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The Ohio State University, Ph.D., 1977
Microbiology

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LEUKEMIA VIRUS AND FELINE LEUKEMIA VIRUS PROTEINS:
A PUTATIVE MODEL FOR IMMUNOSUPPRESSION

DISSERTATION

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

By
Lawrence Eaton Mathes, B.S., M.S.

* * * * *

The Ohio State University
1977

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Major Field: Virology-Immunology

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Introduction

The virus-host relationship of feline leukemia virus (FeLV) is complex. Susceptibility of cats to disease is dependent upon the age of cat at time of FeLV exposure (44), virulence of the virus inoculum (21), route of virus inoculation (21), and strain of virus (21, 24, 43, 44). Moreover, other as yet undefined parameters must also be involved, in that even when all known factors are held constant, a significant variation in susceptibility among different cats persists.

The age of exposure to FeLV appears to be a critical factor in determining susceptibility. Newborn cats may have 100% incidence of tumor formation following an oncogenic challenge with the appropriate dose of virus. Susceptibility changes drastically, however, between two and four months of age. The FeLV challenge dose inducing 100% incidence of tumor in newborns will produce only a 10% or less incidence in four-month-old cats (43).

The principle neoplasm of cats infected with FeLV is a form of leukemia called lymphoma or lymphosarcoma, although other forms of leukemia have been described (54,57). Along with the leukemias, another group of disorders called FeLV-related disease are often found in cats which are FeLV viremic. These diseases, in many cases, are more acute and may cause death prior to onset of leukemia symptoms. The FeLV-related
diseases include: non-regenerative anemia (41,66), infectious peritonitis (38), glomerulonephritis (2), hemobartonellosis (22), and wasting syndrome (3,42).

The correlation of these diseases syndromes with FeLV infection, of course, are not absolute, i.e., cats may have the FeLV-related disease without being infected by FeLV. However, the correlation is much higher than would be expected by random coincidence and is probably a result of a preleukemic condition of immunosuppression characteristically found in FeLV-infected animals (13,81). This condition allows infections to develop to which the host would normally be resistant. The magnitude of immunosuppression in the preleukemic stage of FeLV infections may also be a deciding factor in determining the length of latency of the leukemia and perhaps even the ultimate course of the disease to remission or progression.

The nature of immunosuppression associated with FeLV infections has yet to be completely elucidated. T-lymphocyte functions appear to be altered as demonstrated by prolonged allograph rejection time in leukemic cats (81). Exactly which T-cell functions are being affected, however, are completely unknown.

The present study was undertaken to examine parameters of infection and resistance which may determine susceptibility of cats to FeLV. It was felt that the key to understanding FeLV susceptibility may lie with understanding the mechanism of immunosuppression. Experiments were designed to investigate this process using both in vitro and in vivo methods.

Several recent reports (78,114) provided the basis for the experimental design. Those studies indicated that abrogation of immune functions
occurred in the absence of infection by inoculation of inactivated or attenuated retroviruses. In one study, mice that were inoculated with attenuated murine sarcoma virus (MuSV) and later challenged with infectious MuSV reportedly had higher incidences of malignant disease than control mice that were challenged but not preinoculated with attenuated virus (114).

A similar study in cats immunized with ultraviolet light (U.V.) inactivated FeLV resulted in abrogated tumor immunity and increased tumor incidence in animals that were later challenged with feline sarcoma virus (FeSV) (78).

In two independent studies using in vitro techniques, soluble extracts of MuLV-induced tumor (5,16) were shown to exert suppressive effects on the induction of lymphocyte blast transformation (LBT). Thus, a significant amount of evidence has accumulated which implicates the viral particle itself or a subviral component of the particle as the substance responsible for abrogation of immune function.

The specific objectives of this study, then, were: i) to identify, purify and to characterize the substance responsible for immune abrogation and ii) to examine possible mechanisms by which such immune abrogations might function.
Literature Review

To explore the immunosuppressive properties of feline leukemia virus (FeLV), an understanding of the feline retrovirus system and the complex host-virus relationship is necessary. The following review is intended to provide basic information about the general properties of mammalian retroviruses and the specific characteristics of the feline viruses. The most extensively studied retroviruses, by far, are those derived from mice. Where necessary, the murine leukemia-sarcoma virus (MuLV/ MuSV) group will be substituted for FeLV to provide data which may apply to FeLV but has not yet been experimentally substantiated.

History

Feline leukemia virus (FeLV) was first described as the etiologic agent for spontaneous neoplasm in cats by Jarrett et al. (55,58). In these studies, cats that were inoculated with cell-free supernatants of feline lymphosarcoma tumor homogenates subsequently developed lymphosarcoma (LSA). Since the initial observation, a number of other investigators isolated similar agents associated with cat lymphosarcomas as well as fibrosarcomas (30,61,82,98,99,103). In these studies, the oncogenicity of FeLV was confirmed by carefully designed transmission studies.
Taxonomic Classification

FeLV and feline sarcoma virus (FeSV) are taxonomically classified as retroviruses belonging to the family of retroviridae (27). Retroviruses, also called Type-C RNA viruses, RNA tumor viruses, oncornaviruses and leukoviruses, comprise a large group of physically and chemically similar viruses isolated from reptilian (31,113), avian (20,86) and mammalian (19,33,37) including primate (59,60) species.

Diseases Associated with FeLV/FeSV Infections

Lymphosarcoma or lymphoma (LSA) is the most common neoplasm associated with FeLV infections and, in fact, is the most frequently diagnosed tumor of cats (17,96). The incidence of LSA is elevated in two age groups, one at 2 years and one in older cats (7-8 years) (54).

Spontaneous fibrosarcoma, a neoplasm associated with FeSV, is found much less commonly than lymphosarcoma and occurs primarily in adult or aged animals (73).

The age of cats at the time of exposure to FeLV or FeSV, under experimental conditions, has a substantial effect on the outcome of the infection. Newborn kittens have a high incidence of LSA after FeLV infection while adult cats generally develop resistance (43,44).

The latency period before onset of clinical symptoms varies with viral strains (21) but is generally 6 months to 1 year or longer for FeLV (58) and 3 to 11 weeks for FeSV (98).

Between 2 and 4 weeks following inoculation of FeLV, FeLV viremia develops (infectious virus in plasma). The method described by Hardy et al. (39) to measure FeLV viremia uses indirect immunofluorescence (IFA)
to detect viral antigen in peripheral blood leukocytes (PBL). A comparison of viremia as measured by an *in vitro* focus forming infectivity assay with the IFA procedure demonstrated over 90% concordance making the IFA test a reliable indicator of FeLV viremia.

FeLV viremia persists throughout the latency period (preleukemic stage) with apparently no deleterious effects to the host. Naturally occurring FeLV viremia may persist for years in what appear to be healthy normal cats without any signs of disease. These cats are thought to represent carrier states in the natural population and are probably continually shedding virus (via saliva, urine, feces, etc.) and infecting other cats while remaining symptom-free. The ultimate fate of these cats, however, is unknown. Similar conditions in experimentally infected cats indicate that, though the latency period is greatly extended, all viremic cats eventually develop some kind of FeLV associated disease (unpublished data from our laboratory).

Viremia also occurs in FeSV infected cats. It has been shown that when some cats regress FeSV-induced fibrosarcomas, as has happened under experimental conditions, a state of persistent viremia (FeLV continuously present in blood) may also develop (77).

Other disorders associated with FeLV infection are: feline infectious peritonitis (38), nonregenerative anemia (41,66), glomerulonephritis (2), hemobartonellosis (22), and wasting syndrome (3,42).

**In Vitro Infectivity Characteristics**

FeLV has a wide *in vitro* host range, compared to other mammalian retroviruses, being able to infect cat (49,104), dog (51), hamster (89),
pig (51), ox (62), and human (50, 89) cells. Rat and mouse cells, however, appear to be resistant (21). FeSV has a similar host range.

FeLV is able to infect and replicate, but does not visually transform cat cells in vitro (90). FeSV, however, is genetically defective in its ability to replicate in vitro (88). Only when FeLV is present to provide the missing genetic information is FeSV able to replicate. FeSV, with helper FeLV, will infect and in addition cause transformation of cells grown in vitro (90). Because FeSV has a one-hit kinetic pattern of infection, excess FeLV appears to be present in FeSV pools (88).

The method for assaying FeLV utilized feline embryo cells which were infected with MuSV (28). The infected cells were cloned in order to isolate cells infected by MuSV but free of MuLV. These sarcoma-positive, leukemia-negative (S+L-) cells appeared normal and were non-virus producing. The addition of FeLV helper, however, initiated cell transformation and virus replication. Each S+L- cell that became FeLV infected produced a focus of transformed cells and, thus, provided a means of enumerating infectious units of FeLV present. In this system, FeLV provided the genetic helper activity for MuSV which subsequently initiated transformation.

Physical Characteristics

The physical characteristics of FeLV/FeSV are similar to those of other retroviruses. They are enveloped particles of 90 to 115 nm diameter containing 3 or 4 pieces of single-stranded 70S RNA (53). The diameter of the virus increases to 110-140 nm when the delicately attached surface spike structures are included (21). Packaged with the
viral RNA is the reverse transcriptase (RT) or RNA-dependent DNA polymerase enzyme (100) required in the replication cycle of the virus.

The structural protein make-up of FeLV-FeSV was examined by a number of investigators (34, 35, 48, 74, 92). The virus has an envelope structure composed of at least one glycoprotein of 70,000 daltons molecular weight (GP70) and perhaps a second of approximately 100,000 daltons (34). The core structure was reported to have four proteins, with molecular weights of 27-30,000 (P27), 15,000 (P15), 12,000 (P12) and 10,000 (P10) daltons (34, 35, 74), and possibly a fifth protein with molecular weight of 21,000 daltons (P21) (34).

Murine retroviruses, as do other mammalian retroviruses, have protein make-ups similar to FeLV (GP70, P30, P15, P12, and P10) (14). Recent reports by Ikeda et al. (47) and Ihle et al. (46) have described two P15 proteins in MuLV. One is associated with the envelope of the virus and is designated P15(E). The other is a core component designated P15(C). Similar findings have not yet been reported for FeLV/FeSV.

**Antigenic Characteristics**

Feline retroviruses, as do other retroviruses, have interspecies, species, and type antigenic specificities. Interspecies antigens are those that cross react among all viruses from animal species of a given taxonomic class but not with viruses from animal species of a different class. Thus, all mammalian retroviruses have a common interspecies antigenic determinant as do all avian retroviruses. Avian and mammalian retroviruses interspecies antigen, however, do not have antigenic cross-reactivity. Type specific antigens are unique to a given strain of
virus. These are antigenic differences that exist within a virus group from a given species.

Proteins from retroviruses have a spectrum of interspecies, species and type specificities. The GP70 protein of MuLV has primarily type but also species and interspecies reactivity (102). A similar antigenic pattern may be true of the FeLV GP70. Three type specific antigens (A, B, and C) have been characterized for FeLV/FeSV based on viral interference and neutralization patterns (91).

The P27 protein of FeLV has strong interspecies and species specific antigenic determinants (32,35,93). P15, P12, and P10 of FeLV reportedly have primarily species specific antigenic activity (35,93). However, the finding of strong interspecies antigenic cross-reactivity associated with the P15(E) and P10 proteins of MuLV (4,92) may lead to a similar finding with the feline virus. The reverse transcriptase enzyme protein reportedly has interspecies and species specific antigenic reactivity (1).

**Tumor Specific Antigens**

An apparent tumor specific antigen, associated with FeLV/FeSV infections has been reported (23). The antigen(s), designated feline oncornaivus-associated cell membrane antigen (FOCMA) (23), appears to be transformation specific in that it is detected only on FeLV/FeSV infected cells that have become neoplastic (25). FeLV infected but non-transformed cells reportedly do not express FOCMA (25).

The test for measuring FOCMA antibody was first described by Essex et al. (23) and uses indirect membrane immunofluorescence (IMI) to
detect the immune reaction between cat antibody and a membrane tumor cell antigen. FOCMA is probably composed of a mixture of viral specific as well as tumor cell antigens although this point is controversial at present (83, 21).

**Immunologic Response to FeLV Infection**

Patterns of serologic responsiveness have been well characterized in FeLV-infected cats. Cats may respond to viral infection by producing viral neutralizing antibody (52), and antibody specific for core proteins (11,12,80) and FOCMA (23).

Viral neutralizing antibody can be detected in adult cats which developed immunity after exposure to FeLV (43). The level of FOCMA antibody also appears to correlate with the disease pattern. A direct relationship appears to exist between the presence of high titer FOCMA antibody in the cat and resistance to FeLV infection (21). The presence of antibody to core proteins, however, does not appear to correlate with resistance or susceptibility to FeLV infection (12).

Immunologic resistance to FeLV infection appears to rely at least partially on humoral factors. This finding was evident in studies where nursing dams having high FOCMA and serum neutralizing (SN) antibody titers were able to transmit via colostrum antibody to the kitten. The kittens were subsequently found to be resistant to FeLV infection (45).

Undoubtedly, there are cellular immune functions involved in resistance to FeLV. These functions, however, have not been thoroughly explored.
Immunosuppression Associated with Retrovirus Infection

Studies by many investigators have demonstrated that infection with murine leukemia virus leads to alterations in immunologic response to various antigens (15). The most carefully studied system has been the production of antibody to sheep red blood cells in Freund virus infected mice (75,76,87). These mice show a decreased ability to form hemagglutinating and hemolytic antibody. Both the primary and secondary immune responses seem to be impaired. Analysis of immunoglobulin types indicated that the 7S responses are more inhibited than the 19S response using both the criteria of 2-mercaptoethanol sensitivity of the serum antibody and the direct and indirect Jerne plaque technique (6,7,8,9,10, 106). Similar studies were also performed in the cat. FeLV-infected cats reportedly had longer allograft rejection time than age-matched non-infected controls (81). In the same study, however, both FeLV-infected and control cats had equal primary antibody responses (IgM) to sheep red blood cells. Thus, it appeared that T- but not B-cell immune functions were impaired by FeLV infection.

In an in vitro study, lymphocytes from normal and FeLV-infected cats were measured for phytomitogen-induced blast transformation (13). The FeLV infected cats had sharply reduced Con A stimulation levels. At the same time, the number of circulating T-cells increased (13).

The immunosuppression associated with FeLV infections is believed to be one of the primary factors which determine the outcome of FeLV infections (21,26). The lag period between exposure to FeLV and development of adequate immunologic protection in some instances was apparently enough time for the virus infection to become well entrenched causing
immunologic unresponsiveness. Attempts to shorten the lag period by inoculating young cats with inactivated FeLV were not successful (95,112). Young cats as opposed to adults produced weak neutralizing antibody to FeLV.

In other studies, young cats inoculated with FOCHA containing killed tumor cells were resistant to FeSV-induced fibrosarcoma (56,77,78) but not FeLV/FeSV viremia (77,78). At the same time, cats inoculated simultaneously with killed tumor cells and inactivated FeLV were not protected from FeSV challenge (78). The present of FeLV in these inocula appeared to cause abrogation of immunity (78,95).
Materials and Methods

**Cats**

All cats used in vaccination experiments and as blood donors for the lymphocyte blast transformation assay were taken from The Ohio State University, Department of Veterinary Pathobiology specific-pathogen-free (SPF) cat colony (85). Cats challenged with oncogenic virus were housed in special isolation quarters. Entry to these areas was restricted and personnel were required to wear protective clothing which did not leave the area.

**Serum and Blood Smear**

Blood was taken from cats aseptically by jugular vein puncture and allowed to clot. Serum was collected and stored at -90°C. Blood smears for the FeLV-viremia test were made at the time of bleeding. The blood for lymphocyte isolation was mixed with heparin to prevent clotting.

**Lymphocyte Isolation**

Lymphocytes were isolated from heparinized blood by centrifugation through a Ficoll-Hypaque gradient (Ficoll, Sigma Chemical Co., St. Louis, MO; Hypaque, Winthrop Laboratories, New York, NY) (14). Eight ml of blood was brought to a volume of 28 ml with Eagles' minimum essential medium for suspension cultures (MEM-s) (GIBCO, Grand Island, NY) supplemented
with 20% heat inactivated (56°C for 30 minutes) fetal calf serum (FCS) (K.C. Biological, Lenexa, KS), 2% NaHCO₃ (1M) and 1% penicillin-streptomycin solution (2 x 10⁶ units/ml penicillin and 1 g/100 ml streptomycin. Ten ml of the Ficoll-Hypaque mixture (24 parts 9.5 g/100 ml Ficoll plus 10 parts 33.9% Hypaque to yield a solution of 1.080 specific gravity) was slowly layered beneath the blood using a syringe and 6-inch, 18-gauge needle. The tubes were then centrifuged at 400 x g for 40 minutes in a Sorvall GLC-2 centrifuge (Ivan Sorvall Inc., Norwalk, CT). Lymphocytes which formed a layer at the medium/Ficoll-Hypaque interface were collected by removing first the medium then the cells. The harvested lymphocytes were washed twice by centrifugation (200 x g for 10 minutes) with Hank's balanced salt solution (HBSS) then resuspended in a small volume of complete medium (MEM-s, 20% FCS, 2% 1M NaHCO₃, and 1% penicillin-streptomycin solution). Total white blood cell counts were done with a Coulter counter model ZBI (Coulter Elect., Hialeah, FL). Leukocyte differentials were determined by counting Wright stain smears of the cell mixtures. The final cell concentration of leukocytes was adjusted to 1 x 10⁶ lymphocytes/ml in complete medium.

**Lymphocyte Blast Transformation (LBT) Assay**

The LBT assay was a modification of the technique described by Cockerell (14). Briefly, 0.1 ml of lymphocyte cell suspension (1 x 10⁶ cell/ml) was mixed with 0.05 mls of an optimal concentration of Concana-valin A (Con A) (10 ug/ml) (Sigma Chemical Co., St. Louis, MO) in Microtest plates (Falcon Plastics, Oxnard, CA) then incubated at 37°C for 5 days. For Con A-LBT inhibition assays, 0.05 mls of the protein being assayed was also added. The protein preparations assayed for LBT
inhibition were previously dialized against 3 changes of 400 volumes of complete medium. In the Con A control wells, the protein to be assayed for Con A-LBT inhibition was replaced with 0.05 ml complete medium. In cell control wells, both Con A and inhibitory proteins were replaced with complete medium. During the final 18 hrs of incubation, 0.1 ml of medium containing 0.5 μCi of (3H) thymidine (3H-TdR) (6.7 Ci/m mole New England Nuclear Corp., Boston, MA) was added. Cells were collected on glass filter paper with a semiautomatic multiple processor (Otto Heller Co., Madison, WI) and assayed for radioactivity using a Packard liquid scintillation counter (Packard Instruments Co., Downers Grove, IL). Net counts per minute (CPM) of quadruplicate wells were averaged to obtain mean cpm. The cocktail used in the liquid scintillation counting was Permablend II (Packard Inst. Co.) in toluene (5 g per liter).

**Tissue Culture**

FL-74 cells (provided by Dr. M. Essex, Harvard University) used as target cells in the IM1 and MCT tests were grown in static suspension cultures in 40 mls of McCoy's 5A media (GIBCO, Grand Island, NY) containing 15% fetal calf serum (Reheis Chemical Co., Phoenix, AX) and 1% penicillin-streptomycin solution in 75 cm² plastic flasks (Corning Glass Works, Corning, NY). Cultures were transferred at a ratio of 1 to 4 every 4 or 5 days.

FL-74 cells to be used as vaccine were grown in suspension cultures in 2 liter roller bottles as previously described (69,79). Cells were seeded at a concentration of 1.6-1.8 x 10⁶ cells/ml for a total of 200 ml/bottle. Three days after transfer, the cultures were fed with 200 ml
of fresh McCoy's 5A medium containing 15% FCS plus 1% penicillin-streptomycin solution and at 5 days cells were harvested by centrifugation at 400 x g for 10 min in an International PR 6 centrifuge (International Equipment Co., Needham Heights, MD). Cell counts obtained by this procedure ranged from 7 to 13 x 10^8 cells/bottle.

FL-74 cells and F422 cells (F422 cell line provided by Dr. de Noronha, Cornell University) used for virus production (KT-FeLV and R-FeLV, respectively) were grown in roller bottles as described above.

FeLV Virus Purification

The procedure for purifying FeLV was developed as a part of this study. A detailed report is located in Appendix D (69). Briefly, tissue culture fluids were taken from FL-74 or F422 cell cultures, usually a total of 10 liters from 24 roller bottles and were concentrated by continuous flow molecular filtration (Millipore, Bedford, MA) between 10 and 100 X using a membrane with exclusion range of 1 x 10^6 daltons. Twenty ml of the concentrate was layered onto a preformed 0 to 50% (w/w) linear sucrose gradient (68 ml) in 1 x 3 in cellulose nitrate tubes. Gradients were formed using an ISCO Gradient Form Model 570 (ISCO, Lincoln, NE). Sucrose (Schwartz Mann, Orangeburg, NY) was dissolved in TNE buffer (0.10 M Tris, 0.10 M NaCl, 0.001 M EDTA pH 7.2). The tubes were centrifuged at 57,000 g for 2 hr in a fixed angle Type 21 rotor (Beckman Inst., Palo Alto, CA) using a Beckman L2 65-B ultracentrifuge. The gradients were harvested by bottom puncture and collected in approximately 2 ml fractions. The virus formed an opaque band at between 35 and 40% sucrose. The fractions containing virus were pooled and dialized again in TNE
buffer overnight to remove sucrose. If desired, further purification of
the virus was accomplished by a second sucrose gradient centrifugation
followed the same procedure described above.

**Murine Leukemia Virus**

Murine leukemia virus was provided by the Resources and Logistics
Branch of the Viral Oncology Division of Cancer Cause and Prevention of
NCI, NIH through Pfizer Incorporated (Pfizer, Inc., Maywood, NJ).

**Virus Particle Counts**

The purified virus preparations were mixed with an equal volume of
latex bead (0.109 μ diameter) at a concentration of $1.38 \times 10^{13}/\text{ml}$ and
applied to a copper grid. The grids were stained with phosphotungstic
acid in distilled $\text{H}_2\text{O}$ pH 6.8 and immediately examined with a Phillips
Total virus particle concentration was determined by counting both la-
tex and virus particles per examination field until the latex particle
counts accumulated to 100. This procedure was repeated 5 times from
which the average virus particle count per 100 latex particle could be
determined. The average virus particle count was then multiplied by $1 \times 10^{11}$ to give the original virus particle count/ml.

**SDS-Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis was performed in 12 cm cylindri-
cal gels using 7.5% acrylamide (Bio Rad Laboratories, Richmond, CA) with
5% bisacrylamide (Bio Rad Laboratories) in a Tris acetate buffer con-
taining $0.205 \text{ M Tris}$, $0.205 \text{ M acetic acid}$, $0.1\%$ SDS, pH 6.6 using a Bio
Rad PAGE electrophoresis unit, Model 150A. Protein samples were mixed with equal volumes of a 1:10 dilution of Tris acetate buffer containing 0.1% SDS and 0.1 M dithiothreitol and heated to 100° C for 3 min prior to electrophoresis. Gels were electrophoresed for 5 hrs at 8mA/gel using constant current. Following electrophoresis, gels to be stained were fixed separately in 250 ml of 10% acetic acid and 40% isopropyl alcohol in H2O overnight. Gels were stained separately for 2 hr in 20 ml of freshly prepared 0.25% coomassie blue in 10% acetic acid and 40% isopropyl alcohol. Destaining was carried out over a 48 hr period in 7% acetic acid and 5% isopropyl alcohol. Densitometer scans of stained gels were done at 650 nm on a Gilford 250 spectrophotometer equipped with a Model 2520 gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, OH). Gels to be sliced were sectioned into 1.2 mm slices using a gel slicer (Specialized Medical Instruments, Baltimore, MD).

With each group of gels that were electrophoresed (usually 12), 2 protein standards were included. The first was FeLV and the second was a mixture of proteins of known molecular weight: phosphotase, 98,000 daltons; bovine serum albumin, 65,000 daltons; ovalbumin, 43,000 daltons; chymotrypsinogen, 25,000 daltons; and ribonuclease, 13,700 daltons.

Microcytotoxicity Assay

The microcytotoxicity assay was developed specifically for this study to measure complement-dependent, antibody-mediated, cell lysis. Details concerning the assay are located in Appendix E (68). Briefly, two-fold serial dilutions of cat sera (25 µl) were performed in "U" bottom Microtiter Plates (Cooke Engineering, Alexandria, VA) with 25 µl
Cooke microdiluters using veronal buffer (VB) as diluent (formula for VB located in Appendix C). Added to the test serum were 50 µl of rabbit complement (diluted 1:2) and 25 µl of cell suspensions containing 8 x 10^5 FL-74 cells. FL-74 cells were prepared from a 3-day-old culture and washed once with VB. The plates were incubated at 37°C for 1 hour with periodic agitation. At the end of the incubation period, the plates were centrifuged at 600 x g for 5 minutes in an international PR-6 centrifuge with 276 head (International Equipment Co., Needham Heights, MA) and carrier bucket (Cooke Engineering, Alexandria, VA). The MCT titer was taken as the reciprocal of the highest dilution of serum that produced at least 50% lysis over controls (scored visually) of target cells.

The controls in the MCT are target cells incubated with: i) complement alone and ii) test serum alone under MCT conditions.

**Indirect Membrane Immunofluorescence (IMI) Test for FOCMA**

The IMI test for FOCMA antibody was developed by Essex (23). In this procedure, live FL-74 cells grown in suspensions from 3- to 5-day-old cultures were used as target cells. The cells were washed twice with HBSS and resuspended at a concentration of 1 x 10^6 cell/ml. Sera was 2-fold diluted in microtiter plate using 50 ul diluters. Fifty µl of cell suspension was added to each well and the plates were incubated for 30 minutes at 37°C. Next, the plates were centrifuged and the cell pellet washed twice with HBSS.

Fifty µl of fluorescent isothiocyanate (FITC) conjugated rabbit antiserum to cat gamma globulin (Miles Laboratories Inc., Elkhart, IL) was then added to each well followed by incubation as before. Finally,
the cells were washed twice, as before, and read for membrane fluorescence using a Zeiss Universal fluorescence microscope (Carl Zeiss Inc., New York, NY). Cell samples for microscopic examination were prepared by transferring one drop of the cell suspension from each well of the microtiter plate on a microscope slide, then covering the sample with a cover slip. The end point titer was the last dilution of serum for which membrane fluorescence could be detected.

**Test for FeLV Viremia**

The test for FeLV viremia was developed by Hardy et al. (39). In this procedure, methanol fixed blood smears on microscope slides were tested for the presence of FeLV antigen in peripheral blood leukocyte using indirect immunofluorescence. The primary reagent was hyperimmune goat anti-FeLV serum which had been repeatedly absorbed with normal cat blood until no antibody reactivity could be detected with normal cat leukocytes. The secondary reagent was FITC conjugated rabbit anti-goat gamma globulin (Miles Laboratories, Inc.). The primary reagent was incubated on the blood smear for 1 hr at 37°C in a humidified chamber. The slides were then washed twice for 5 minutes in phosphate buffered saline (PBS) pH 7.2, and dipped in H₂O before drying. The secondary reagent was incubated with the blood smear for an additional hour.

The smears were washed as before then counter-stained for 7 minutes with 0.5% Evans Blue in H₂O. Excess Evans blue was removed by five minute washes in PBS and H₂O. The slides were then dried and a cover slip mounted with 50% glycerin in PBS. Cats were considered to be FeLV viremic if positive fluorescence was detected in any of the blood leukocyte.
**Serum Neutralization Assays**

Serum neutralization (SN) assays were performed by the procedure of Schaller et al. (94). Two-fold dilution of serum (cat or goat) in one ml volumes were made in test tubes using McCoy's 5A growth medium containing 5% FCS and 1% penicillin-streptomycin solution. The dilution was then inactivated at 56°C for 30 minutes to destroy complement. Two-tenth ml/well of each serum dilution was added to three wells of sterile flat bottom microtiter plates (Falcon Plastic, Oxnard, CA) leaving one set of wells for a cell control and another set for virus plus cell control. Twenty-five µl of FeLV suspension was added to each well except the cell controls. The FeLV virus suspension was adjusted so that 8 infectious foci would be produced per well as determined by previous titration.

The plates containing FeLV and serum were incubated at 4°C for 1 hr before the cell suspension was added. Three-day-old 81C cells (sarcoma positive, leukemia negative)feline embryo cell line (81C cell line was provided by Dr. P. Fischinger, NIH) were released from surface attachment by trypsinization. The cells were suspended in McCoy's 5A growth medium and washed twice by centrifugation using McCoy's 5A growth medium. The final cell pellet was resuspended in 0.001% DEAE-Dextran (Sigma, St. Louis, MO) in McCoy's growth medium and allowed to stand for 30 min at room temperature. Excess DEAE-Dextran was then removed by 2 x washing using the centrifugation procedure described above. The final cell suspension was adjusted to 5 x 10⁶ cell/ml in McCoy's 5A growth medium and added in 0.1 ml volumes to each well. The plates were subsequently incubated at 37°C in CO₂ incubator (National Appliance Co., Portland, OR)
for seven days. At day 3 and 5 post-infection, the medium was changed. At day 7, the cells were fixed with 10% formalin for 3 hrs, dried, and stained with 2% Giemsa for 45 min. Foci were scored microscopically. The serum neutralizing end point titer was the dilution at which there was a 50% or greater reduction in foci counts.

**Chloramine T Radio-iodination**

The chloramine-T iodination procedure was that of Greenwood et al. (36). By this procedure, proteins to be separated by column chromatography were radiiodinated to facilitate their recovery from column fractions. Ten microliters of protein were mixed with 20 µl of phosphate buffered saline (PBS), pH 7.6 and 1 mCi of carrier-free radioactive iodine (125I or 131I) (New England Nuclear, Billerica, MA). The reaction was started by the addition of 10 µl of chloramine T (Eastman Kodak, Co., Rochester, NY) solution (1 mg/ml) and allowed to go for 1 minute. The reaction was stopped by the addition of 25 µl of sodium metabisulfite (Allied Chemical Co., Morristown, NJ) solution (1 mg/ml). Unbound iodine, chloramine T and sodium metabisulfite were removed from the protein mixture by liquid column chromatography. The radioactive mixture was applied to the top of a 1 x 5 cm column packed with Bio Gel P10 (Bio Rad Laboratories, Richmond, CA) and equilibrated with PBS. Four drop fractions were collected. Iodinated protein was found in the first of two radioactive peaks to elute.

**Lactoperoxidase Radio-iodination**

The lactoperoxidase iodination procedure was described by Marchalonis (67) and Witte (107). This technique was used as a gentle method of
iodinating the surface components of intact viruses. In the procedure, 100 μCi of iodine 125 was added to 400 μl of virus suspension containing 45 μg of lactoperoxidase (Sigma, St. Louis, MO). To the mixture was added 40 μl of 10 μM H2O2, in 4 doses, added over a period of 30 minutes at 10 minute intervals. Unbound iodine as well as lactoperoxidase and H2O2 were separated from radioiodinated virus by sucrose density gradient centrifugation as described in the virus purification section of Materials and Methods.

**Ultraviolet Light Inactivation of FeLV**

Ultraviolet light (UV) inactivation of FeLV was accomplished by exposing the solution of virus, spread to a thickness of 1 mm in petri dishes, to a surface dose of 150 ergs/sq mm/sec of UV for an accumulated total of 35,000 ergs/sq mm (111). The petri dishes were laying atop an ice tray that was attached to a rotary table. The virus solution was agitated throughout the inactivation procedure by the circular motion of the rotary table (Belco Glass Co., Vineland, NJ).

**FL-74 Cell Inactivation**

FL-74 cells to be used in the vaccine protocol were heat killed as described by Heding et al. (40). The cells were centrifuged into a cell pellet and the medium removed. The cell pellet was heated to 56°C in a water bath for 4 minutes with constant agitation. This procedure caused cell death with minimal cell lysis while FOCMA antigenicity was retained.
Vaccine Preparation

Two vaccines were used in this study. One was killed FL-74 cells prepared as described by Olsen et al. (77) and the other was killed FL-74 cell plus the FeLV P15 protein (purification of FeLV P15 described in Results). Both inocula were emulsified with complete Freund's adjuvant (CFA) just prior to inoculation. Control animals were inoculated with TNE buffer emulsified in CFA. The FL-74 cell vaccine was composed of $5 \times 10^8$ heat-killed FL-74 cells (approximately 0.5 ml) mixed with 0.5 ml TNE buffer and 1 ml CFA. The FL-74 cell-P15 vaccine was composed of $5 \times 10^8$ heat-killed FL-74 cell mixed with 0.5 ml of P15 solution (either 300 or 100 ug) and 1 ml CFA. Vaccines were inoculated intramuscularly into cats three times on a biweekly schedule.

Ouchterlony Tests

Ouchterlony tests were performed on glass microscope slides coated with a 1 mm layer of 0.7% agarose in veronal buffer (Appendix C) containing 1% Triton X-100. One mm diameter wells were cut using an LKB die (LKB Instruments Inc., Rockville, MD) with a 9 well pattern. To each well was added 10-20 μl of the appropriate reagent (see Results).

Lymphocyte Viability

Lymphocyte viability tests were carried out in microtiter plates. Fifty μl of 0.25% solution of trypan blue in Hank's balanced salt solution was added to each well of the 5-day-old cultures. The plates were mixed and allowed to stand for several minutes. Viability was determined by counting a portion of the cell suspension from each well in a hemocytometer.
Results

Measurement of Immune Repression by In Vitro Techniques

Phytomitogen stimulation of lymphocytes in the lymphocyte blast transformation (LBT) assay has become an accepted means of measuring non-specific immune responsiveness (29).

A number of investigators have used inhibition of LBT for measuring the immunosuppressive nature of certain substances and have correlated this in vitro repression with in vivo immune unresponsiveness (64,109). Similar experimental designs were applied in the following series of experiments. Immune repression was first characterized by in vitro methods then confirmed with the in vivo system.

Inhibition of LBT with Inactivated FeLV

To evaluate the inhibitory effect of FeLV virus in the LBT assay, varying dilutions of UV inactivated virus were incubated with lymphocytes from three adult specific-pathogen-free (SPF) cats. Virus protein concentrations were 18.75, 7.5, 0.75, 0.075, and 0.0075 ug per microtiter plate well. Figure 1 depicts the percent repression from normal Con A-LBT stimulation values of the three cats at the indicated concentrations of viral protein. Significant inhibition of Con A stimulation was evident for all three cats at a viral protein concentration of 18.75 ug/well and for two of the three cats at a concentration of 7.5 ug/well.
Figure 1. Con A-LBT assays of lymphocytes from three normal SPF cats incubated with various concentrations of U.V. inactivated FeLV. Dilutions of FeLV (100 ul volume) and an optimum dose of Con A (10 ug in 50 ul) were incubated in quadruplicate with $1 \times 10^5$ lymphocytes for 5 days. $^3$HTdR was added on the fourth day. Each point represents the mean value of quadruplicate tests. Inhibition was expressed as percent repression of $^3$HTdR uptake measured PBL cultures incubated without FeLV. Vertical lines represent the standard deviation of the mean.
Figure 1

Percent Repression of Con A-LBT

FeLV Concentration (μg protein/well)
Inhibition of LBT by Subcomponents of FeLV

Based upon the results with UV inactivated FeLV, experiments were performed to ascertain what component in the preparation of purified virus was responsible for the abrogation of feline lymphocyte response to mitogen.

Whole FeLV was fractionated into crude sub-component preparations based on solubility following the purification scheme shown in Figure 2. The four crude fractions (A through D) from this scheme were assayed for inhibition of LBT as previously described for virus.

The results of these tests were summarized in Table 1. Fractions A, B, and C were not significantly inhibitory (less than 10%) while fraction D inhibited 40%. This was comparable to whole virus which inhibited an average of 43%.

Polyacrylamide Gel Electrophoresis Analysis of Crude FeLV Fractions

Protein samples from whole FeLV and fractions A, B, C, and D were separated by SDS-PAGE to determine their protein make-up. Plates I, II, III and IV show densatometer scans of these gels compared to whole FeLV virus. The FeLV gel displayed protein markers of known molecular weight (34,35,74) for comparison with the proteins from Fractions A, B, C, and D. A second gel on which other protein standards were electrophoreosed, was also used as a standard for comparison (see Plate VIII). Fraction A was composed of primarily higher molecular weight proteins with the exception of the 12,000 dalton (P12) protein of FeLV.

Fraction B contained a mixture of proteins which, under the extraction conditions, were insoluble. Fraction C was composed of several
Figure 2. Scheme for Fractionating FeLV.

FeLV was twice frozen-thawed to disrupt the virus envelope. The frozen-thawed virus was centrifuged at 100,000 x g for 1 hr. in an L2 65B ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) using an SW27 rotor with 39 ml buckets. Following the centrifugation, the supernatant (Fraction A) was collected and stored at -90°C. The pellet was resuspended with TKE-D-Tx buffer (Appendix A) and incubated at 37°C for 1 hr. Proteins that become soluble by treatment with TKE-D-Tx buffer were separated from insoluble proteins (Fraction B) by centrifugation at 100,000 g for 1 hr as described above. Following centrifugation, the supernatant was collected and extracted ten times with ether to remove the Triton X-100 from the aqueous phase. When the Triton X-100 was removed some of the protein became insoluble. Residual ether was removed from the aqueous phase by bubbling nitrogen gas through the liquid. The soluble (Fraction C) and insoluble protein (Fraction D) found in the aqueous phase after extraction, were separated by centrifugation at 100,000 x g for 1 hr.
FeLV
1×10^11 PARTICLES / ml PURIFIED
BY 2x SUCROSE DENSITY
GRADIENT CENTRIFUGATION

FROZEN THAWED 2x
ULTRACENTRIFUGATION
100,000 G FOR 1 HOUR

PELLET
RESUSPEND IN EXTRACT BUFFER
INCUBATE AT 37°C FOR 1 HOUR
ULTRACENTRIFUGATION
100,000 G FOR 1 HOUR

PELLET
SUPERNATANT
FRACTION A
FRACTION B

SUPERNATANT
ETHER EXTRACT 10x
REMOVE ETHER BY N₂ BUBBLING
ULTRACENTRIFUGATION
100,000 G FOR 1 HOUR

PELLET
SUPERNATANT
FRACTION C
FRACTION D

Figure 2
Table 1. Repression of Con A LBT by FeLV and FeLV Protein Fractions.

<table>
<thead>
<tr>
<th>Protein(a)</th>
<th>Percent Repression(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole FeLV(c)</td>
<td>43.1 ± 12.5(d)</td>
</tr>
<tr>
<td>Fraction A</td>
<td>1.4 ± 9.3</td>
</tr>
<tr>
<td>Fraction B</td>
<td>3.5 ± 8.5</td>
</tr>
<tr>
<td>Fraction C</td>
<td>1.5 ± 1.5</td>
</tr>
<tr>
<td>Fraction D</td>
<td>41.1 ± 13.3</td>
</tr>
</tbody>
</table>

(a) Proteins used to repress Con A LBT.

(b) Mean percent repression of Con A LBT cultures incubated with the indicated protein. Repression was determined by dividing the \(^3\)H TdR uptake in the test culture by that of parallel control cultures incubated without the indicated protein.

(c) Ultraviolet inactivated FeLV.

(d) Standard deviation of the mean expressed as a percent.
Plate I. Densitometer scans of PAGE gels on which whole FeLV (figure A) and Fraction A protein (figure B) were separated. Gels consisted of 7.5% acrylamide with 5% bisacrylamide in Tris acetate buffer containing 0.1% SDS. Sample volume was 100 µl. Electrophoresis was carried out for 4.5 hr and 8 mA per column. Gels were stained with 0.25% coomassie blue in 10% acetic acid and 40% 150 isopropyl alcohol.
Plate II. Densitometer scans of PAGE gels on which whole FeLV (figure A) and Fraction B protein (figure B) were separated.
Plate III. Densitometer scans of PAGE gels on which whole FeLV (figure A) and Fraction C protein (figure B) were separated. Fraction C contained lower molecular weight proteins, primarily P27.
Plate IV. Densitometer scans of PAGE gels on which whole FeLV (figure A) and Fraction D proteins (figure B) were separated. Fraction D consisted of lower molecular weight proteins, primarily P15.
lower molecular weight proteins but primarily the 27,000 dalton protein (P27). Fraction D was also made-up of lower molecular weight proteins with the major protein being of 15,000 daltons molecular weight (P15).

**Purification of P15 Protein**

The Con A-LBT inhibition experiments with crude protein fractions of FeLV indicated that Fraction D contained the inhibitory component (Table 1). In that the major component of Fraction D was the P15 component of FeLV, further purification of that protein was the next step. Purification was accomplished by descending liquid column chromatography using a 2.6 x 90 cm column containing Sephacryl S200 (Pharmacia Fine Chemicals, Upsala, Sweden) at the matrix. The column was equilibrated with TKE-D-Tx buffer (see Appendix A). Prior to being chromatographed, a small amount of the Fraction D protein mixture was radiolabeled with $^{125}$I by the Chloramine-T method (see Materials and Methods) to facilitate identification of protein peaks.

Figure 3 is a chromatogram showing the separation of radioiodine labeled protein. Two major radioactive peaks were evident (Peaks I and II) along with a smaller void volume peak. PAGE analysis of each peak indicated that Peak I contained purified P15 (Plate V) while Peak II (not shown) contained no detectable protein and was probably free iodine not removed from the preparation during the iodination procedure (see Materials and Methods).

**Purification of P27 Proteins**

Purified P27 was prepared to provide a protein control for the in vitro Con A-LBT inhibition studies. P27 was purified from Fraction C by descending liquid, column chromatography using a 2.5 x 90 cm column
Figure 3. Chromatographic separation of Fraction D protein on a 2.6 x 90 cm Sephacryl S200 column equalibrated with TKE-D-Tx buffer (see Appendix A) Sample and fraction volumes were 2 mls.
Plate V. Densitometer scans of PAGE gels on which Fraction D proteins (figure A) and protein from S200 chromatographic peak (figure B) were separated. Peak I consisted of highly purified P15 protein.
of Sephadex G200, equilibrated with TKE-D buffer (see Appendix B). Two
mls of Fraction C was applied to the top of the column and fractions
were monitored for protein by measuring optical absorbance at 280 nM.
Figure 4 is a chromatogram of the protein separation. Peak #2 contained
P27 along with a small amount of lower molecular weight proteins. Fur­
ther purification of P27 was accomplished by rechromatographing Peak
#2 proteins through the same column. Plate VI is a SDS-PAGE scan of the
twice chromatographed P27 protein.

Inhibition of Con A-LBT by Purified P15 and P27

In this test, highly purified P15 or P27 was added to Con A-LBT as­
says to determine if either protein had inhibitory properties similar to
whole FeLV and Fraction D. Lymphocytes from six normal SPF cats were
used with P15 and two normal SPF cats with P27. P15 and P27 were used
at concentrations of 5, 2, 0.2, 0.02, and 0.002 ug per well. Results
of tests in which 5 ug of protein were used are summarized in Table 2.
P15 reduced the level of stimulation 45 to 95% of normal values. P27,
however, was not significantly inhibitory, reducing the level of stimu­
lation less than 20%. Figure 5 shows the mean of inhibition values over
the full range of P15 and P27 concentrations. P15 was significantly in­
hibitory at concentrations of 0.2 ug and greater.

Characterization of P15 Inhibition

Measurement of P15 Toxicity

One explanation for the inhibitory effect of P15 in the Con A-LBT
assay was that P15 was toxic to cat lymphocytes and that lymphocyte
killing occurred prior to the additional ³H-TdR. To determine if P15
Figure 4. Chromatographic separation of Fraction C protein on a 2.5 x 90 cm Sephadex G150 column equilibrated with TKE-D buffer (see Appendix B). Peak 2 protein was rechromatographed to yield purified P27.
Plate VI. Densitometer scans of PAGE gels on which Fraction C protein figure (A) and rechromatographed Peak 2 protein from G200 separation of Fraction C (figure B) were separated.
Plate VI
Table 2. Inhibition of Con A LBT by P15 and P27 from FeLV.

<table>
<thead>
<tr>
<th>Cat #</th>
<th>Inhibitory Protein</th>
<th>(a) Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>550</td>
<td>P15</td>
<td>51.8 ± 8.8(b)</td>
</tr>
<tr>
<td>555</td>
<td>P15</td>
<td>45.1 ± 6.6</td>
</tr>
<tr>
<td>281</td>
<td>P15</td>
<td>92.0 ± 17.9</td>
</tr>
<tr>
<td>679</td>
<td>P15</td>
<td>92.9 ± 16.3</td>
</tr>
<tr>
<td>795</td>
<td>P15</td>
<td>61.5 ± 7.9</td>
</tr>
<tr>
<td>760</td>
<td>P15</td>
<td>67.3 ± 11.4</td>
</tr>
<tr>
<td>550</td>
<td>P27</td>
<td>15.2 ± 8.7</td>
</tr>
<tr>
<td>555</td>
<td>P27</td>
<td>20.8 ± 10.2</td>
</tr>
</tbody>
</table>

(a) Percent repression of Con A control value  
(b) Standard deviation of the mean expressed as a percent.

Cat lymphocytes were incubated with and without FeLV P15 (5 ug/well) for 5 days. Tritiated thymidine was added on the 4th day. Percent repression was determined by dividing the CPM's from cultures incubated with P15 by the CPM's from cultures incubated without P15 (X 100).
Figure 5. Con A-LBT Inhibition assays. Purified P15 or P27 proteins at concentration of from 0.002 to 5.0 ug/well were added to Con A-LBT tests. •—, P15; o—o, P27. Each point represents the mean repression value of tests run on PBL from 6 cats for P15 and 2 cats for P27. PBL from each cat were tested in quadruplicate. The vertical line represents the standard error of the mean. Only P15 was repressive.
Figure 5

Percent Repression of Con A-LBT

µg Protein / Well
was toxic, trypan blue dye exclusion tests were run on cat lymphocytes from two cats that were incubated with P15 for five days. Table 3 summarizes the results. For all dilutions of P15 used, the total cell viability was approximately equivalent to control cell counts of cat lymphocytes incubated without P15. Thus, P15 was not toxic to dormant cat lymphocytes. The results from the above experiment, however, do not exclude the possibility that P15 in conjunction with Con A may be cytotoxic to dividing cells.

Effect of Adding P15 at Various Times During Con A-LBT Incubation

The next experiment was designed to determine the effect of adding P15 at various times during the incubation period of the Con A-LBT test. Lymphocytes from two cats were set up in parallel cultures with an optimum concentration of Con A. A series of concentrations of P15 were added at 0, 24, 48, 72 and 96 hours. Tritiated thymidine was also added at 96 hrs and the cells were harvested on day 5.

Plate VII shows a series of graphs depicting the level of repression of the Con A response at the indicated concentration of P15 for tests in which P15 was added at different times during the incubation period. Repression was evident in all tests though at a much higher level when added during the first 48 hours of incubation.

Table 4 summarizes the results of tests in which 5 ug of P15 was added at the indicated time. When P15 was added at 0, 1 or 2 days, the amount of repression was approximately equal. Between the second and third day, however, the level of repression dropped suddenly, almost to background (Figure 6). Statistical analysis of the data using the
Table 3. Viability of Peripheral Blood Lymphocytes Incubated with FeLV-P15.

<table>
<thead>
<tr>
<th>Concentration of P15 (ug)</th>
<th>PBL Concentration/well x 10^5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cat # 687</td>
</tr>
<tr>
<td>0</td>
<td>1.48</td>
</tr>
<tr>
<td>5</td>
<td>1.58</td>
</tr>
<tr>
<td>2</td>
<td>1.82</td>
</tr>
<tr>
<td>0.2</td>
<td>1.87</td>
</tr>
<tr>
<td>0.02</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Cat PBL's were seeded at a concentration of 1 X 10^5 cells/well in microtiter plated. FeLV P15 was added at concentration ranging between 0 and 5 ug protein. Cell viability was determined by Trypan blue exclusion after 5 days incubation.
Plate VII. Con A-LBT assays to which P15 was added at various times during the incubation period. (0 day o—o, 1 day •—•, 2 day □—□, 3 day ■—■, and 4 day Δ—Δ.) Figures A and B represent tests run with PBL from cat 760 and cat 795 respectively. On the indicated days, P15 was added at concentrations of from 0.002 to 5 μg/well. Each point is a mean value of quadruplicate testing.
Percent Repression of Con A-LBT

μg of Inhibitory Protein/Well

Plate VII

Percent Repression of Con A-LBT

CAT # 795

CAT # 760
Table 4. Effect of Adding FeLV P15 at Various Times during the Con A LBT Incubation.

<table>
<thead>
<tr>
<th>Day of Addition of P15&lt;sup&gt;(a)&lt;/sup&gt;</th>
<th>Percent Repression of Con A-LBT</th>
<th>Cat #760</th>
<th>Cat #795</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(b)&lt;sup&gt;66.7 ± 11.4&lt;/sup&gt;&lt;sup&gt;(c)&lt;/sup&gt;</td>
<td>60.6 ± 7.9</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>57.2 ± 10.2</td>
<td>74.7 ± 5.3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>59.0 ± 7.6</td>
<td>62.4 ± 14.2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>38.6 ± 11.3</td>
<td>25.1 ± 13.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>28.8 ± 13.7</td>
<td>19.4 ± 16.4</td>
<td></td>
</tr>
</tbody>
</table>

(a) P15 protein added at a concentration of 5 ug/well.
(b) Percent repression of normal con A-LBT values as determined from quadruplicate tests.
(c) Standard deviation of the mean expressed as a percent.

FeLV P15 (5 ug/well) was added to Con A LBT cultures at 0, 1, 2, 3, and 4 days of culture. ³H TdR was added on the 4th day. Cultures were harvested on the 5th day. PBL's from 2 cats were used as cell donors. Percent repression was determined by dividing the CPM's from cultures incubated with P15, by the CPM's from cultures incubated without P15.
Figure 6. Percent repression of Con A-LBT assay for lymphocytes from 2 cats to which 5 μg/well of P15 was added on various days of incubation. Each point represents the mean of quadruplicate tests. The vertical line represents the standard error of the mean. Cat #795, •—•; cat #760 o—o. The level of repression remained approximately the same during the first 2 days but dropped on the 3rd and 4th days.
student-t test indicated the most significant difference in levels of repression existed between the second and third day of incubation. Figure 7 is a block graph showing the changes in level of immune repression between tests in which P15 was added on different days. It appeared that P15 had its inhibitory effect during the first 48 hours of incubation.

P15 Cytotoxicity of Con A Stimulated Dividing Lymphocytes

The above experiment (Figure 6) also provided data pertaining to the possible toxicity of P15. Earlier, P15 was shown not to be toxic to dormant lymphocytes. The possibility still remained, however, that P15 was toxic to dividing cells, such as those stimulated by Con A. Results from the above experiment indicated that Con A stimulated dividing cells, to which P15 was added on the 3rd or 4th day of culture incubation, had stimulation values close to those of parallel cultures to which P15 was not added. Thus, Incubating P15 with dividing lymphocyte cultures for 24 hours and perhaps longer did not appear to cause cell killing.

Molecular Weight Determination of P15 by Column Chromatography

Column chromatographic separation of P15 indicated its molecular weight to be somewhat higher than 15,000 daltons. In order to estimate the molecular weight of Peak I (Figure 3), five molecular weight standards were chromatographed under the same conditions as Fraction D. The molecular weight standards chosen were Dextran blue 2000 (2,000,000 daltons), aldolase (158,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen
Figure 7. Changes in percent repression of Con A-LBT caused by adding P15 on different days of incubation. The greatest change in percent repression occurred between the second and third day. The sign indicates if repression increased (+) or decreased (-). t = the student t test value; df = degrees of freedom.
Figure 7: On which pig was added difference between days

Differences in % repression

CAT 795

CAT 760
(25,000 daltons), and ribonuclease (13,700 daltons). A composite drawing showing the separation of these standards is shown in Figure 8. The elution volume for Dextran blue 2000 (Figure 8) provided the value for the void volume of the column. The elution volumes for each of the other protein standards were used to calculate standard values of protein elution (Kav) using the formula:

$$Kav = \frac{\text{sample volume} - \text{void volume}}{\text{column volume} - \text{void volume}}$$

The Kav values for each standard was determined and plotted against its molecular weight as shown in Figure 9. Peak I from the Fraction D separation had a Kav of 1.1 which was approximately the same as that for aldolase. Thus, the molecular weight for Peak I was calculated to be approximately 160,000 daltons.

**Molecular Weight Determination of P15 by SDS-PAGE Analysis**

With each batch of gels used to analyze viral proteins was included a gel containing molecular weight standards. These standards were phosphotase (98,000 daltons), bovine serum albumin (65,000 daltons), ovalbumin (43,000 daltons), chymotrypsinogen (25,000 daltons) and ribonuclease (13,700 daltons). By comparing the migration distances of the protein standards with the migration distance of a given viral protein, the molecular weight of the viral protein could be estimated. The low molecular weight proteins of FeLV have been reported to have molecular weights of 27,000, 15,000, 12,000, and 10,000 daltons (74). The results of this study agreed closely with those molecular weight determinations. The major protein of Fraction D and the protein taken from Peak I of
Figure 8. Chromatographic separation of protein standard on the 2.6 × 90 cm Sephacryl S-200 column used to purify P15. The figure is a composite of 3 chromatographic separations. The first was dextran blue 2000 (2,000,000 daltons) to determine void volume. The second chromatographic run separated a mixture of aldolase (158,000 daltons) and chymotrypsinogen (25,000 daltons). The third separated ovalbumin (45,000 daltons) and ribonuclease (13,7000 daltons). Sample and fraction volumes were 2 mls. The Kav values were determined by using the formula:

\[ Kav = \frac{\text{Sample volume} - \text{void volume}}{\text{Column volume} - \text{void volume}} \]
Optical Density (280 nm)

- DEXTRAN BLUE 2000
- ALDOSE
- OVALBUMIN
- CHYMOTRYPSINOGEN
- RIBONUCLEASE

Figure 8
Figure 9. Graph of Kav values against molecular weight of the protein standards used to equilibrate the S200 column. Protein standards were Aldolase, Ovalbumin, Chymotrypsinogen and Ribonuclease.

\[
Kav = \frac{\text{Sample volume} - \text{void volume}}{\text{Column volume} - \text{void volume}}
\]
chromatographed Fraction D both migrated as a protein of 15,000 daltons molecular weight (Plate VIII).

**Characterization of P15**

A 15,000 dalton protein from FeLV had been previously described, however, the physical and antigenic characteristics of this protein were still unknown. Murine leukemia virus proteins, by contrast, are well characterized and include two 15,000 dalton proteins (47). One is a protein located on the outer envelope of the virus designated P15(E). The other is a core-protein designated P15(C). P15(E) has a strong interspecies antigenic determinant and will stimulate neutralizing antibody in xenotropic species (46). P15(C) has mostly type specific antigenic determinants (46).

In order to characterize the FeLV-P15 protein, MuLV P15 proteins were used as models. One of the traits of MuLV P15(E) had already been ascribed to FeLV-P15, that being the strong tendency to aggregate when in solution free of detergents. This characteristic was used as the basis for separation in the purification scheme (Figure 2).

**Antigenic Analysis of FeLV-P15**

Ouchterlony tests were used to determine the antigenic nature of P15. Plate IX (figure A) shows the results of these tests. A line of identity was clearly seen between FeLV, FeLV-P15, and MuLV with antisera prepared against FeLV. A second line of identity was evident between FeLV, FeLV-P27, and MuLV. This precipitin line, however, does not extend to P15. Thus, two separate interspecies lines can be identified, one which reacts with P15 and the other with P27. In a second
Plate VIII. Densitometer scans of PAGE gels on which purified P15 protein (figure A) and protein standard were separated. The protein standards were phosphatase, 98,000 daltons; bovine serum albumin, 65,000 daltons; ovalbumin, 43,000 daltons; chymotrypsinogen, 25,000 daltons; and ribonuclease, 13,700 daltons. Protein standards were run with P15 to show that the molecular weight of P15 as determined by SDS-PAGE was approximately 15,000 daltons.
Plate VIII

A

BOVINE SERUM ALBUMIN

PHOSPHATASE

OVALBUMIN

CHYMOTRYPSINOGEN

RIBONUCLEASE

B

PI5

CENTIMETERS

Plate VIII
Plate IX. Ouchterlony tests characterizing interspecies antigenic cross-reactivity of FeLV-P15 protein. (A) Triton X-100 disrupted FeLV; (B) Purified P15 in TKE-D-Tx buffer; (C) Triton X-100 disrupted MuLV; (D) Purified P27 in TKE-D-Tx buffer. Immunodiffusion was carried out using 0.7% agarose in TKE-D-Tx buffer.
test (Plate IX, figure B), antiserum specific for FeLV-P27 was used instead of anti-whole FeLV. The characteristic interspecies precipitin line appeared with FeLV, MuLV, and FeLV-P27, but not with P15, again indicating the P15 and P27 did not contain the same interspecies antigenic determinate.

Lactoperoxidase Iodination of Whole FeLV

To determine if P15 was an outer envelope component of FeLV, freshly harvested and purified FeLV was radiiodinated with $^{125}$I by the lactoperoxidase method as described in the Materials and Methods (67). Following lactoperoxidase treatment, the iodinated virus was mixed with 10 ml of unlabeled FeLV and purified a second time by density gradient centrifugation. After a two hour centrifugation, the gradient was fractionated. Each fraction was assayed for optical density at 280 nm, radioactivity and density. Figures 10 and 11 show the results of these determinations for the Kawakami-Thellen and Rickard strains of FeLV, respectively. KT-FeLV banded at a density of 1.17 g/cm$^2$ (38% sucrose) and R-FeLV banded at a density of 1.14 g/cm$^2$ (33% sucrose).

SDS-PAGE analysis of the lactoperoxidase labeled virus was performed to determine which proteins were iodinated. With this procedure, gels were sliced into 1.2 mm sections as described in Materials and Methods and each section counted separately in a Beckman Biogamma II (Beckman Instruments, Palo Alto, CA) gamma counter. Plates X and XI show the results of this procedure for KT-FeLV and R-FeLV, respectively. KT-FeLV showed 2 areas of radioactivity. One corresponding to a protein of approximately 70,000 dalton molecular weight, while the other was at a
Figure 10. Density gradient separation of $^{131}$I lactoperoxidase labeled FeLV (Kawakami-Thellen strain) on linear 0 to 50% sucrose gradient. The virus formed a band at a density between 1.15 and 1.18 g/cc. The gradient was fractionated into approximately 1.2 ml volumes. Each fraction was assayed for radioactivity, optical observation and density.
Figure 10

\[ ^{131}I \text{ Counts / Minute} \times 10^{-6} \]

Optical Density (280nm)

Density (g/cc)

Percent Sucrose

Gradient Fraction Number
Figure II. Similar to Figure 19. Density gradient separation of $^{125}\text{I}$ lactoperoxidase labeled FeLV (Rickard strain) on gradient. The virus formed a band at a density between 1.13 and 1.15 g/cc.
Figure 11

\[ {^{125}}I \text{ Counts / Minute } \times 10^{-5} \]

Optical Density (280 nm)

Gradient Fraction Number

Percent Sucrose

Density (g/cc)
Plate X. SDS-PAGE separations of $^{131}$I lactoperoxidase labeled KT-FeLV. One gel was stained with coomassie blue. A second gel was sliced into 1.2 mm sections and counted for radioactivity in a gamma counter. Densitometer scan of stained gel (figure A); radioactive profile of sliced gel (figure B).
Plate XI. Similar to Plate IX. SDS-PAGE separation of $^{125\text{I}}$ lactoperoxidase labeled R-FeLV. Densitometer scan of stained gel (figure A); Radioactive profile of sliced gel (figure B).
region at the end of the gel which did not correspond with any viral protein and probably represented unbound iodine. The pattern for R-FeLV was similar, but there was a smaller peak at the 70,000 dalton molecular region. In either case, however, P15 was not iodinated and, thus, was apparently not available at the outer envelope for labelling.

**Neutralization of FeLV by Antiserum Specific for P15**

In this experiment, a rabbit was twice inoculated with 100 ug of purified P15 mixed with complete Freund's adjuvant. Serum was collected prior to the first inoculation and two weeks after the final inoculation, FeLV serum neutralization assays were set up with pre- and post-inoculation rabbit serum and hyperimmune goat anti-FeLV serum of known neutralizing titer. Results of these assays are shown in Table 5. Pre-inoculation rabbit serum, as expected, had no measurable neutralizing titer (1:2). Rabbit anti-P15 serum, however, was only weakly neutralizing having a titer of 1:4. By comparison, goat anti-FeLV was strongly neutralizing with a titer of 1:80.

**In Vivo P15 Immunosuppression**

This aspect of the study was undertaken to determine if inoculation of P15 could induce an immunosuppressive effect in cats being immunized with tissue culture grown tumor cells (FL-74 cells) as was the case in previous studies where UV-Inactivated FeLV was included with a FL-74 cell vaccine (78). The *in vivo* immunosuppression studies were carried out using 2 experimental protocols. The first was a preliminary study to test the hypothesis that P15 caused abrogation of immunity. Four litters of cats were divided into control and vaccination groups such
Table 5. Serum Neutralization Assays.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Serum Neutralization Titer&lt;sup&gt;(a)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td></td>
</tr>
<tr>
<td>Prebleed&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>&lt;1:2</td>
</tr>
<tr>
<td>Hyperimmune&lt;sup&gt;(c)&lt;/sup&gt;</td>
<td>1:4</td>
</tr>
<tr>
<td>Goat anti-FelV&lt;sup&gt;(d)&lt;/sup&gt;</td>
<td>1:80</td>
</tr>
</tbody>
</table>

(a) Serum neutralizing titers were the highest dilution that reduced viral infectivity as measured by focus formation, 50%.

(b) Rabbit 132 serum taken before inoculation with P15.

(c) Rabbit 132 serum taken after 2 inoculations of P15 (100 ug) mixed with complete Freund's adjuvant.

(d) Serum from goat inoculated 6 times over a period of 16 weeks with U.V. inactivated FelV (1 x 10<sup>11</sup>) mixed with complete or incomplete Freund's adjuvant.
that half of each litter fell in each group. The control group was inoculated with Tris buffer while the vaccination group received a mixture of P15 (300 ug in the first inoculation and 100 ug in the second and third inoculation) and heat killed FL-74 cells (5 x 10^8 cells). All immunogens were emulsified in complete Freund's adjuvant. Immunization started at four weeks of age, all cats were challenged with an oncogenic dose of FeSV which gave 50% incidence of progressive fibrosarcomas in age matched cats. (Progressive fibrosarcomas are defined as fibrosarcoma which continue to increase in size and eventually cause death).

The results of this study are summarized in Table 6. Three of 7 or 42% of control cats developed progressive tumors. In the vaccinated group, no progressive tumors were observed. The incidence of viremia, however, in the two groups was approximately the same, 2 of 5 or 40% for the vaccinated and 2/7 or 42% for the control group. When the mean FOCMA antibody titers of all vaccinated cats at a given weeks post first inoculation were plotted, the profile of the graph resembled that previously reported for cats immunized with FeLV and FL-74 cells (78) (Figure 12). Thus, FOCMA titers resulting from immunization with FL-74 cells appeared to be markedly reduced by including P15 with the inoculum.

The second in vivo immunosuppression experimental protocol was designed to compare the immune response of litter mate cats inoculated with and without P15. The rationale for conducting this experiment was to use cats as closely matched as possible to establish P15 immunosuppression. By designing the experiment in this way, the criticism that cats from different litters may respond differently, could be avoided. Two cat litters were internally divided into 2 vaccination groups. One
Table 6. Biologic Response of FL-74 Cell + P15 Immunized or Control Cats following FeSV Challenge.

<table>
<thead>
<tr>
<th>Cat Number</th>
<th>FL-74 + P15(a)</th>
<th>Control(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immunized</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumor</td>
<td>Viremia</td>
</tr>
<tr>
<td>274-1</td>
<td>R</td>
<td>+/-</td>
</tr>
<tr>
<td>-2</td>
<td>R</td>
<td>+/-</td>
</tr>
<tr>
<td>-3</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>417-1</td>
<td>R</td>
<td>+/-</td>
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<tr>
<td>-3</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>176-1</td>
<td>NT(e)</td>
<td></td>
</tr>
<tr>
<td>-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>154-2</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of Progressor Cats/Total: 0/5, 3/7

% of Cats with Progressive Tumors: 0%, 42%

Number of Viremic Cats/Total: 2/5, 3/7

% Viremic Cats: 40%, 42%

(a) Cats Immunized with $5 \times 10^8$ heat killed FL-74 cells plus 100-300 ug of FeLV P15 protein emulsified in CFA.
(b) Control cats immunized with Tris buffer emulsified in CFA.
(c) R = tumor regressed.
(d) NT = not tested.
(e) +/- = temporarily viremic.
(f) P = progressive fibrosarcoma.
Figure 12. FOCMA antibody responses, as measured by indirect membrane immunofluorescence (IMI) of cats immunized with heat-killed FL-74 cells \(5 \times 10^8\) cells + P15 protein (300 or 100 ug). Each point represents a mean antibody titer value (LOG2). The arrows indicate the time at which cats were inoculated. Vertical lines represent standard deviation of the mean. Titers are expressed in Log2.
Figure 12

IMI FOCMA TITER (LOG₂)

AGE OF CAT (WEEKS)
group received P15 plus killed FL-74 cells, as before, and the other group received killed FL-74 cells alone. All inocula were emulsified with complete Freund's adjuvant. The age of cat, time of inoculation and dose of immunogen were identical to the earlier experiment. The FeSV challenge, however, was different.

The results are summarized in Table 7. Three of 4 or 75% of cats immunized with the FL-74 + P15 preparation developed progressive fibrosarcomas. In the cats inoculated with FL-74 cells alone, only 1 of 5 (20%) had a progressive disease. The incidence of viremia was also different for the two groups. Four of 4 (100%) of FL-74-P15 cats and 3 of 5 (60%) of the FL-74 cats became persistently viremic (viremic throughout the observation period). Figure 13 compares the mean FOCMA antibody titers at various times during vaccination of the two groups of cats. Cats in the FL-74 cell group developed higher FOCMA antibody titers earlier than the FL-74 cell + P15 group. At 2 weeks post-first inoculation, the mean FOCMA antibody titers for the two groups were significantly different at the 0.05 level of confidence. In later serum samples, however, the differences in mean antibody titers was not found to be significant. It, thus, appears that inoculating cats with FL-74 cells plus P15 causes FOCMA antibody production to be retarded. This suppressive effect was eventually (week 9) overcome with time.

Serologic Tests

The nature of the immune abrogation induced by inoculating cats with P15 is unknown. Immunosuppression associated with FeLV infections, however, have been shown to affect T-cell but not B-cell immune functions
Table 7. Biologic Response following FeSV Challenge of Cats Immunized with FL-74 Cells or FL-74 Cells + P15.

<table>
<thead>
<tr>
<th>Number</th>
<th>FL-74 + P15(a)</th>
<th></th>
<th>FL-74(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor</td>
<td>Viremia</td>
<td>Tumor</td>
</tr>
<tr>
<td>618-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td>P(c)</td>
<td>+</td>
<td>R</td>
</tr>
<tr>
<td>-3</td>
<td>R(d)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td></td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>-5</td>
<td></td>
<td></td>
<td>R</td>
</tr>
<tr>
<td>687-1</td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>-2</td>
<td>P</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>-3</td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>-4</td>
<td>R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of Cats with Progressive Tumor/Total

<table>
<thead>
<tr>
<th></th>
<th>FL-74 + P15(a)</th>
<th></th>
<th>FL-74(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3/4</td>
<td></td>
<td>1/5</td>
</tr>
</tbody>
</table>

% Progressive Tumor

<table>
<thead>
<tr>
<th></th>
<th>FL-74 + P15(a)</th>
<th></th>
<th>FL-74(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>75%</td>
<td></td>
<td>20%</td>
</tr>
</tbody>
</table>

Number of Viremic Cats/Total

<table>
<thead>
<tr>
<th></th>
<th>FL-74 + P15(a)</th>
<th></th>
<th>FL-74(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4/4</td>
<td></td>
<td>3/5</td>
</tr>
</tbody>
</table>

% Viremic Cats

<table>
<thead>
<tr>
<th></th>
<th>FL-74 + P15(a)</th>
<th></th>
<th>FL-74(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100%</td>
<td></td>
<td>60%</td>
</tr>
</tbody>
</table>

(a) Cats immunized 3X with 5 x 10^8 heat killed FL-74 cells + FeLV P15 (100-300 ug) emulsified in CFA.
(b) Cats immunized 3X with 5 x 10^8 heat killed FL-74 cells in CFA.
(c) P = progressive fibrosarcoma.
(d) R = tumor regressed
(e) +/- = temporarily viremic.
Figure 13. FOCSMA antibody responses, as measured by indirect membrane Immunofluorescence (IMI), of cats from divided litters immunized with heat-killed FL-74 cells alone (o—o) or with heat-killed FL-74 cells + P15 (e—e). Each point represents the mean of FOCSMA antibody titers for 5 cats for FL-74 cells alone and 4 cats for FL-74 cells + P15. Vertical line represents the standard error of the mean. Arrows indicate the times of inoculation. Titers are expressed in LOG2.
(81). How T-cell function abrogations ultimately makes the cat more susceptible to lymphosarcoma (LSA) was of interest in this study. One T-cell function is controlling the type of immunoglobulin produced after antigenic stimulation.

Part of this study was concerned with measuring one of the possible anti-tumor functions of FOCMA antibody, that being complement dependent cell cytotoxicity. The objectives were: i) to compare cytotoxic antibody levels found in FeLV-infected cats that became resistant with the antibody level found in cats that became diseased and ii) to correlate FOCMA antibody as measured by IMI with cytotoxicity.

**Cytotoxic Antibody in FeLV-Infected Cats**

Thirty-five cats from various age groups ranging from 2 to 72 weeks were inoculated I.P. with a dose of FeLV known to induce lymphosarcoma in 100% of cats inoculated neonatally. Cats were tested, usually bi-weekly, for cytotoxic antibody and for FeLV viremia. Cytotoxic antibody was measured using the microcytotoxicity (MCT) assay (68) as described in Appendix E. Table 8 summarizes the results. The highest cytotoxic antibody titer attained in the period post-challenge is shown along with the cat number, age at time of inoculation, and viremic state. Of 35 cats inoculated with FeLV, 17 were FeLV viremic and developed lymphosarcoma while the remaining 18 were resistant. The highest cytotoxic antibody titers attained for the LSA-positive cats range from 1:2 to 1:32 with a mean value of 1:4.7. Cytotoxic antibody levels for resistant cats ranged from 1:4 to 1:128 with a mean value of 1:27.4. Statistical analysis of values using the student t test showed a significant
Table 8. Cytotoxic Antibody Titers of Cats Inoculated with FeLV.

<table>
<thead>
<tr>
<th>Cat Number</th>
<th>Age at Time of Infection (wks)</th>
<th>Highest Titer After Challenge</th>
<th>FeLV Viremia</th>
<th>Cat Number</th>
<th>Age at Time of Infection (wks)</th>
<th>Highest Titer After Challenge</th>
<th>FeLV Viremia</th>
</tr>
</thead>
<tbody>
<tr>
<td>932-1</td>
<td>2</td>
<td>16</td>
<td>+</td>
<td>687-1</td>
<td>4</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>-3</td>
<td>2</td>
<td>8</td>
<td>+</td>
<td>-5</td>
<td>4</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>-4</td>
<td>2</td>
<td>8</td>
<td>+</td>
<td>875-1</td>
<td>4</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>35R-1</td>
<td>4</td>
<td>16</td>
<td>+</td>
<td>-2</td>
<td>4</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>-2</td>
<td>4</td>
<td>8</td>
<td>+</td>
<td>410 B</td>
<td>8</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>-3</td>
<td>4</td>
<td>32</td>
<td>+</td>
<td>411 B</td>
<td>8</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>687-2</td>
<td>4</td>
<td>&lt;2</td>
<td>+</td>
<td>137 R</td>
<td>13</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>-3</td>
<td>4</td>
<td>16</td>
<td>+</td>
<td>150 R</td>
<td>13</td>
<td>128</td>
<td>-</td>
</tr>
<tr>
<td>-4</td>
<td>4</td>
<td>&lt;2</td>
<td>+</td>
<td>122 R</td>
<td>16</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>875-3</td>
<td>4</td>
<td>16</td>
<td>+</td>
<td>132 R</td>
<td>16</td>
<td>128</td>
<td>-</td>
</tr>
<tr>
<td>187 B</td>
<td>8</td>
<td>16</td>
<td>+</td>
<td>114 R</td>
<td>17</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>188 B</td>
<td>8</td>
<td>&lt;2</td>
<td>+</td>
<td>128 R</td>
<td>17</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>189 B</td>
<td>8</td>
<td>&lt;2</td>
<td>+</td>
<td>129 R</td>
<td>17</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>191 B</td>
<td>8</td>
<td>16</td>
<td>+</td>
<td>917 R</td>
<td>49</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>457 B</td>
<td>8</td>
<td>&lt;2</td>
<td>+</td>
<td>919 R</td>
<td>49</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>145</td>
<td>1</td>
<td>&lt;2</td>
<td>+</td>
<td>889 R</td>
<td>53</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>907</td>
<td>1</td>
<td>&lt;2</td>
<td>+</td>
<td>910 R</td>
<td>53</td>
<td>64</td>
<td>-</td>
</tr>
</tbody>
</table>

Mean FOCM Titer: 1:4.7

(a) Reciprocal of end point dilution.
difference to exist at the 0.001 levels of confidence indicating that the titer differences are significant.

As described earlier, the age of the cat at the time of inoculation was one of the prime factors in determining susceptibility. As shown in Table 8, 3 of 3 two-week-old, 7 of 11 four-week-old, 5 of 7 eight-week-old, and 2 of 14 one-week-old cats developed LSA. Of interest is the fact that those cats in the 4- and 5-week-old group that were resistant to FeLV infections were able to develop cytotoxicity antibody levels equivalent to that of the older cats thus indicating that age-related susceptibility was not simply the inability of younger cats to respond immunologically.

Comparison of Cytotoxic and Non-Cytotoxic FOCMA Antibody

The direct relation between high levels of FOCMA antibody as measured by IMI and resistance to FeLV infection has been established (21). To explore this problem, serial serum samples from cats inoculated between 13 and 72 weeks of age with FeLV were tested for FOCMA antibody by the IMI and MCT tests. Also included in these tests were single bleed serum samples taken from 43 healthy conventional cats in households where virus-excretion cats were known to be present. These sera were provided by Dr. Max Essex of Harvard University. Table 9 summarizes the results of the assays. Of the 16 SPF cats tested, 13 were concordantly positive or negative by the two assays for FOCMA antibody (MCT and IMI) (81%). By contrast, when samples from 43 conventional cats from leukemic households were examined, only 23 of 42 (55%) gave concordant results. For both groups, the largest number of discordant samples were those that were IMI positive and MCT negative (Table 9).
Table 9. Correlation of Microcytotoxicity Assay with Fluorescent Antibody Test on FL-74 Cells.

<table>
<thead>
<tr>
<th>Cats Tested</th>
<th>IMI Positive(^{(a,b)})</th>
<th>IMI Negative(^{(a)})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCT Positive</td>
<td>MCT Negative</td>
</tr>
<tr>
<td>SPF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R-FeLV Inoculated</td>
<td>11/14</td>
<td>3/14</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>Conventional</td>
<td>18/34</td>
<td>16/34</td>
</tr>
</tbody>
</table>

(a) Expressed as MCT reaction over IMI reaction
(b) IMI titers of $\geq 1:4$ were considered positive
(c) MCT titers of $\geq 1:2$ were considered positive
(d) Healthy conventional animals in households where virus-excretor cats were known to be present. Provided by Dr. Max Essex of Harvard University.
Samples from the SPF-infected cats described in Table 9 were also checked by both IMI and MCT at various times post-inoculation for positive or negative concordance (Figure 14). At 5 weeks post-inoculation, 11 of 11 samples that were positive by IMI were also positive by MCT. After 10 weeks post-inoculation, however, while most animals either remained positive or converted to positive by IMI, the number that were also positive by MCT substantially decreased. By 19 weeks post-inoculation, only 6 of the 13 samples that were positive by IMI remained positive by MCT.

Characterization of Immunoglobulin Involved in Cytotoxicity

The immunoglobulin class responsible for cytotoxicity could be of some significance if it were shown to be IgG (7S). The conversion from IgM to IgG in the immune response has been characterized as a T-cell function. If T-cell functions were being abrogated, IgG and perhaps cytotoxic antibody may not be produced.

A serum sample from an adult female SPF cat whose kittens had been inoculated with FeLV and who had developed a FOCA cytotoxicity antibody titer of 1:64 was fractionated on a G200 2.5 x 45 cm Sephadex column. Three protein peaks (as determined by optical density at 280 nM) were resolved as shown in the chromatograph in Figure 15. The central fractions of each peak were separately pooled and concentrated to the original relative volume. Each concentration was then assayed for cytotoxic antibody. Table 10 shows these results. Peak II (7S) had the greatest activity with a titer of 1:128 while Peak I (19S) was less, with a titer of 1:32. Peak III had no detectable cytotoxic titer.
Figure 14. Comparison of percent of cats that had positive antibody titers for FOCMA as measured by IMI (o—o) and MCT (●—●) following FeLV challenge. The age of the cats at time of challenge ranged from 13 to 72 weeks. Samples were considered positive for IMI and MCT tests with titers of 1:4 and 1:2, respectively. At 6 weeks, all 11 IMI positive cats were MCT positive. By 19 weeks, however, only 6 of 16 (37%) cats were MCT positive, while 13 of 16 (81%) remained IMI positive.
Figure 14
Figure 15. Chromatographic separation of cat serum on Sephadex G200 column. A 1 ml sample of serum from cat Q150 was applied to the top of a 2.5 x 45 cm column of G200 equilibrated with veronal buffer. Elutes were monitored at 280 nm with an ultraviolet analyzer and collected in 1 ml portions. Peaks I and II represent enriched preparations of 19S and 7S, respectively.
Thus, it appeared that both 7S and 19S fractions contain some cytotoxic activity, although the greatest activity was in the 7S peak.

**Cytotoxic Antibody in Cats Immunized with P15 + FL-74 Cells and FL-74 Cells Alone**

MCT assays were performed using sera from the cats Immunized in an earlier experiment with P15 + FL-74 cells or FL-74 cells alone. When sera from cats on the first protocol (half given FL-74 cells + P15; half given Tris buffer) were tested, only 1 of 6 had detectable cytotoxic antibodies while 5 of 6 produced FOCMA antibody detectable by IMI. Table 11 shows the highest FOCMA antibody level attained for these cats as measured by IMI and MCT assays. It is clear that cats responded immunologically to the FL-74 cells but did not produce measurable levels of antibody capable of mediating cytotoxicity. When sera taken from cats on the second immunization protocol (FL-74 cells + P15 and FL-74 cells alone) were tested for cytotoxic antibody, the results were somewhat different from those observed in the first experiment. The results are summarized in Table 12. Cats inoculated with FL-74 cells + P15 developed higher MCT titer than before (See Table 11), however these titers were significantly lower than those observed for cats immunized with FL-74 cells alone (Table 12).

In Figure 16, the geometric mean of the MCT titers for the cats inoculated with FL-74 cells + P15 or FL-74 cells (second protocol) are plotted at various times through the immunization period before challenge. Cytotoxic antibody titers for the cats given FL-74 cells + P15 are consistently lower throughout the observation period than those of
Table 10. Microcytotoxicity Activity to FL-74 Cells of Whole Feline Serum and Fractions Therefrom.

<table>
<thead>
<tr>
<th>Material Tested</th>
<th>Microcytotoxic Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Serum</td>
<td>1:64</td>
</tr>
<tr>
<td>Gel Filtration(^{(a)})</td>
<td></td>
</tr>
<tr>
<td>Peak I (19S)</td>
<td>1:32</td>
</tr>
<tr>
<td>Peak II (7S)</td>
<td>1:128</td>
</tr>
<tr>
<td>Peak III (Albumin)</td>
<td>1:2</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Serum fractionated on a Sephadex G200 column (2.5 x 45 cm) at a constant flow rate of 5.0 ml/hr. Eluates were monitored at 280 nm with an ultraviolet analyzer and collected in 1 ml portions. Protein peaks were concentrated to the original volume by dialysis against an aqueous solution of Aquacide III. Peaks I and II represent enriched preparations of 19S and 7S respectively.
Table II. Comparison of MCT and IMI FOCMA Titers of Cats Immunized with FL-74 Cells + P15.

<table>
<thead>
<tr>
<th>Cat #</th>
<th>Highest MCT Titer Attained Before Challenge</th>
<th>Highest IMI Titer Attained Before Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>247-1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>247-2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>417-1</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>417-2</td>
<td>16</td>
<td>512</td>
</tr>
<tr>
<td>176-1</td>
<td>2</td>
<td>128</td>
</tr>
<tr>
<td>154-2</td>
<td>2</td>
<td>32</td>
</tr>
</tbody>
</table>

Mean FOCMA Titer 1:1.6 1:28.4

Cats were inoculated at 4, 6 and 8 weeks of age with heat killed FL-74 cells (5 x 10⁸ cells) plus FeLV P15 (300 µg in the first inoculation, 100 µg in the second and third inoculation) emulsified in complete Freund's adjuvant. FeSV challenge was at 12 weeks of age.
Table 12. Comparison of MCT and IMI FOCHA Titers of Cats Immunized with FL-74 Cell + P15 or FL-74 Cell Alone.

<table>
<thead>
<tr>
<th>Cat Number</th>
<th>FL-74 Cell + P15</th>
<th>FL-74 Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Highest FOCHA Antibody Attained Before Challenge</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCT</td>
<td>IMI</td>
</tr>
<tr>
<td>618-1</td>
<td>≥256</td>
<td>128</td>
</tr>
<tr>
<td>-2</td>
<td>128</td>
<td>64</td>
</tr>
<tr>
<td>-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>678-1</td>
<td>&lt;2</td>
<td>32</td>
</tr>
<tr>
<td>-2</td>
<td>32</td>
<td>256</td>
</tr>
<tr>
<td>-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean Titer 1:38 1:91 1:294 1:169
Figure 16. FOCHA antibody responses as measured by MCT of cats from split litters immunized with heat-killed FL-74 cells alone (o—o) or heat-killed FL-74 cells + P15 (o—o). Each point represents the mean of MCT antibody titers for 5 cats for FL-74 cells alone and 4 cats for FL-74 cells + P15. Vertical lines represent the standard deviation of the mean. Arrows indicate the time of inoculation. Titers are expressed in LOG₂.
Figure 16

MCT TITER (LOG₂)

AGE OF CAT (WEEKS)
cats given FL-74 cells alone. It is of interest to note that cytotoxic antibody titers reach their peak at 4 weeks post-inoculation then decline rapidly. FOCHA antibody measured by IMI reach their peak at about 5 weeks but tend to persist at approximately the same level for the remainder of the observation period.
Discussion

The phenomenon of immunosuppression has been widely associated with retrovirus infections (15). Until recently, the virus infection was thought to impair the immune system by direct infection and perhaps transformation of lymphoid tissue. Recent evidence, however, suggested an alternate hypothesis. It was found that preinoculating animals with certain attenuated or inactivated retroviruses appeared to make the animal more susceptible to oncogenic challenge (95, 108). This phenomenon was demonstrated in the murine and feline systems. In a related study, cats developed reduced tumor antibody levels when inoculated simultaneously with killed tumor cells and inactivated FeLV (78).

These observations became the basis for hypothesizing that FeLV and perhaps other retroviruses have the capacity to mediate immune repression in the absence of infection, and that the resulting immunosuppression is responsible for not only allowing FeLV-related diseases to develop, but also may account for the inability of the host to immunologically respond to the neoplasm.

The objective of this study was to identify and characterize, by in vitro and in vivo methods, the FeLV associated substance(s) responsible for mediating immune abrogation or immunosuppression.
In Vitro Experiments

The *in vitro* experimental design was similar to that used successfully by others to characterize the immunosuppressive properties of certain proteins (64, 109). The initial study was intended to show the feasibility of using such a test with the FeLV system.

When inactivated FeLV was incubated with Con A stimulated normal feline lymphocytes in the LBT assay, the amount of $^3$H-TdR incorporation was reduced by an average of 43%. FeLV or some subcomponent of FeLV appeared to interact directly or indirectly with the *in vitro* cultured lymphocytes rendering them unresponsive to Con A stimulation.

In an attempt to identify a subcomponent of FeLV capable of interfering with the Con A LBT assay, whole virus was broken into 4 fractions (Fractions A, B, C, and D) based on solubility and sedimentation. Each fraction was analyzed by SDS-PAGE to determine their protein make-up.

Fraction A consisted of proteins released from the virus by twice freeze-thawing which disrupts the virus envelope. This fraction contained primarily higher molecular weight proteins (as determined by PAGE) with the exception of the 12,000 dalton protein (P12) (Plate I). Treating the virus pellet (resulting from centrifugation of the freeze-thawed virus preparation) with TKE-D-Tx buffer, which contains a reducing agent capable of breaking disulfide bonds (dithiothreitol), and a non-ionic detergent (Triton X100) which would help solubilize hydrophobic proteins produced by a soluble fraction (later divided into Fractions C and D) and an Insoluble fraction (Fraction B) (see Figure 2). Fraction B was shown to contain a mixture of proteins (Plate II). When the soluble fraction was ether extracted to remove the Triton X-100, a
portion of the protein became soluble. The soluble (Fraction C) and insoluble (Fraction D) proteins were separated by centrifugation. Fraction C contained several low molecular weight proteins but primarily P27 (27,000 daltons molecular weight) (Plate III). Fraction D contained primarily a protein of 15,000 daltons molecular weight although other low molecular weight proteins were also present (Plate IV). The fact that to remain soluble the Fraction D proteins required Triton X-100 to be present, indicated that the Fraction D proteins probably had hydrophobic regions. Removing the Triton X-100 apparently caused the hydrophobic regions to form a tertiary and, perhaps, a quaternary structure which became insoluble.

Each protein fraction was dialyzed against MEMs, then evaluated for their ability to interfere with the Con A-LBT assays. Only Fraction D was able to reduce $^{3}$H-TdR uptake to a significant level. Con A-LBT cultures were repressed an average of 40% in such tests. It became evident that Fraction D appeared to contain a sub-component of FeLV which inhibited normal Con A stimulation of feline lymphocytes in that Fraction D was composed mostly of P15. The next step was to purify this protein and retest it for Con A inhibition.

P15 was purified from Fraction D by liquid column chromatography using a Sephacryl S-200 column equilibrated with modified TKE-D-Tx buffer. The first large peak (Peak I) (discounting the variable, void volume peak) contained purified P15 as determined by SDS-PAGE.

Purified P15 was tested for inhibitory properties in Con A-LBT assays using lymphocytes from 6 donor cats. In the LBT assays, $^{3}$H-TdR uptakes were reduced 45 to 92 percent.
Thus, the data appeared to implicate P15 as the protein of FeLV responsible for interfering with Con A stimulation of feline lymphocytes. Because of the methodology used in the in vitro assay, however, several alternate interpretations must be examined. One possibility is that adding any protein to the Con A-LBT assay might cause reduced $^{3}$H-TdR uptake. This, however, was not shown to be the case in parallel Con A-LBT assays in which P27 was substituted for P15 as the inhibitory protein. In these assays, P27 was not found to be inhibitory.

Another explanation concerned the possibility that the extraction or column buffers used in purifying P15 might have affected the cultured lymphocytes. This possibility was dispelled after examining the inhibitory properties of Fractions C and D described earlier. Though both fractions were derived by extraction with TKE-D-Tx buffer, only Fraction D, containing P15, was inhibitory.

One interpretation of the results was that P15 was toxic rather than suppressive to lymphocyte cultures. Two experiments were conducted to determine if P15 was toxic. In the first experiment, P15 was incubated with feline lymphocyte cultures for 5 days then measured for viability by the Trypan blue dye exclusion method (Table 3). The results of these tests revealed no cytotoxicity. Thus, P15 did not appear to be toxic to dormant lymphocytes. However, not discounted was the possibility that P15 was only toxic to dividing cells such as those stimulated by Con A. Experiments to be discussed later, in which P15 was added on various days of incubation of the Con A-LBT assay, indicated that P15 was not toxic to Con A stimulated lymphocytes (Figure 6, Table 4). When P15 was added on the 3rd or 4th day of incubation, only a slight reduction
in Con A stimulation occurred so that normal cell division, expressed in $^3$H-TdR uptake, appeared to be proceeding. If P15 were cytotoxic, significant reduction in $^3$H-TdR uptake would have occurred even when added as late as the 3rd or 4th day.

P15 interference of the Con A-LBT assay may be accounted for by several mechanisms. It is possible that P15 acts directly or indirectly on the in vitro cultured lymphocytes leaving them unresponsive to Con A stimulation. Alternately, P15 may interfere with Con A-lymphocyte interaction by blocking the site of Con A attachment at the lymphocyte membrane or by binding to Con A thus preventing it from attaching to the lymphocyte. Further studies will be required to establish the exact cause of P15 interference.

**P15 Characterization**

Part of this study dealt with characterizing the biological, physical and chemical properties of P15. Biologically, P15 was shown to interfere with Con A stimulation of feline lymphocytes, as discussed earlier (Figure 5). Also discussed was the fact that P15 did not appear to be cytotoxic. Of interest was the time requirement for optimal P15 biological interaction with lymphocytes. Knowing at what point P15 interfered with Con A stimulation of lymphocytes would help to identify the cellular function being affected. If P15 acted during the late stages of the Con A-LBT assay, one could postulate that cellular functions such as nucleic acid synthesis were being blocked. Early interference of Con A-LBT might indicate cell membrane involvement.
An experiment was conducted to determine the time required for P15 to have its inhibitory effect on Con A-LBT cultures. Lymphocytes from two cats were stimulated with Con A. P15 was added at various times ranging from 0 to 4 days of cell incubation. The results indicated that adding P15 at 0, 1 or 2 days essentially gave the same level of repression. Adding P15 at day 3 or 4, however, proved to be ineffective at repressing the ConA-LBT cultures (Plate VII). The data indicated that an early event in the Con A stimulation process was being affected by P15. Such actions as interfering with Con A binding or preventing membrane capping could occur in this time period. Further experiments would be necessary to determine which cellular functions are affected.

While characterizing the physical properties of P15, a discrepancy in molecular weight determination occurred. Though P15 migrated as a protein of approximately 15,000 daltons in SDS-PAGE, during liquid column chromatography, purified P15 eluted as a protein of approximately 160,000 daltons molecular weight. Apparently P15, under the conditions of liquid column chromatography, formed a complex of between 10 and 12 units which eluted as a single peak. This complex appeared to be uniform in size in that only a single narrow protein peak was detected. It was still possible that P15 complexes of other sizes existed but were low in concentration and did not form detectable peaks. The void volume peak could be a complex of many more than 12 units of P15. This peak, however, varied in size from run to run (in some preparations, no void volume peak was detected) and probably represented a variety of aggregated protein subunits rather than a uniform complex of P15.
The fact that two different detergents were used in the two methods of determining molecular weight (chromatography and SDS-PAGE) might account for the different affinity properties of the proteins. SDS, being an ionic detergent, might interfere with the formation of P15 complex while Triton X-100, a non-ionic detergent would not. Though highly speculative, it is possible that P15, in its native state, exists as a complex of 10-12 units and that this complex represents a protein subunit of the virus.

To characterize other physical and biochemical properties of P15, a comparison was made with the well characterized murine retrovirus system. In the murine system, two P15 proteins have been identified (46, 47). One was the envelope constituent, P15(E). This protein reportedly has a strong interspecies antigenic determinant and is difficult to maintain in solution because of its tendency to aggregate. The second P15 was a soluble core protein (P15(C)) with primarily species specific antigenic determinants.

Several experiments were conducted to determine if the FeLV-P15 was similar to either of the murine P15 proteins. In the first experiment, Ouchterloney tests were conducted to determine if FeLV, MuLV and purified FeLV-P15 had antigenic cross reactivity. This was shown to be the case when antisera prepared against FeLV was used. Thus, P15 appeared to contain an interspecies determinant. A second test was conducted to determine if the P15 interspecies antigenic determinant cross reacted with the P27 interspecies determinant. In these tests, antiserum specific for P27 did not form a precipitin line with purified P15 but did form a line of identity with FeLV, MuLV and purified P27. It was evident
from this experiment that P15 had an interspecies determinant similar to that reported for MuLV-P15(E), but different from the major interspecies determinant associated with P27.

To ascertain if FeLV-P15 was an envelope protein, two experiments were conducted. The first entailed radioliodinating the surface envelope proteins of whole virus using the lactoperoxidase labeling procedure. A small portion of freshly purified Rickard or Kawakami-Theilen strains of FeLV was iodinated then mixed with unlabeled virus and purified on a second sucrose gradient. By rebanding the virus, it was possible to separate the labeled intact virus from free iodine and labeled and unlabeled soluble protein released from disrupted virus. With both virus strains, though to a greater degree with KT virus, only a protein of 70-80,000 daltons was iodinated (Plates X and XI). By this method, FeLV-P15 was not labeled. In the second experiment, antisera to P15 was assayed for viral neutralizing antibody using the FeLV-81C cell system (see Materials and Methods). Antisera were taken from a rabbit before and after inoculation with P15 plus complete Freund's adjuvant. Only a weak neutralizing antibody titer (1:4) was detected in the post-immunization serum while no neutralizing antibody was measurable in the prebleed serum.

The data from the two experiments seemed to discount P15 being a surface envelope component. The lactoperoxidase labeling experiments indicated that only the 70-80,000 dalton protein and not P15 was exposed at the surface of the virus. The second experiment seemed to bear out this conclusion. Antibody to P15 did not neutralize the virus to any significant degree. The experiments, however, did not discount the
possibility that P15 was buried in the viral envelope such that it would not be exposed to iodine or antibody. It is also possible that the immunosuppressive nature of P15 interfered with the immune response of the rabbit so that only low antibody titers developed. With repeated immunizations it might be possible to attain higher neutralizing antibody titers.

At this time, based on the fact that P15 has an interspecies antigen determinant, the FeLV-P15 protein purified in this study appears to be more like the MuLV-P15(E) than P15(C). Further testing, however, will be required to characterize the exact physical location of FeLV-P15.

The Effect of Pig from FeLV on the Immune Response

In previous experiments, kittens inoculated with heat-killed FL-74 cells developed high titered antibody to FOCMA and were protected from FeSV challenge (77). However, when killed FL-74 cells plus inactivated FeLV were inoculated at the same time, the FOCMA antibody titers were significantly reduced, and protection from FeSV challenge was almost non-existent (78).

The objective of the in vivo P15 experiments was to determine if inoculating P15 into cats would cause abrogation of immunity as did FeLV in an earlier (78). FL-74 cells were included with the inoculum to provide an indicator antigen (FOCMA) for which antibody could be measured using the MCT or IMI assays for FOCMA. The antibody response of cats to FL-74 cells was well characterized in previous studies (77,78), thus providing a basis for comparison with the results of current experiments. The experimental protocol was identical to that used in the FeLV-FL-74 cell vaccine (78) (see Materials and Methods). Four litters of cats were divided into vaccine and control groups. The vaccinated group received
heat-killed FL-74 cells plus P15, while the control group received Tris buffer. Both inocula were emulsified in CFA. An important feature of the experimental design was the use of intra-litter controls. In that the cats used were outbred, this design was necessary to control for genetic variability from litter to litter.

The antibody responses to FOCMA of the FL-74 cell-P15 inoculated cats were similar to that reported for kittens inoculated with FL-74 cell-FelV (78). The highest mean FOCMA antibody titers for both groups was approximately 1:32. By comparison, cats inoculated with FL-74 cells alone reportedly had titers of 1:1024 (78).

The P15 vaccination experiment, however, did not provide enough data concerning abrogation of tumor immunity (protection from FeSV challenge). Only 3 of 7 control animals developed progressive tumors (42%) while in the vaccinated group, 0 of 5 developed progressive tumors. The data would seem to indicate that cats inoculated with FL-74 P15 were protected from FeSV challenge, however, inoculation of FL-74 cells alone was known to induce protection. The question was not whether cats were protected by the FL-74-P15 inoculations but whether the protection was to the same degree as would be instilled by inoculating FL-74 cells alone. It was not possible to answer this question from the data reported above.

Of interest was the fact that the percentage of cats with FelV viremia was approximately the same between the two groups, a point which may be of more significance than the fate of the primary tumor. Though the FL-74-P15 inoculated group was void of progressive tumors, their immunity did not prevent viremia in 2 of 5 cats.
The second *in vivo* vaccination experiment was designed similar to the first except, instead of inoculating control cats with Tris buffer, they were inoculated with killed FL-74 cells. In this experiment, an FeSV challenge of higher infectivity (*in vivo*) was used so that a higher incidence of progressive tumors would be expected.

The cats inoculated with FL-74 cells + P15 developed a 75% (3 of 4) incidence of progressive tumors while cats inoculated with FL-74 cells alone developed only a 20% incidence (1 of 5). The incidence of viremica, though not as dramatic, was also different between the two groups.

When IMI-FOCMA antibody responses were examined, titer differences were noted in the early serum samples during the vaccination period. Cats inoculated with FL-74-P15 had significantly reduced titers in the 6 and 8-week-old serum samples. This response pattern, however, was overcome by the 10th week of age (2 wks after the last inoculation) when both groups attained approximately the same mean titer levels.

The results appeared to indicate that P15 caused a retardation of the normal antibody response to FL-74 cells. The cats were still able to respond immunologically, but at a slower rate. Eventually, comparable IMI FOCMA antibody levels were attained in both groups. The maximum IMI antibody level was probably determined and maintained by a feedback mechanism such that the antibody concentration did not rise above a certain point. Thus, theoretically, even with fewer committed antibody producing cells (which might be the case in the cats inoculated with FL-74-P15), it was possible to attain the same antibody level but at a slower rate.

These experiments seem to implicate P15 as a protein capable of interfering with the normal immune response to tumor cells. In looking at
the in vivo experiments alone, however, one must be careful to include other possible mechanisms to explain the different biological and immunological response patterns between the two vaccine groups. One such mechanism might be antigen competition between P15 and FL-74 cells. Though possible, this mechanism is not likely because of the low quantity of P15 added to the inoculum (100 to 300 ug) as compared to the quantity of FL-74 cells present (5 x 10^8 cells). Another possibility is that immunizing with P15 stimulates blocking antibody that is capable of interfering with the cell mediated destruction of neoplastic cells. At present, however, there is no evidence that P15 is expressed at the cell membrane surface nor is there evidence that P15 contains a target antigen for cell mediated immunity. Further experimentation will be required to determine if blocking antibody is present in this system.

Another point which must be addressed in this discussion is the affect of complete Freund's adjuvant (CFA) on the immune system during the immunization period. CFA is a strong stimulator of both cellular and humoral responses. It is quite possible that the immunosuppressive effect of P15 was being counteracted by the stimulatory effect of CFA. CFA was included in the original protocol so that direct comparisons could be made between previously reported immune and biological responses of cats inoculated with FL-74-FeLV and those of cats on the current experiments.

Cytotoxic Antibody

The next phase of the study involved measuring complement dependent cytotoxic antibody levels in FeLV exposed cats that either became leukemic
or developed resistance. Much evidence has been accumulated to show a direct relationship between the level of FOCMA antibody and the degree of resistance to FeLV (21). An even more definitive proof of the role of antibody in resistance, however, was shown in passive transfer studies. Kittens which received colostrum transfer of antibody from FeLV immune dams were resistant to FeLV infection (45). Antibody, therefore, appeared to play a direct role in resistance to neoplasm.

The mechanism by which antibody might mediate tumor resistance is still speculative. One possibility is antibody-dependent cell cytotoxicity (21) while another is complement-dependent antibody cytotoxicity (21). The scope of this study was limited to measuring complement-dependent cytotoxic antibody levels.

In the initial cytotoxic antibody study, 35 cats of various ages, which had received an oncogenic challenge of FeLV (Table 8) were assayed for cytotoxic antibody at various times after challenge. In the group of cats that developed lymphosarcoma (LSA), the mean of the highest titers was 1:27. These results indicate that the direct relationship that existed between FeLV resistance and IMI-FOCMA antibody might also exist with cytotoxic FOCMA antibody. When serial serum samples from the cats that became resistant to FeLV challenge (LSA negative group) were tested for IMI and MCT FOCMA antibody, 13 of 16 cats were either positive or negative for both tests. Thus, the concordance between the two tests was 81% (Table 9). Further tests using single serum samples from 42 conventional cats from households in which at least one FeLV positive cat had resided, however, showed only a 55% concordance between the two tests (Table 9).
The discrepancy of concordance percentages between the FeLV infected, LSA negative group and the conventional group was explained by the fact that only single serum samples from the conventional group as compared to serial serum samples from the FeLV-infected group were tested. This was shown to be the case when the cats that were MCT and/or IMI positive were compared over a 19 week period post-FeLV challenge. At 6 weeks post-inoculation, the concordance between the two tests was 100% (Figure 14), but by the 19th week, the concordance of positive and negative was reduced to only 56% (Figure 14). Cytotoxic antibody appeared to reach a peak between 6 and 10 weeks post-challenge then to decay in the next 9 weeks. The decay pattern of cytotoxic antibody in the later weeks might be due to lack of antigen stimulation or to conversion from one antibody type to another.

From these experiments, one could conclude that cytotoxic antibody was present and reached a higher titer in FeLV resistant as compared to LSA positive cats. Also, cytotoxic antibody appeared to be short-lived even though FOCHA antibody, as measured by IMI, persisted.

The class of antibody involved in cytotoxicity using a late serum sample from an FeLV exposed cat and found to be primarily 7S although cytotoxic 19S was also detected. This finding was not only important from the standpoint of showing the cytotoxicity test to be antibody mediated but also because it showed that IgG was involved. The presence of IgG indicated a T-cell mediated immune response. Interference with cytotoxic antibody production might be due to interference with T-cell
function. In FeLV infected cats, T-cell functions were suppressed as shown by prolonged allograft rejection responses (81) while primary antibody responses to sheep red blood cells (a B-cell function) were not affected. Interference with T-cell functions may account for the poor cytotoxic antibody responses in FeLV infected cats (Table 8).

In the final experiment of this study, serial serum samples from the cats inoculated with FL-74 cells plus P15 or FL-74 cells alone were tested for the presence of cytotoxic antibody. In the first experimental cat group given FL-74 cells plus P15, only 1 of 6 cats developed cytotoxic antibody (Table 11) and the mean of the highest cytotoxic antibody attained was 1:1.6. In the same group, 5 of 6 cats developed IMI antibody with a mean of highest titers of 1:28 (Table 11).

When the second experimental group of cats, which received FL-74 cells-P15 or FL-74 cells alone, were compared for cytotoxic antibody responses, a significant difference was noted. The cats that received FL-74-P15 developed mean of highest cytotoxic antibody titers of 1:38 while the cats that received FL-74 cells alone developed mean of highest titers of 1:294 (Table 12). The mean of highest IMI-FOCMA antibody titers attained for cats that received the two inoculums, however, were only slightly different (1:91 vs 1:169).

P15 seemed to have its effect, as previously described, in the early stages of the immune response such that the antibody responses were retarded. Ultimately, the level of IMI antibody in the FL-74-P15 cats did reach that of cats receiving FL-74 cells alone.

The reason for the large cytotoxic antibody difference between the two groups was due to cytotoxic antibody being expressed early in the
immune response at the time when P15 had its strongest immunoretardative effect. By the time IMI antibody titers in the FL-74-P15 inoculated cats had attained the same level as cats given FL-74 cells alone (9-10 weeks), cytotoxic antibody had reached its peak level and had started to decay.

An alternate explanation for this type of immune response may be that P15, in a free form, stimulates a different type of antibody not capable of mediating complement dependent antibody cytotoxicity. If P15 were expressed on the cell membrane and was a target antigen for cytotoxicity, non-cytotoxic antibody might interfere with the attachment of cytotoxic antibody. To determine if this is a viable mechanism, tests to measure the presence of antibody to P15 need to be conducted.

Conclusion

The results of this study taken as a whole support a new hypothesis concerning the mechanism of immunosuppression associated with FeLV-FeSV induced neoplasms in cats. It is generally accepted that FeLV-related immunosuppression was a result of the FeLV infection, probably of lymphoid tissues, causing loss of immunologic function (13,21). In this study, however, an FeLV virion protein of 15,000 daltons molecular weight appeared to have immunosuppressive properties by both in vitro and in vivo methods in cat cells. These data along with results from other studies presented earlier in the discussion provided the basis for the hypothesis that FeLV-P15 as an unbound protein released from infected cells or complexed on the surface envelope of the virus particle may provide the initial immunosuppression required, either locally or systemically, for successful onset of feline leukemia. There are undoubtedly many other
parameters affecting FeLV infections (age of cat, strain of virus, environmental factors) which interplay to determine the final outcome of the disease. It is still possible and perhaps probable that FeLV-infected lymphoid tissue become immunologically inoperative. However, in the critical period during the first 4 weeks of infection, before general viremia and widespread infection develops, a mechanism to insure escaping from immunological surveillance would be advantageous to the virus. P15 immunosuppression may provide such a mechanism.

Upon initial contact with FeLV, the lymphocyte would become hyporesponsive. Infected cells shedding virus in their immediate area would be protected from invading effector lymphocyte and would be allowed to proliferate.

If substantiated by further studies, the implications of this hypothesis are far reaching. P15 or P15-like proteins from either ecotropic or endotropic viruses might exist for other tumor systems including those of man and may account, in part, for the inability of the animal to respond immunologically to the tumor cells.
APPENDIX A

FORMULA FOR TKE-D-Tx BUFFER
Appendix A

TKE-D-Tx Buffer, pH 7.6

Tris Base (Tris (hydroxymethyl) amino methane) (Sigma Chemical Co., St. Louis, MO) 0.057 M
Tris HCl (Tris (hydroxymethyl) amino methane) (Sigma Chemical Co.) 0.0115 M
EDTA (Disodium dihydrogen ethylenediaminetetra-acetate dihydrate) (G. Frederick Smith Chemical Co., Columbus, Ohio) 0.01 M
KCl (Potassium chloride) (Mallinckrodt, Inc., St. Louis, MO) 0.6 M
DTT (Dithiothreitol) (Bio Rad Laboratories, Richmond, CA) 0.01 M
Triton X-100 (Sigma Chemical Co.) 1.0%

TKE-D-Tx Buffer (Modified) pH 7.6

Tris Base 0.0115 M
Tris HCl 0.0023 M
EDTA 0.01 M
KCl 0.3 M
DTT 0.01 M
Triton X-100 1.0%
APPENDIX B

FORMULA FOR TKE-D BUFFER
Appendix B

TKE-D Buffer, pH 7.6

Tris Base (Tris (hydroxymethyl) aminomethane) (Sigma Chemical Co., St. Louis, MO) 0.0115 M

Tris HCl (Tris (hydroxymethyl) aminomethane hydrochloride) (Sigma Chemical Co.) 0.0023 M

EDTA (Disodium dihydrogen ethylenediaminetetra-acetate dihydrate) (G. Frederick Smith Chemical Co., Columbus, OH) 0.01 M

KCl (Potassium Chloride) (Mallinckrodt, Inc., St. Louis, MO) 0.3 M

DTT (Dithiothreitol) (Bio Rad Laboratories, Richmond, CA) 0.01 M
APPENDIX C

FORMULA FOR VERONAL BUFFER
Appendix C

**Veronal Buffer, pH 7.35**

- Sodium chloride (Fisher Scientific Co., Fair Lawn, NJ) 0.14 M
- Sodium-5,5-diethylbarbiturate (Mallinckrodt Chemical Works, St. Louis, MO) 0.0055 M
- Hydrochloric acid (J. T. Baker Chemical Co., Phillipsburg, NJ) 0.003458 N
- Magnesium chloride (Fisher Scientific Co.) 0.0005 M
- Calcium chloride (Fisher Scientific Co.) 0.00015 M
APPENDIX D

PURIFICATION OF FELINE LEUKEMIA VIRUS
Appendix D

Purification of Feline Leukemia Virus

In order to accomplish the objectives of this study, large amounts of purified virus were needed for purification of subviral proteins. The initial step in recovering virus from tissue culture media, usually a concentration procedure which reduces the volume of these fluids to a size that could be conveniently accommodated by conventional gradient centrifugation. Some of the methods used to reduce the volumes include (NH₄)₂SO₄ precipitation (18, 84, 97), polyethylene glycol concentration (63, 65, 97, 110), Con A precipitation (101) and centrifugation (72, 97). All of these techniques are effective means of concentrating virus but may have a dilatory effect on viral infectivity. Loss of infectivity may be due to disruption or alteration of the surface membrane structures characteristic of C-type RNA viruses. Using unaltered virus (infectious), if possible, was felt to be important in characterizing any possible immunosuppressive properties that whole virus might have.

A relatively new method of concentrating virus using the principle of continuous flow molecular filtration appeared to have promise as an alternate procedure.

With continuous flow molecular filtration, fluids are continuously passed between two membrane filters. Molecules with molecular weights
smaller than the exclusion size of the membrane can pass across the membrane (filtrate) while larger molecules are retained (retentate) and cycled back into the starting reservoir. As the fluids recycle through the filtration unit, the retentate volume continuously decreases.

For this study, the molecular filtration system (Millipore, Bedford, MA) was equipped with one five-square-foot membrane cassette having an exclusion size of approximately 1,000,000 daltons. A 20 liter closed flask with input and output connections was attached to the unit and a high volume paristaltic pump (Cole-Parmer, Chicago, IL) was placed in line on the output side of the flask. The discard filtrate was collected in a second flask. The filtration unit was decontaminated by pumping 2% formalin in normal saline into the system and allowing it to stand for one hour, or continuously during periods when the unit was not in use. Just prior to use, the formalin was flushed from the unit by pumping three liters of sterile distilled water through the system. During operation, the molecular filtration system was housed in a 40 constant temperature room.

The F422 or the FL-74 cell lines used to produce virus were grown in suspension culture and used as the source of the Rickard Kawakami-Theilen strain of feline leukemia virus respectively. Cells were seeded at a concentration of 1.6-1.8 x 10^6 cell/ml in 2 liter roller bottles, containing 200 ml of McCoy's 5-A suspension medium (GIBCO, Grand Island, NY) with 20% fetal bovine serum (FBS, Reheis Chem. Co., Phoenix, AZ). At 3 days, the cultures were fed with 200 ml of fresh McCoy's 5-A media containing 15% FBS and at 5 days the tissue culture fluids were separated
from the cell by centrifugation at 400 x G for 10 min. Usually 24 roller bottles containing a total of 9600 ml were processed each time.

Tissue culture fluids were concentrated by recycling fluids through the system until the volume was reduced 10 to 100X. The entire concentration step was accomplished in approximately 3 hours. Twenty ml of the concentrate was then applied to the top of each 0 to 50% preformed sucrose gradient (68 ml) in TNA (0.01 mTris, 0.1 ml NaCl, 0.001 m EDTA) buffer, pH 7.6. The preformed gradients were produced in 1.5 x 4" cellulose nitrate centrifuge tubes (Beckman, Palo Alto, CA) using the Isco Model 570 gradient former (Isco, Lincoln, NE). The gradients were centrifuged at 4° C for 2 hours in a 21 rotor at 45,000 x G using a model L2 65B ultracentrifuge (Beckman). The opaque virus band was harvested from the gradients by bottom puncture and collection of 2.5 ml fractions (Fig.17). The purified virus suspension was dialyzed overnight against 50 volumes of TNE buffer to remove the sucrose.

Viral infectivity assays (Table 13) revealed no loss of infectivity during concentration. However, after one banding, the infectivity titer was reduced approximately one log10. No infectious virus was detected in the filtrate.

Loss of infectivity of oncornaviruses during purification has been a serious problem. The delicate membrane structures are known to be easily denatured by changes in osmotic pressure, the sheering forces of centrifugation and freeze-thawing (70,71). By using the molecular filtration system, it was possible to concentrate virus from large volume tissue culture fluid as much as 100-fold without measurable loss of
Figure 17. Feline leukemia virus tissue culture fluids, concentrated 10X, were purified on 0 to 50% sucrose gradients. Twenty ml of viral concentrate was layered onto the 68 ml gradient in 1.5 x 4 in. cellulose nitrate centrifuge tubes. The gradients were centrifuged for 2 hours at 45,000 X G in a 21 rotor. The virus band formed between 32 and 36% sucrose (density 1.14 to 1.16) optical density, o-o-o % sucrose.
Table 13. Infectivity of Feline Leukemia Virus Preparations Taken at Various Steps during Purification

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>Titer FFU/ml</th>
<th>Concentration Factor</th>
<th>Infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unconcentrated T.C. Fluids</td>
<td>9600</td>
<td>$1.32 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>100 x Concentrated T.C. Fluids</td>
<td>100</td>
<td>$1.20 \times 10^6$</td>
<td>96</td>
</tr>
<tr>
<td>Once Banded Virus</td>
<td>80</td>
<td>$1.82 \times 10^5$</td>
<td>120</td>
</tr>
<tr>
<td>Filtrate</td>
<td>9500</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>
infectivity. Following density gradient centrifugation, however, viral infectivity was reduced by approximately one log10.

We have routinely processed 10 liter batches of virus by the molecular filtration procedure and find the method superior to other techniques. The fact that the entire procedure can be accomplished in a single day and that the system is relatively inexpensive to operate makes it ideal for routine virus purification. Because the molecular filtration system is closed during operation, the concentrate remains sterile. The closed system also prevents aerosolized infectious virus from escaping. This aspect is of particular importance when moderate or high risk agents are being purified.
APPENDIX E

MICROCYTOTOXICITY ASSAY (MCT)

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Evaluation of Various Animal Species of Complement .......... 144
Appendix E

Measuring cytotoxic antibody in the cat system required developing a test which was specific for tumor cells but did not react with possible allogenic antigens present on the target cell. In that the cats used in these studies were outbred, the possibility of alloantibody was real.

Characterization of MCT Assay

Before the MCT assay could be used as a reliable test for cytotoxic antibody in the cat, it first had to be characterized as to its antigen specificity and complement dependency as well as to the species of complement required.

Specificity of MCT Assay for FOCHA

As a control against the possibility that cat sera contained natural antibody to allogeneic antigens present on the target cells, serum from 137 normal cats from The Ohio State University SPF cat colony were tested by the MCT assays. None of these sera were positive by either test.

The possibility still remained that antibody detected by MCT might be stimulated by antigens present in the virus inoculum rather than by antigens derived de nova due to an oncogenic event. This point was clarified by an experiment in which an adult female cat (Q150) was contact-exposed to its nursing kittens which had been experimentally infected with FeLV-R at birth. The dam was not inoculated. Figure 18 represents the MCT antibody response of Q150. Circulating antibody to FOCHA appeared after 80 days of contact, and persisted for over 100 days.
Figure 18. Cytotoxic antibody response of a female cat (Q150) during contact exposure to its nursing kitten which had been inoculated with an oncogenic dose of Rickard FeLV at birth. Day 0 was the day of kitten birth. Q150 was an SPF cat which had never been inoculated or vaccinated with feline oncornaviruses.
To confirm the specificity of the MCT assay, Q150 serum was adsorbed with $8 \times 10^6$ feline lymphocytes and with a 50% feline tissue (kidney, liver) homogenate with no loss of MCT activity. However, when the same cat serum was adsorbed with $8 \times 10^6$ FL-74 cells, all cytotoxic activity to FL-74 cells was lost (Table 14).

**Complement Dependency of MCT Assay**

The complement dependency of the MCT assay was established in tests where varying dilutions of complement were used. As the complement dilution increased, the cytotoxic titers of the reference serum decreased (Table 15).

Complement alone was not cytotoxic to FL-74 cells. The possibility that the specific cytotoxic factor was of complement origin and that feline sera contained a secondary factor, i.e. heat stable components of complement, was ruled out by results from MCT tests in which the order of reagents was reversed, and added separately. In these tests, complement was incubated with FL-74 cells before the MCT positive feline antibody was added. The cells were washed free of complement before incubation with positive cytotoxic serum. All tests performed in this manner were negative (1:12).

**Evaluation of Various Animal Species of Complement in the MCT Assay**

Rabbit, cat and guinea pig complements were tested for lytic potency in the MCT assay. Rabbit complement demonstrated the strongest toxic activity, whereas guinea pig complement was weakly cytotoxic and cat complement had no detectable cytotoxicity (Table 15). When trypan blue dye
Table 14. Effects of Adsorption on Antibody Titer as Determined by Microcytotoxicity and Indirect Membrane Immunofluorescence Tests on FL-74 Cells (a)

<table>
<thead>
<tr>
<th>Material Used for Adsorption</th>
<th>Antibody Titer by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCT</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>1:16</td>
</tr>
<tr>
<td>Feline Lymphocytes</td>
<td>1:16</td>
</tr>
<tr>
<td>Feline Tissue Homogenate</td>
<td>1:16</td>
</tr>
<tr>
<td>FL-74 Cells (2 x 10&lt;sup&gt;6&lt;/sup&gt; cell/ml)</td>
<td>&lt;1:2</td>
</tr>
</tbody>
</table>

(a) Serum from Rickard FeLV contact exposed adult.
(b) Not tested.

Centrifuged pellets of each absorbant (0.1 ml) were mixed with 1 ml of serum and incubated at 40°C for 24 hours.
Table 15. FL-74 Microcytotoxicity Test: Comparison of Complements from Various Species.

<table>
<thead>
<tr>
<th>Dilution of Complement (a)</th>
<th>Cytotoxic Antibody Titer with Complement from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbit</td>
</tr>
<tr>
<td>1:2</td>
<td>1:32</td>
</tr>
<tr>
<td>1:4</td>
<td>1:8</td>
</tr>
<tr>
<td>1:8</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

(a) All complement species controls were non-cytotoxic at ≥ 1:2.
exclusion, rather than lysis, was used to evaluate cytotoxicity of FL-74 cells, the results were similar.
Bibliography


