ABSTRACT

The 15,000-molecular-weight polypeptide (p15) of feline leukemia virus (FeLV) was shown to impair normal lymphocyte function in vitro and to abrogate immunity to feline oncornaivus disease in vivo. FeLV p15 suppressed concanavalin A-induced blast formation of normal feline lymphocytes by 68%, while other virion proteins had no effect. p15 suppression was not due to toxicity, nor was p15 a competitive inhibitor of concanavalin A binding. Capping of receptors for concanavalin A on normal feline lymphocytes was also inhibited by either inactivated FeLV or FeLV p15.

Groups of cats were immunized with either killed feline oncornaivus-associated cell membrane antigen bearing tumor cells or tumor cells plus FeLV p15. After challenge with feline sarcoma virus, three of four p15-treated cats developed progressive fatal fibrosarcoma as compared to one of five non-p15-treated cats. The cats receiving p15 also had lower cytotoxic antibody titers against feline oncornaivus-associated cell membrane antigen (mean peak titer, 1:6) than did the non-p15 group (1:74). These data support the hypothesis that the immunosuppression in cats infected with FeLV is mediated by FeLV p15.

INTRODUCTION

The immunosuppression associated with retrovirus infections of several species (for review, see Ref. 5) generally has been attributed to impaired lymphoid function secondary to viral infection of lymphoid tissues. Several recent reports, however, indicated that impaired immune function can be induced by inactivated or attenuated retroviruses in the absence of infection. In one study, mice inoculated with attenuated MuSV2 and later challenged with infectious MuSV had a higher incidence of malignant disease than did control mice (24). Likewise, immunization of cats with UV-inactivated FeLV caused abrogation of immunity to fibrosarcoma induced by challenge with FeSV (19, 22). Recent in vitro studies have shown that inactivated murine and feline leukemia viruses suppress at least one normal lymphocyte function, that being phytomitogen-induced LBT (9, 12).

The present study was undertaken to investigate further the lymphocyte unresponsiveness caused by exposure to inactivated FeLV and to identify possible subviral component(s) which may have a similar effect.

MATERIALS AND METHODS

Cats. All cats used in vaccination experiments and as blood donors for the LBT assay were taken from The Ohio State University, Department of Veterinary Pathobiology, SPF cat colony (21).

LBT Assay. The LBT assay was a modification of that described by Cockerell et al. (2). Enriched lymphocyte preparations were obtained by centrifugation of heparinized blood through Ficoll-Hypaque gradients. One-tenth ml of the cell suspensions (1 x 10^8 cells/ml) was mixed in microtest plates (Falcon Plastics, Oxnard, Calif.) with 0.05 ml of an optimal concentration of Con A (Sigma Chemical Co., St. Louis, Mo.) (10 μg/well) and 0.05 ml of the protein being assayed for LBT inhibition. The plates were incubated at 37° for 5 days. The protein preparations being assayed for LBT inhibition previously had been dialyzed 3 times against 400 volumes of minimal essential medium (suspension) containing 1% antibiotics. In the Con A control wells, the inhibitory protein 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 hr of incubation, 0.1 ml of medium containing 0.5 μCi of [3H]dThd (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added. Cells were collected on glass filter paper with a semiautomatic multiple processor (Otto Heller Co., Madison, Wis.) and assayed for radioactivity by liquid scintillation counter. Net cpm of quadruplicate wells were averaged to obtain mean cpm.

Virus. Production and purification of Rickard FeLV had been described (17). The only addition to the procedure was that purified virus was banded a second time on linear sucrose gradient.

Virus Fractionation. The technical aspects of the procedure for purification of p15 from FeLV are described in another report. In this method (see Chart 1), purified FeLV (10^9 particles/ml) was twice freeze-thawed and centrifuged
at 100,000 x g for 90 min with a SW27 rotor in a Beckman L2-65-B ultracentrifuge (Beckman Instruments, Palo Alto, Calif.). The liquid was collected and stored at -90° (Fraction A). The protein pellets were resuspended and partially solubilized in 2 ml of TKE-D-Tx buffer, pH 7.2, and incubated at 37° for 1 hr. The soluble protein was separated from the insoluble material by centrifugation at 100,000 x g for 90 min. The insoluble pellet was resuspended in TKE-D-Tx buffer and stored at —90° (Fraction B). The liquid phase solubilized in 2 ml of TKE-D-Tx buffer, pH 7.2, and incubated at 37° for 1 hr. The soluble protein became insoluble. Follow

Fig. 1. PAGE analysis of FeLV protein. Electrophoresis was in 7.5% polyacrylamide gel in the presence of 0.1% SDS. 1, FeLV; 2, Fraction A; 3, Fraction B; 4, Fraction C; 5, Fraction D; 6, purified p15; 7, purified p27; 8, ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400.

Purification of FeLV p15. p15 was purified from Fraction D by liquid column chromatography using a 2.5- x 90-cm Sephacryl 200 (Pharmacia, Uppsala, Sweden) column equilibrated with modified TKE-D-Tx buffer (0.01 M Tris-HCl, 0.3 M KCl, 0.01 M EDTA, and 1% Triton X-100), pH 7.2. A small amount of Fraction D was radiolabeled with 125I using the chloramine-T method (10) to facilitate identification of protein peaks. A single major peak of highly purified p15 was resolved by electrophoresis in TKE-D-Tx buffer.

Purification of FeLV p27. p27 was purified from Fraction C by liquid column chromatography using a 2.5- x 90-cm Sephadex G-150 column equilibrated with 0.01 M Tris-0.3 M KCl-0.01 M EDTA-0.01 M dithiothreitol, pH 7.2.

Preparation of FL-74 and FL-74 plus p15 inocula. The FL-74 cell line is feline lymphoblastoid cells, originally established by Theilen et al. (23) from a FeLV-induced lymphoma. FL-74 cells were grown in suspension cultures in 2-liter roller bottles as described previously (20). Cultures were initiated at a concentration of 1.6 to 1.8 x 10^6 cells/ml with 200 ml/bottle. Three days after transfer, an additional 200 ml of McCoy’s Medium 5A containing 15% fetal calf serum were added. At 5 days, the cells were harvested by centrifugation; final cell counts were 7 to 13 x 10^8 cells/bottle.

FL-74 cells were killed but not lysed by heating in a 56° water bath for 4 min by the procedure of Heding et al. (13). The FL-74 cell inoculum was prepared by emulsifying 5 x 10^9 heat-killed FL-74 cells (approximately 0.5 ml) with 0.5 ml 0.01 M Tris-HCl, pH 7.0-0.1 M NaCl-0.001 M EDTA buffer and 1 ml complete Freund’s adjuvant. The FL-74 cell plus p15 inoculum was prepared by emulsifying 5 x 10^9 heat-killed FL-74 cells (0.5 ml) and either 300 or 100 μg of p15 (0.5 ml) with 1 ml complete Freund’s adjuvant. Cats were given i.m. injections 3 times at biweekly intervals.

Lymphocyte Viability. Viability of lymphocytes was determined by their capacity to exclude trypan blue dye.

Polyacrylamide Gel Analysis. PAGE was performed in 12-cm slab gels using 7.5% acrylamide with 0.375% bisacrylamide in a Tris-acetate buffer containing 0.205 M Tris, 0.205 M acetic acid, and 0.1% SDS, pH 6.6. Protein samples were mixed with equal volumes of 0.2 M Tris, 0.02 M acetic acid, 0.1% SDS, and 0.1 M dithiothreitol and heated to 100° for 3 min prior to electrophoresis. Gels were electrophoresed for 5 hr at 100 V.

Virus Inactivation. UV inactivation of FeLV was accomplished with a surface dose rate of 150 ergs/sq mm/sec for an accumulated total of 35,000 ergs/sq mm (25).

51Cr Cytotoxicity Assay. 51Cr-labeled FL-74 cells were prepared by incubating 8 x 10^6 cells in 1 ml of McCoy’s Medium 5A (3-day-old cultures) with 0.2 ml of Na^51CrO_4 solution (approximately 200 μCi) at 37° for 1 hr with constant agitation. After 1 hr, the labeled cells were washed with 40 ml of cold McCoy’s Medium 5A by centrifugation. The cell pellets were resuspended in 40 ml of cold media and incubated at 4° for 30 min. Finally, the cells were centrifuged, and the pellets were resuspended in 1 ml of media.

Serial 2-fold dilutions of feline serum (25 μl) were made in U-bottomed microtiter plates (Cooke Engineering, Alexandria, Va.) with 25-μl Cooke microdiluters in McCoy’s Medium 5A containing 10% fetal calf serum as diluent. Added to the test serum were 50 μl of rabbit serum as a source of complement (diluted 1:2) and 25 μl containing 2 x 10^6 51Cr-labeled FL-74 cells. The plates were incubated at 37° for 1 hr with periodic agitation. At the end of the incubation period, the plates were centrifuged at 600 x g for 5 min in an International PR-6 centrifuge with No. 276 head (International Equipment Co., Needham Heights, Mass.) and carrier bucket (Cooke Engineering). Tissue culture fluid containing released 51Cr was collected using a Titertek supernatant collection system (Flow Laboratories, Rockville, Md.). The amount of 51Cr released was determined by γ scintillation counting with a Biogamma II counter (Beckman Instruments, Palo Alto, Calif.). The cytotoxic antibody titer was the reciprocal of the highest serum dilution that produced 50% release of cell-bound 51Cr.

Controls included target cells incubated with either com-
plement alone or test serum alone.

**Indirect Membrane Immunofluorescence Test for FOCMA.** The indirect membrane immunofluorescence test for antibody to FOCMA was developed by Essex et al. (8). 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 Hanks' balanced salt solution and resuspended at a concentration of $1 \times 10^6$ cell/ml. Serial 2-fold dilutions of sera were made in microtiter plates using 50-µl diluters. Fifty µl of cell suspension were added to each well, and the plates were incubated for 30 min at 37°C. The plates were then centrifuged, and the cell pellet was washed twice with Hanks' balanced salt solution.

Fifty µl of FITC-conjugated rabbit antiserum to γ-globulin (Miles Laboratories, Inc., Elkhart, Ind.) were then added to each well and incubated as above. The cells then were washed twice and examined for membrane fluorescence with a Zeiss Universal fluorescence microscope (Carl Zeiss Inc., New York, N. Y.). 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 a modification of the indirect immunofluorescence procedure developed by Hardy et al. (11). The presence of FeLV group-specific antigens in circulating leukocytes and/or platelets correlates the presence of infectious virus in plasma. Blood smears were fixed in absolute methanol. 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 (14). The secondary reagent was FITC-conjugated rabbit anti-goat γ-globulin (Miles Laboratories, Inc.). The counterstain was 0.5% Evans blue in H₂O. Cats were considered to be FeLV viremic if fluorescence was detected in any of the blood leukocytes.

**FITC-Con A Labeling of Lymphocytes for Capping.** The procedure used to assay capping of Con A receptors on feline lymphocytes was that of Dunlap et al. (7). Lymphocytes obtained by Ficoll-Hypaque gradient centrifugation of peripheral blood were suspended at a concentration of $2 \times 10^6$ cells/ml in minimum essential media (Grand Island Biological Co., Grand Island, N. Y.) containing FITC-Con A (Miles-Jeda, Israel), 50 µg/ml. Various concentrations of UV-inactivated FeLV or purified FeLV p15 were incubated with the cells for 15 min at 37°C. Controls were incubated without virus or virus protein. Following incubation, the cells were washed twice with fresh minimum essential media. Capping was determined by examining the cell suspensions by UV microscopy. The percentage of cells undergoing capping was calculated from counts of 100 to 200 cells.

**RESULTS**

**Inhibition of LBT with Inactivated FeLV and Subviral Components of FeLV.** Inactivated FeLV and subviral fractions of FeLV were assayed for their ability to inhibit Con A-stimulated LBT. Four subviral fractions of FeLV were derived, based on solubility, according to the fractionation scheme shown in Chart 1 (see “Materials and Methods”). Crude fractions of viral protein were used in this initial study so that minor components in the whole virus prepa-
ratiation would not be lost in the process of ultrapurification of specific proteins.

Lymphocytes of SPF cats were incubated with an optimum concentration of Con A and with either inactivated FeLV or one of the 4 subviral fractions. The amount of [3H]dThd incorporated into these cultures was compared to that of cultures from the same cats incubated without the indicated protein (Table 1). Inactivated FeLV reduced the incorporation of [3H]dThd by an average of 43% (Table 1). The Fraction D preparation also repressed LBT, in this case, by an average of 41% (Table 1). Fractions A, B, and C, however, did not significantly repress LBT (Table 1).

**Polyacrylamide Gel Electrophoretic Analysis of Crude FeLV Fractions.** Protein samples from whole FeLV and Fractions A, B, C, and D were analyzed by SDS-PAGE to determine their protein composition. Fraction A was composed of primarily higher-molecular-weight proteins (Fig. 1). Fraction B contained a mixture of proteins which were insoluble under the extraction conditions in TKE-D-Tx buffer. Fraction C contained p27 as its chief component in addition to several minor component proteins. Fraction D contained p15 as its major component. Ultrapurification of p15 from Fraction D and p27 from Fraction C were accomplished as described in “Materials and Methods.”

**Inhibition of LBT by Purified p15 and p27.** Highly purified p15 or p27 were added to assays as previously done with virus to determine if either protein had inhibitory properties similar to whole FeLV or to FeLV Fraction D (Table 2). Lymphocytes from 6 normal SPF cats were used for p15, and those from 2 normal SPF cats were used for p27. p15 reduced the level of stimulation by a mean value of 68%. p27 inhibited only 18%.

**Lymphocyte Viability after incubation with p15.** To determine if the reduction of [3H]dThd uptake in lymphocytes treated with p15 was due to a cytotoxic effect of the protein, lymphocytes from 2 cats were incubated under LBT conditions, but without Con A, for 5 days with 5 μg of p15 per well. In each case (Table 3), lymphocyte counts for tests incubated with p15 remained at approximately the same number per well as did those of controls without p15.

**Test for Competitive Inhibition of Con A by p15.** To determine if p15 was a competitive inhibitor for Con A binding, the amount of Con A per test was increased by a factor of 2.5 and 5 (Table 4). The suppressive effect of p15 was not altered by higher concentrations of Con A.

**Effect of Inactivated FeLV and p15 on Capping of FITC-Con A-treated Lymphocytes.** Ten to 33% of normal cat lymphocytes treated with FITC-Con A underwent visible capping after a 15-min incubation at 37°C. Capping was reduced when either UV FeLV or FeLV p15 was included in the incubation mixture (Table 5). Lymphocytes from 6 cats incubated with FITC-Con A plus FeLV (210 μg/ml) had 2 to 7% capping (50 to 80% reduction), whereas lymphocytes from 4 cats incubated with FITC-Con A plus p15 (50 μg/ml) had 5 to 8% capping (50 to 83% reduction).

Incubation of lymphocytes with UV FeLV or p15 did not interfere with FITC-Con A binding. The number of fluorescing cells was not diminished as compared with controls (data not shown).

**Immunosuppressive Effects of p15 in Cats.** This aspect of the study was undertaken to determine if inoculating p15 into cats that were simultaneously being immunized with...
killed tumor cells (FL-74 cells) would alter the immune response of the cats to the tumor cells. The criteria used to evaluate the tumor cell immune response were (a) cytotoxic antibody response to FOCMA and (b) resistance to FeSV carcinogenesis.

Kittens from each of 2 litters were apportioned equally into either of 2 treatment groups receiving (a) heat-killed FL-74 cells or (b) heat-killed FL-74 cells plus FeLV p15. All kittens received 3 inoculations on a biweekly schedule beginning at 4 weeks of age. At 12 weeks of age, all cats were challenged with FeSV at a dose previously shown to produce a 75% incidence of progressive fibrosarcoma in age-matched cats.

Table 5
Effect of UV FeLV and FeLV p15 on the capping reaction of Con A receptors on cat lymphocytes

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a Percentage of cells undergoing capping for control cultures from each cat incubated with FITC-Con A but without inhibitory protein.
b NT: not tested.

The tumor incidence in the cats given p15 plus FL-74 cells (3 of 4) was greater than that in cats vaccinated with FL-74 cells alone (1 of 5) (Table 6). The mean peak cytotoxic antibody titers before and after challenge differed significantly between the 2 groups. The highest mean titer for the cats receiving p15 plus FL-74 cells was 1:6 before challenge and 1:3 after challenge as compared with 1:74 and 1:18, respectively, for the cats receiving FL-74 cells alone. Chart 2 shows the mean cytotoxic antibody titers of the 2 groups of cats over the 8-week period prior to challenge. The cytotoxic antibody titers were significantly different throughout this period. The incidence of viremia in the 2 groups was not significantly different (4 of 4 for the p15 plus FL-74 cell group and 3 of 5 for the cats given FL-74 cells alone). High cytotoxic antibody titers in cats given FL-74 cells alone appeared to correlate with capacity to clear FeLV viremia (Table 6).

DISCUSSION

In this study, inactivated whole FeLV and subviral components of FeLV were analyzed for their inhibitory effect on the Con A LBT response using lymphocytes from normal SPF cats. Interference with lymphocytic function as determined in the LBT assay was found with whole virus and with Fraction D containing the p15 protein of FeLV, but not with other protein fractions of FeLV including purified p27. The inhibition appeared not to be due to p15 toxicity or to competition for Con A binding sites. Although Fraction B appeared to contain p15 by PAGE analysis (Fig. 1), its insoluble form, due to association or aggregation with other proteins, apparently prevented the p15 from having biological effects. Alternately, the p15 protein appearing in Fraction B may be a different protein than that in Fraction D, but having the same migrating pattern in SDS-PAGE. The fact that 2 p15 proteins (p15E and p15C) have been
described for murine leukemia virus (15, 16) lends credence to this possibility.

FeLV and FeLV p15 caused alterations of cell membrane functions as indicated by interference of FeLV p15 with the normal capping process.

The artificial nature of the in vitro test for lymphocyte functions makes it difficult to compare inhibition of LBT with in vivo immunosuppression associated with FeLV infections. Therefore, in order to determine if p15 had biological activity in vivo, cats were given injections of p15 at the same time they were being vaccinated with heat-killed FL-74 cells. Vaccinating cats with FL-74 cells had previously been shown to stimulate high FOCMA antibody levels and to protect cats from FeSV challenge (18) as was evident in the control group of cats in this study. However, when p15 was included with the FL-74 cell vaccine, the normal cytotoxic antibody response to FOCMA was reduced significantly, while the incidence of progressive fibrosarcoma was significantly increased after FeSV challenge.

The combined results of the in vitro and in vivo studies indicate that FeLV p15 causes alterations in lymphocyte function which ultimately can affect the magnitude of the immune response. This immunosuppressive property of p15 would be beneficial to FeLV in terms of survival in nature and pathogenesis of infection and disease. p15 expression at the initial site of viral replication could suppress local immunological functions, allowing FeLV-infected cells to escape surveillance. With the development of FeLV viremia, immunosuppression would become systemic and enhance the growth and survival of neoplastic clones of cells. Other investigators have detected immunosuppressive substances in serum and tumor homogenates from retrovirus-infected animals (1, 3, 4, 6). Therefore, it is plausible that FeLV and perhaps other exogenous (and possibly endogenous) retroviruses carry a structural component which serves the second role of interfering with local immune function at the site of tumorigenesis and eventually may mediate overall immunosuppression.

ACKNOWLEDGMENTS

We gratefully acknowledge Ken Milliser, Jo Ellen Dunlap, and Lilly Romvary for their excellent technical assistance.

REFERENCES

Immunosuppressive Properties of a Virion Polypeptide, a 15,000-Dalton Protein, from Feline Leukemia Virus


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