welsh RM, Biron CA, Bukowski JF *et al* (1984) Role of natural killer cells in virus infection of mice. Surv Synth Path Res 3:409-431.

31. Welsh RM, Doe WF (1980) Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice: Natural killer cell activity in cultured spleen leukocytes concomittant with T-cell dependent immune interferon production. Infect Immun 30:473-483.

32. Zoumbos NC Gascon P, Djell JY, *et al* (1985) Circulating activated suppressor T lymphocytes in aplastic anemia. N Eng J Med 312:257-265.

33. Hooks JJ, Haynes BF, Detrick-Hooks B, et al (1982) Gamma (immune) interferon production by leukocytes from a patient with Tγ cell proliferative disease. Blood 59:198-201.

34. Rojko JL, Hoover EA, Finn Bl, Olsen RG (1981) Determinants of susceptibility and resistance to feline leukemia virus infection. II. Susceptibility of feline lymphocytes to productive feline leukemia virus infection. J Natl Cancer Inst 67:899-910.

35. Moore JL, Poiesz BJ, Zamkoff KW, et al (1985) Inverse relationship between constitutive γ-interferon production and human T-cell lymphoma/leukemia virus expression in cultured T lymphocytes. J Virol 53:440-446.

BIBLIOGRAPHY

Abkowitz JL, Adamson JW (1985) Kinetics of marrow failure in feline leukemia virus (FeLV-C)-induced pure red cell aplasia (PRCA). Blood 66(Suppl 1):118A.

Abkowitz JL, Oft RL Nakamura JM et al (1985) Feline glucose-6-phosphate dehydrogenase cellular mosaicism. Application to the study of pure red cell aplasia. J Clin Invest 75:133-140.

Abkowitz JL, Pwell JS, Nakamura JM et al (1986) Pure red cell aplasia: Response to therapy with antithymocyte globulin. AM J Hematol 23(4):363.

Abkowitz JL, Ott RL, Holly RD, Adamson JW (1987) Lymphocytes and antibody in retrovirus-induced feline pure red cell aplasia. J Natl Cancer Inst 78:135-139.

Anderson LJ, Jarrett WFH, Jarrett O, Laird HM (1971) Feline leukemia-virus infection of kittens: Mortality associated with atrophy of the thymus and lymphoid depletion. J Natl Cancer Inst 47:807-817.

Atrah HI, Crawford RJ, Babra GS (1985) Modulation of suppressor T-cells for the treatment of aplastic anaemia. Lancet ii:339-340.

Bacigalupo A, Podesta M, Frassoni F et al (1985) In vitro tests in severe aplastic anaemia (SAA): A prospective study in 46 patients treated with immunosuppression. Brit J Haematol 59:611-616.

Bagby GC, Rigas VD, Bennett RM et al (1981) Interaction of lactoferrin, monocytes, and T lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. J Clin Invest 68:56-73.

Baron S, Buckler CE, McCloskey RV, et al (1966) Role of interferon during viremia. I. Production of circulating interferon. J Immunol 96:12-16.

Beckman B, Mirand E, Fisher JW (1980) Effects of beta adrenergic agents and prostaglandin E_1 on erythroid colony (CFU-E) growth and cyclic AMP formation in Friend erythroleukemic cells. J Cell Physiol 105:355-361.

Blanden RV (1974) T-cell response to viral and bacterial infection. Transplant Rev 19:56-88.

Blank KJ, Murasko DM (1980) Induction of interferon in AKR mice by murine leukaemia viruses. Nature 283:494-495.

Blank KJ, Murasko DM (1981) Effect of anti-interferon serum on Friend virus infection and FV-1-mediated resistance. J Interferon Res 1:437-442.

Boyce JT, Hoover EA, Kociba GJ, Olsen RG (1981) Feline leukemia virus-induced erythroid aplasia: In vitro hemopoietic culture studies. Expl Hematol 9:990-1001.

Boynton AL, Whitfield JF (1983) The role of cyclic AMP in cell proliferation: A critical assessment of the evidence. Adv Cyc Nucl Res 15:193-294.

Bridges K, Levenson R, Housman D, Cantley L (1981) Calcium regulates commitment of murine erythroleukemia cells to terminal differentiation. J Cell Biol 90:542-545.

Brown JE, Adamson JW (1977)[c] Modulation of in vitro erythropoiesis: The influence of β-adrenergic agonists on erythroid colony formation. J Clin Invest 60:70-77.

Brown JE, Adamson JW (1977) Studies of the influence of cyclic nucleotides on in vitro haemoglobin synthesis. Brit J Haemat 35:193-208,

Broxmeyer HE, Lu L, Puatzer E, Feit C, Juliano L, Rubin BY (1983) Comparative analysis of the influences of human gamma, alpha, and beta interferons on human multipotential (CFU-GEMM), erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor cells. J Immunol 131:1300-1305.

Buchmeier MJ, Welse RM, Dukto FJ et al (1980) The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv Immunol 30:275-313.

Camitta B, O'Reilly RJ, Sensenbrenner L et al (1983) Antithoracic duct lymphocyte globulin therapy of severe aplastic anemia. Blood 62:883-888.

Carter WA, Johnson FH (1981) Induction and production of interferon with porcine, bovine, and equine leukocytes. Meth Enz 78:48-54.

Champling R, Ho W, Gale RP (1983) Antithymocyte globulin treatment in patients with aplastic anemia. N Engl J Med 308:113-118.

Cillo C, Sekaly RP, Magli MC, Odartchenko N (1985) Early hemopoietic progenitor cells: Direct measurement of cell cycle status. Ann NY Acad Sci 459:150-161.

Cockerell GL, Hoover EA, Krakowka S (1976) Lymphocyte mitogen reactivity and enumeration of circulating B- and T-cells during feline leukemia virus infection in the cat. J Natl Cancer Inst 57:1095-1099.

Cunningham AL, Merigan TC (1984) Leu-3+ T cells product γ-interferon in patients with recurrent Herpes labialis. J Immunol 132:197-202.

De Maeyer E, De Maeyer-Guignaro J, Hall WT, et al (1972) Induction of interferon synthesis by mammary tumor virus preparations in mice. In Recherches Fundamentals sur les Tumeurs Mammaires, 119-126, ed. by J. Mouriquand. Edition S Inserm.

Dunlap JE, Nichols WS, Hebebrand LC, Mathes LE, Olsen RG (1979) Mobility of lymphocyte surface membrane concanavalin A receptor of normal and feline leukemia virus-infected viremic felines. Cancer Res 39:956-958.

Engelman RW, Fulton RW, Good RA, Day NK (1985) Suppression of gamma interferon production by inactivated feline leukemia virus. Science 227:1368-1370.

Epstein LB (1984) The special significance of interferon-gamma. Interferon 2:185-220.

Gallicchio VS, Chen MG, Murphy MG (1982) Modulation of murine in vitro erythroid and granulopoietic colony formation by cuabain, digoxin, and theophylline. Expt Hematol 10:682-690.

Gascon P, Zoumbos NC, Scala G et al (1985) Lymphokine abnormalities in aplastic anemia: Implications for the mechanism of action of antithymocyte globulin. Blood 65:407-413.

Gasper PW, Hoover EA (1983) Feline leukemia virus strain specific abatement of erythroid burst forming units (BFU-E). Leuk Red Intl 1:71-72.

Glasgow LA, Friedman SB (1969) Interferon and host resistance to Rauscher virus-induced leukemia. J Virol 47:99-105.

Graf T, Beug H, Roussel M, Saule S, Stehelin D, Hayman MJ (1980) Avian leukemia viruses and hematopoietic cell differentiation. Brit J Cancer 41:659-661.

Greco B, Bielory L, Stephan D et al (1983) Antithymocyte globulin reacts with many normal human cell types. Blood 62:1047-1054.

Gresser I, Tovey MG, Maury C, et al (1976) Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. J Exp Med 144:1316-1323.

Hagberg H, Nilsson P, Nisell J (1980) Treatment of pure red cell anemia with antilymphocytic globulin. Scand J Haematol 24:360-364.

Harada M, Gale RP (1982) Evaluation of antithymocyte globulin for human bone marrow transplantation. Transplantation 33:625-630.

Hart CC, Murray HW (1984) Cellular immunity and the acquired immune deficiency syndrome: Failure to secrete lymphokines and gamma interferon. Surv Synth Path Res 3:397-408.

Herrmann F, Griffin JD, Meuer SG, et al (1986) Establishment of an interleukin 2-dependent T cell line derived from a patient with severe aplastic anemia, which inhibits in vitro hematopoiesis. J Immunol 136:1629-1634.

Heyworth MF (1981) Effects of antilymphocytic globulin in human subjects. Immunology 43:793-802.

Hooks JJ, Haynes BF, Detrick-Hooks B, et al (1982) Gamma (immune) interferon production by leukocytes from a patient with T_γ cell proliferative disease. Blood 59:198-201.

Hoover EA, Perryman LE, Kociba GJ (1973) Early lesions in cats inoculated with feline leukemia virus. Cancer Res 33:145-152.

Hoover EA, Kociba GJ, Hardy WD, Yohn DS (1974) Erythroid hypoplasia in cats inoculated with feline leukemia virus. J Natl Cancer Inst 53(5):1261-1265.

Hoover EA, Mathes LE, Rojko JL et al (1978) Modification of the immunofluorescence assay for feline leukemia virus group-specific antigen. Am J Vet Res 39:1877-1880.

Hoover EA, Kociba GJ, Olsen RG (1981) Experimental erythroid aplasia in cats induced by feline leukemia virus. Proceedings of the Conference on Aplastic Anemia: A stem cell disease, p 306. National Institutes of Health Publication No. 81-1008, June.

Hosoi T, Ozawa K, Urabe A, Takaku F (1985) Effects of recombinant interferons on the clonogenic growth of leukemic cells and normal hemopoietic progenitors. Int J Cell Cloning 3:304-312.

Hunter RF, Mold NG, Mitchell RG et al (1985) Differentiation of normal marrow and HL60 cells induced by antithymocyte globulin. Proc Natl Acad Sci USA 82:4823-4827.

Inglot AD, Oleszak E (1978) Application of antisera to interferon in studying oncogenic viruses. Arch Immunol Ther Exp 26: 529-536.

Jameson P, Essex M (1983) Inhibition of feline leukemia virus replication by human leukocyte interferon. Antiviral Res 3:115-120.

Jarrett O, Golder MC, Toth S, Onions DE, Stewart MF (1984) Interaction between feline leukaemia virus subgroups in the pathogenesis of erythroid hypoplasia. Int J Cancer 34:283-288.

Johnson HM, Dianzani F, Georgiades JA (1981) Large-scale induction and production of human and mouse immune interferons. Meth Enz 78:158-162.

Kantor HS, Hampton TN (1979) Indomethacin of submicromolar concentrations inhibits cyclic AMP-dependent protein kinase. Nature 276: 817-820.

Kirchner H (1986) The interferon system as an integral part of the defense system against infections. Antiviral Res 6:1-17.

Klimpel GR, Fleischmann WR, Klimpel KO (1982) Gamma interferon (IFN-γ) and IFN α/β suppress murine myeloid colony formation (CFU-C): Magnitude of suppression is dependent upon level of colony-stimulating factor (CSF). J Immunol 129:76-129.

Kociba GJ, Lange RD, Dunn CDR, Hoover EA (1983) Serum erythropoietin changes in cats with feline leukemia virus-induced erythroid aplasia. Vet Pathol 20:548-552.

Kociba GJ (1987) Lymphocyte coculture studies of retrovirus-induced feline erythroid aplasia. In preparation.

Lands AM, Arnold A, McAuliff JP, Luduena FP, Brolon TG (1967) Differentiation of receptor systems activated by sympathomimetic amines. Nature (Lond) 214:597-598.

Lewis MG Determination of a mechanism of feline retroviral suppression of the feline immune system. PhD Dissertation, Ohio State University, 1983, pp 30-49.

Lewis MG (1985) Reversal of feline retroviral suppression by indomethacin. Leuk Red 9:1451-1456.

Lewis MG, Fertel RH, Olsen RG (1985) The reversal of feline retroviral-induced suppression of lymphocyte Con A receptor mobility by indomethacin and PGE_2. Leuk Res 9:1105-1109.

Liu WT, Good RA, Trang LQ, et al (1984) Remission of leukemia and loss of feline leukemia virus in cats injected with Staphylococcus protein A associated with increased circulating interferon and complement-dependent cytotoxic antibody. Proc Natl Acad Sci 81:6471-6475.

Lobue J (1984) Viral-associated alterations in hematopoiesis in the mouse. Blood Cells 10:211-222.

Mamus SW, Beck-Schroeder S, Zanjani ED (1985) Suppression of normal human erythropoiesis by gamma interferon in vitro. J Clin Invest 75:1496-1503.

Mamus SW, Oken MM, Zanjani ED (1986) Suppression of normal human erythropoiesis by human recombinant DNA-produced alpha-α-interferon in vitro. Exp Hematol 14:1015-22.

Mangan KF, Shadduck RK (1984) Successful treatment of chronic refractory pure red cell aplasia with antithymocyte globulin: correlation with in vitro erythroid culture studies. Am J Hematol 17:417-426.

Mangan KF, Zidar B, Shadduck RK, Zeigler Z, Winkelstein A (1985) Interferon-induced aplasia: Evidence for T-cell-mediated suppression of hematopoiesis and recovery after treatment with horse antihuman thymocyte globulin. Am J Hematol 19:401-413.

Mangan KF, O'Allessandro L, Mullaney MT (1986) Action of antithymocyte globulin on normal human erythroid progenitor cell proliferation in vitro: Erythropoietic growth-enhancing factors are released from marrow accessory cells. J Lab Clin Med 107: 353-365.

Marmont A, Peschle C, Sanguineti M et al (1975) Response of three patients to cyclophosphamide and antilymphocyte globulin and demonstration of two types of serum IgG inhibitors to erythropoiesis. Blood 45:247.

Mathes LE, Yohn DS, Olsen RG (1977) Purification of infectious feline leukemia virus from large volumes of tissue culture fluids. J Clin Microbiol 5:372-374.

Mathes LE, Olsen RG, Hebebrand LC, Hoover EA, Schaller JP (1978) Abrogation of lymphocyte blastogenesis by a feline leukemia virus protein. Nature (Lond) 274:687-689.

Mazur EM, Richtsmeier WJ, South K (1986) Alpha-interferon: Differential suppression of colony growth from human erythroid, myeloid, and megakaryocytic hematopoietic progenitor cells. J Interferon Res 6:199-206.

Miller WJ, Branda RF, Flynn PJ et al (1983) Antithymocyte globulin treatment of severe aplastic anemia. Brit J Haematol 55:17-25.

Misiti J, Spivak JL (1979) Erythropoiesis in vitro. Role of calcium. J Clin Invest 64:1573-1579.

Mookerjee BK, Azzolina L, Poultar L (1974) Interaction of antithymocyte serum with hematopoietic stem cells. J Immunol 112: 822-829.

Moore JL, Poiesz BJ, Zamkoff KW, et al (1985) Inverse relationship between constitutive γ-interferon production and human T-cell lymphoma/leukemia virus expression in cultured T lymphocytes. J Virol 53:440-446.

Nagasawa T, Abe T, Hanada T (1982) Inhibitory effects of T-cells on in vitro granulopoiesis and immunoglobulin production in patients with aplastic anemia. Scand J Haematol 28:389-398.

Netzel B, Rodt H, Hoffmann-Fezer G et al (1978) The effect of crude and differently absorbed anti-human T-cell globulin on granulocytic and erythropoietic colony formation. Exp Hematol 6:410-420.

Neumann HA, Fauser AA (1982) Effect of interferon on pluripotent hemopoietic progenitors (CFU-GEMM) derived from human bone marrow. Exp Hematol 10:587-590.

Nickerson M, Collier B (1975) Drugs inhibiting adrenergic nerves and structures innervated by them. In The Pharmacological Basis of Therapeutics. LS Goodman, A Gilman, editors. MacMillan Publ Co Inc., NY, pp 533-546.

Olsen RG, Mathes LE, Tarr MJ, Blakeslee JR (1986) Oncogenic viruses of domestic animals. Vet Clin N Am:Sm Anim Pr 16:1129-1143.

Onions D, Jarrett O, Testa N, Frassoni F, Toth S (1982) Selective effect of feline leukaemia virus on early erythroid precursors. Nature 296:156-158.

Orosz CG, Zinn NE, Olsen RG, Mathes LE (1985) Retrovirus-mediated immunosuppression. I. FeLV-UV and specific FeLV proteins alter T lymphocyte behavior by inducing hyporesponsiveness to lymphokines. J Immunol 134:3396-3403.

Orosz CG, Zinn NE, Olsen RG, Mathes LE (1985) Retrovirus-mediated immunosuppression. II. FeLV-UV alters in vitro murine T lymphocyte behavior by reversibly impairing lymphokine secretion. J Immunol 135:584-590.

Ortega JA, Ma A, Shore NA, Dukes PP, Merigan TC (1979) Suppressive effect of interferon on erythroid cell proliferation. Exp Hematol 7:145-150.

Perryman LE, Hoover EA, Yohn DS (1972) Immunologic reactivity of the cat: Immunosuppression in experimental feline leukemia. J Natl Cancer Inst 49:1357-1365.

Prasad KN (1980) Butyric acid: A small fatty acid with diverse biological functions. Life Sci 37:1351-1358.

Preble OT, Friedman RM (1983) Biology of Disease: Interferon-induced alterations in cells: Relevance to viral and nonviral diseases. Lab Invest 49(1):4-18.

Preble OT, Rook AH, Quinnan GU et al (1984) Role of interferon in AIDS. Ann NY Acad Sci 437:65-75.

Reed PW, Laroy HA (1972) A23187: A divalent cation ionophore. J Biol Chem 247:6970.

Riedel N, Hoover EA, Gasper PW et al (1986) Molecular analysis and pathogenesis of the feline aplastic anemia retrovirus. feline leukemia virus C-sarma. J Virol 60:242-250.

Rojko JL, Hoover EA, Mathes LE, Schaller JP, Olsen RG (1979) Pathogenesis of experimental feline leukemia virus infection. J Natl Cancer Inst 63:759-778.

Rojko JL, Hoover EA, Finn Bl, Olsen RG (1981) Determinants of susceptibility and resistance to feline leukemia virus infection. II. Susceptibility of feline lymphocytes to productive feline leukemia virus infection. J Natl Cancer Inst 67:899-910.

Rojko JL, Olsen RG (1984) The immunobiology of the feline leukemia virus. Vet Immunol Immunopath 6:107-165.

Rozengurt E (1986) Early signals in the mitogenic response. Science 234:161-166.

Rubin BY, Gupta SL (1980) Differential efficacies of human Type I and Type II interferons as antiviral and antiproliferative agents. Proc Natl Acad Sci USA 77:5928-5931.

Sarma PS, Log T (1973) Subgroup classification of feline leukemia and sarcoma viruses by viral interference and neutralization tests. Virology 54:160-169.

Seamon KB, Daly JW (1981) Forskolin: A unique diterpene activator of cyclic AMP-generating systersm. J Cyc Nucl Res 7:201-224.

Sheppard H, Wiggan A (1971) Different sensitivities of the phosphodiesterases (adenosine-3',5'-cyclic phosphate 3',phosphohydrolase) of DDG cerebral cortex and erythrocytes to inhibition by synthetic agents and cold. Biochem Pharmacol 20:2128-2130.

Snyder DS, Desforges JF (1986) Lipoxygenase metabolites of arachidonic acid modulate hematopoiesis. Blood 67:1675-1679.

Socinski MA, Ershler WB, Tosato G (1984) Pure red blood cell aplasia associated with chronic Epstein-Barr virus infection: Evidence for T cell-mediated suppression of erythroid colony-forming units. J Lab Clin Med 104:995-1006.

Stewart WE. The Interferon System. Springer-Verlag, NY. 1979, p. 30.

Tambourin PE, Wendling F, Jasmin C, Smadja-Joffe F (1979) The physiopathology of Friend leukemia. Leuk Res 3:117-129.

Templeton JG, Cocker JE, Crawford PJ (1985) Fcy-receptor blocking antibodies in hyperimmune and normal pooled gammaglobulin. Lancet i:1337.

Testa NG, Onions D, Jarrett O, et al (1983) Haemopoietic colony formation (BFU-E, GM-CFC) during the development of pure red cell hypoplasia induced in the cat by feline leukaemia virus. Leuk Res 7:103-116.

Thomas JM, Williams AJ, Thomas FT et al (1978) T-cell-specific activity in rabbit anti-human thymocyte globulin. Transplantation 25:97-102.

Tompkins MB, Cummins JM (1982) Response of feline leukemia virus-induced nonregenerative anemia to oral administration of an interferon-containing preparation. Feline Pract 12(3):6-15.

Torok-Storb B, Doney K, Sale G et al (1985) Subsets of patients with aplastic anemia identified by flow microfluorometry. N Engl J Med 312:1015-1022.

Tosato G, Magrath I, Koski I, Dooley N, Blaese M (1979) Activation of suppressor T cells during Epstein-Barr virus-induced infectious mononucleosis. N Engl J Med 301:1133-1137.

Vegh P, Erdos E, Janossy T et al (1978) Correlation of the in vivo and in vitro activities of antithymocyte sera (ATS) with the immunizing antigen dose. Transplantation 25:324-327.

Weiser MG, Kociba GJ (1983) Erythrocyte macrocytosis in feline leukemia virus associated anemia. Vet Pathol 20:687-697.

Wellman ML, Kociba GJ, Lewis MG, Mathes LE, Olsen RG (1984) Inhibition of erythroid colony-forming cells by a M_r 15,000 protein of feline leukemia virus. Cancer Res 44:1527-1529.

Wellman ML, Kociba GJ (1987) Characterization of normal feline bone marrow fibroblast colony forming units. Am J Vet Res, In press.

Welsh RM, Doe WF (1980) Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice: Natural killer cell activity in cultured spleen leukocytes concomittant with T-cell dependent immune interferon production. Infect Immun 30:473-483.

Welsh RM, Biron CA, Bukowski JF et al (1984) Role of natural killer cells in virus infection of mice. Surv Synth Path Res 3:409-431.

Whitfield JF, Boynton AL, MacManus JP, Rixon RH, Sikorska M, Tsang B, Walter PR, Swierenga S (1980) The roles of calcium and cyclic AMp in cell proliferation. Ann NY Acad Sci 339:216.

Young N, Mortimer P (1984) Viruses and bone marrow failure. Blood 63:729-737.

Zack PM, Kociba GJ (1987) Antithymocyte globulin treatment of retrovirus-induced feline erythroid aplasia: In vivo and in vitro studies. Submitted for publication.

Zack, PM, Kociba GJ (1987) Antithymocyte globulin treatment of retrovirus-induced feline erythroid aplasia: In vivo and in vitro studies. Submitted for publication.

Zoumbos NC, Djell JY, Young NS (1984) Interferon is the suppressor of hematopoiesis generated by stimulated lymphocytes in vitro. J Immunol 33:769-774.

Zoumbos NC, Gascon P, Djell JY et al (1985) Circulating activated suppressor T lymphocytes in aplastic anemia. N Engl J Med 312:257-265.

Zoumbos NC, Gascon P, Djell JY, Young NS (1985) Interferon is a mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo. Proc Natl Acad Sci 82:188-192.