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Authors
Pedersen, NC
Johnson, L
Birch, D
et al.

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POSSIBLE IMMUNOENHANCEMENT OF PERSISTENT VIREMIA BY FELINE LEUKEMIA VIRUS ENVELOPE GLYCOPROTEIN VACCINES IN CHALLENGE-EXPOSURE SITUATIONS WHERE WHOLE INACTIVATED VIRUS VACCINES WERE PROTECTIVE

N.C. PEDERSEN, 1 L. JOHNSON, 1 D. BIRCH, 2 and G.H. THEILEN 2

1Departments of Medicine and 2Surgery, School of Veterinary Medicine, University of California, Davis, California 95616 (U.S.A.)

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ABSTRACT

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Kittens immunized with purified native FeLV-gp70 or -gp85 envelope proteins developed ELISA, but not virus neutralizing, antibodies in their serum to both whole FeLV and FeLV-gp70. Kittens vaccinated with envelope proteins and infected with feline sarcoma virus (FeSV) developed smaller tumors than nonvaccinates, but a greater incidence of persistent retroviremia. Similarly, FeLV-gp70 and -gp85 vaccinated kittens were more apt to become persistently retroviremic following virulent FeLV challenge exposure than nonvaccinates.

Kittens vaccinated with inactivated whole FeLV developed smaller tumors after FeSV inoculation and had a lower incidence of persistent retroviremia than nonvaccinates. The protective effect of inactivated whole FeLV vaccine against persistent retroviremia was also seen with FeLV challenge-exposed cats. Protection afforded by inactivated whole FeLV vaccine was not associated with virus neutralizing antibodies, although ELISA antibodies to both whole FeLV and FeLV-gp70 were induced by vaccination.

INTRODUCTION

Feline leukemia virus (FeLV) infection is one of the most devastating diseases of domestic cats. As many as 1 to 6% of free-roaming household cats and 30% of cats in some catteries and multiple cat homes are chronically infected (Hardy, 1981; Pedersen et al., 1980). Feline leukemia virus infection induces a myriad of different diseases, and is directly or indirectly responsible for about one-third of all chronic illness in domestic cats in some parts of the United States (Pedersen et al., 1980). Prevention of FeLV infection is, therefore, a high priority for veterinary researchers. Similar to other viral infections, vaccination appears to be the most logical approach to prevention. The fact that a majority of older cats in nature recover from viremia after infection indicates that cats can mount an active immune response to FeLV under appropriate conditions (Hardy, 1981; Pedersen et al., 1977, 1978).
Over the last decade there have been numerous reports dealing with FeLV vaccination. These included the use of crude vaccines made up of soluble products of virus infected cells (Lewis et al., 1981; Mathes et al., 1981), whole live or dead FeLV infected tumor cells (Grant et al., 1980; Jarrett et al., 1975, 1976; Olsen et al., 1976), inactivated whole FeLV (Hoover et al., 1977; Olsen et al., 1977; Pedersen et al., 1978; Yohn et al., 1976), live virus (Hardy, 1981; Pedersen et al., 1978), and viral envelope subunit proteins (Salerno et al., 1978). The efficacy of these various vaccines was tested by measuring for the acquisition of virus neutralizing antibodies (Salerno et al., 1978), by challenging cats with fibrosarcoma virus preparations (Grant et al., 1980; Olsen et al., 1977; Yohn et al., 1976) or virulent FeLV (Hardy, 1981; Hoover et al., 1977; Jarrett et al., 1975, 1976), or by contact exposure with chronically infected cats (Pedersen et al., 1978). Some of these vaccines have protected cats against infection with virulent virus, while others have not.

The present emphasis of most FeLV vaccine research is on genetically engineered viral subunit proteins, either in the form of bacterial or yeast expressed products or synthetic oligopeptides. Among the various virus structural proteins, the 70,000 dalton envelope glycoprotein (FeLV-gp70) has been thought to be responsible for evoking protective immunity in cats (Hardy, 1981; Salerno et al., 1978). In mice, murine leukemia virus gp71 covalently linked to its 15,000 dalton transenvelope anchoring protein (gp71-pl5E or gp85) has been shown to be even more immunogenic than gp71 alone (Hunsmann et al., 1981). Using genetic engineering, all or parts of these proteins can be synthesized by bacteria or yeast, or chemically produced as small immunogenic polypeptides containing pertinent antigenic epitopes (Lerner, 1983; Nunberg et al., 1984).

There is some previous experience with retrovirus subunit vaccines. Hunsmann and coworkers (1975, 1981) have immunized mice against Friend murine leukemia virus using purified viral envelope proteins gp71 or gp85 complexes. A similar preparation, FeLV-gp85, has been used to immunize cats against Snyder-Theilen fibrosarcoma virus (ST-FeSV) induced tumors (Hunsmann et al., 1983). Feline leukemia virus gp85 was immunogenic in cats, and immunized cats developed smaller tumors than non-vaccinated animals. No data was presented, however, on the retroviremia that accompanied tumor growth. Salerno and associates (1978), failed to induce neutralizing antibodies in cats with a dose of purified FeLV-gp70 that produced good neutralizing antibody titers in guinea pigs. The efficacy of this vaccine against virulent FeLV challenge exposure was not tested, however.
It is essential for the success of genetically engineered subunit FeLV vaccines to first determine whether native viral subunits are immunogenic in themselves, and more importantly, whether cats immunized with native viral subunit proteins are protected against virulent FeLV challenge exposure. It was for these reasons that the following experiments were conducted. In these experiments cats were immunized with immunoaffinity column purified FeLV-gp70 or chemically purified FeLV-gp85 and then challenged in various ways with virulent FeLV or FeSV. As controls for these studies, cats were either left unimmunized, or were vaccinated with formalin inactivated purified whole FeLV. Formalin inactivated whole FeLV vaccines have previously been shown by our laboratory to be effective immunogens in preventing persistent FeLV infection following natural exposure (Pedersen et al., 1978). Experimental studies reported herein indicated that infection with virulent FeLV may have been actually enhanced by preimmunization with FeLV-gp70 or -gp85 vaccines in challenge-exposure situations where inactivated whole FeLV vaccines were protective.

MATERIALS AND METHODS

Experimental animals

Kittens 9 to 14 weeks of age were obtained from the specific pathogen free and conventional FeLV free breeding colonies of the Feline Leukemia Research Laboratory, University of California, Davis. Animals were housed in isolation facilities of the Animal Resources Services, University of California, Davis.

Virus strains.

The Kawakami-Theilen strain of FeLV was produced by cultures of FL74 feline lymphoblastoid cells (Theilen et al., 1969). This strain is in actuality a mixture of subgroup A, B, and C viruses (Sarma et al., 1973). The Snyder-Theilen strain of FeLV (ST-FeLV) was isolated from a cat that remained retroviremic after having recovered from a tumor induced by the ST-feline sarcoma virus (ST-FeSV) (Pedersen et al., 1984). Buffy coat cells from the blood of this cat were cocultivated with primary fibroblasts cells and the resulting infected culture was designated FF280/ST-FeLV. ST-FeLV is the naturally occurring helper virus present in ST-FeSV stocks and belongs to the interference subgroup B (Sarma et al., 1973). The ST-FeLV isolate is nontransforming to normal feline fibroblasts and does not induce fibrosarcomas. The CT600 strain of FeLV (CT600-FeLV) was a naturally occurring subgroup A retrovirus isolated from a leopard cat that lived in the same household as FeLV infected domestic cats (Rasheed et al., 1981). The virus is replicated in a chronic fashion by CT600 leopard cat
testicle cells. The Rickard strain of FeLV (R-FeLV) (Rickard et al., 1969) was originally obtained as a cell-free tumor homogenate from Dr. Richard Olsen, Ohio State University. This isolate has been classified as subgroup A (Sarma et al, 1973).

**Preparation of inactivated whole virus vaccine.**

Cultures of FF280/ST-FeLV cells were grown to confluency in 500 ml plastic flasks. Initial culture media was Eagle's minimum essential media (Earle's base) supplemented with an equal part of Leibowitz's-15 (L15) media and 10% fetal bovine serum. After the cells reached a confluent state the original media was replaced with Eagle's MEM containing no fetal bovine serum but supplemented with 1 ug/ml of hydrocortisone phosphate. Tissue culture media was collected 48 hours later and concentrated 50 to 200 fold in a millipore Pellicon apparatus with 100,000 dalton exclusion filters. Formalin was then added to the concentrate at a final concentration of 0.8% formaldehyde and allowed to react for 24 hours at 4°C. Each 1 ml of the final vaccine contained about 0.15 to 0.6 mg of ST-FeLV.

Vaccines (0.5 ml) were adjuvanted with Freund's incomplete adjuvant (0.6 ml), alhydrogel (0.2 ml), alhydrogel (0.2 ml) and Quil-A (15 ug) (9), or ethylene malic anhydride (0.3%).

**FeLV-gp70, -p27 and -p15E assays**

All three of these FeLV structural proteins were quantitated by a double sandwich type enzyme linked immunosorbent assay (ELISA). The assay procedure for FeLV-p27 was identical to that described by Lutz and coworkers (1983). Similar assays were used for the quantitation of FeLV-gp70 and -p15E. The FeLV-gp70 assay utilized polyclonal goat anti-FeLV-gp70 immunoglobulin (NIH 815-210) for the "catcher" and monoclonal mouse anti-FeLV-gp70 (25-SD) IgG conjugated to horse radish peroxidase (HPASE) for the second antibody. The ELISA for FeLV-p15E utilized monoclonal mouse anti FeLV-p15E (29-2C) IgG as the "catcher" and monoclonal mouse anti-FeLV-p15E (29-2F) IgG conjugated to HPASE as the second antibody. The IgG fraction was purified from serum or ascites fluid by the procedure of Bruck and coworkers (1982). These assay procedures were used to follow FeLV-gp70 through various steps in purification to assure its separation from FeLV-p27 and -p15E. The FeLV-p27 assay was also used to detect virus in the blood of FeLV infected cats (Lutz et al., 1983).

**Purification of FeLV-gp70**

FeLV-gp70 was prepared from KT-FeLV, ST-FeLV, and CT600-FeLV using immunoaffinity column chromatography. Virus containing cell culture
supernatants were clarified of particulate debris by low speed centrifugation and then concentrated 40 fold on a Milipore Pellicon apparatus. Virus was pelleted at 18,000 rpm's for 120 min. in a Beckman Type 21 rotor (Beckman Instruments, Palo Alto, CA). Pelleted virus was resuspended in a small amount of TEN buffer (0.1 M tris, 0.001 M edta, 0.1 M NaCl, pH 7.2), and then placed over a 10-50% continuous sucrose gradient and centrifuged at 25,000 rpm for 4 hr in a Beckman SW27 rotor. Gradient fractions were collected and assayed for FeLV-gp70 by ELISA; fractions containing the largest amounts of this protein were saved (Fig 1).

FIGURE 1 - Continuous 10 to 50% sucrose gradient purification of FeLV pelleted from concentrated and clarified tissue culture media. FeLV-gp70 protein, as determined by an ELISA assay, was concentrated near the 1.16 g/ml region of the gradient and corresponded with the area where whole virions banded.

Virus was removed from sucrose by diluting gradient fractions 1:10 with TEN buffer and ultracentrifugation. Pellets were resuspended in TEN buffer containing 0.1% Triton. Triton disrupted virus was then passed over an immunoaffinity column. Immunoaffinity columns were made according to the procedure of Cuatrecases (1971) using cyanogen bromide activated sepharose-4B and mouse monoclonal IgG against 3 different epitopes of FeLV-gp70 (monoclonal antibodies 25-5D, 1-3B, and 1-3C). Nonbinding proteins were washed through the columns with large volumes of TEN buffer until the eluting material was free of FeLV-p27 (Fig. 2).
FIGURE 2 - Immunoaffinity column purification of FeLV-gp70. The column was made of sepharose 4B covalently linked to anti-FeLV-gp70 mouse monoclonal IgG. The starting material on the column was detergent disrupted whole FeLV. FeLV-p27 appeared in the column wash-through, and when the wash buffer was free of this protein, the 3M KSCN gradient was started. FeLV-gp70 eluted between 1 and 2M KSCN.

FeLV-gp70 was then eluted with a continuous gradient of 0 to 3M sodium thiocyanate in 50 mM borate buffer, pH 8.5. The resulting eluate fractions were then assayed for FeLV-gp70 and fractions containing the greatest amounts of this material were pooled (Fig. 2). The FeLV-gp70 rich eluate was then desalted on a Sephadex G25 column equilibrated with 50 mM ammonium bicarbonate buffer, pH 8.5.

**Purification of FeLV-gp85**

Rosettes made up of covalently linked FeLV envelope proteins gp70 and p15E were produced from KT-FeLV by the procedure described by Ransmann and coworkers (1983), and Schneider and associates (1980). Rosettes were assayed by SDS-PAGE, and a major protein band was demonstrated by Coomassie blue stain at 85,000 daltons. When rosettes were run on reducing gels (Laemmli, 1970), the 85,000 dalton protein was reduced to a 70,000 (gp70) and 15,000/ 17,500 (p15E) dalton protein.

**Antibody assay procedures**

Serum antibodies to whole FeLV and FeLV-gp70 were measured by an ELISA
procedure in 96 well microtiter plates (Imulon I, Dynatech Laboratories, Inc., Alexandria, VA). Wells were coated for 3 hr at 37 C with either 250 ng of gradient purified 5% sodium dodecylsulphate disrupted FeLV, 25 ng of immunoaffinity column purified FeLV-gp70, or 25 ng of purified FeLV-gp85, in 0.1M Na₂CO₃ coating buffer, pH 9.6. Serum to be tested was diluted 1:200 (whole FeLV) or 1:8 (FeLV-gp70 or -gp85) in tris-edta saline buffer with 0.05% tween 20 and 0.1% bovine serum albumin, pH 7.4. Diluted serum was incubated in the antigen coated wells for 1 hr at 37 C. After washing, a 1:500 dilution of rabbit anti-cat IgG horse radish peroxidase conjugate (Cappel Laboratories, Cochranville, PA) was allowed to react for 45 min. Wells were then washed and the enzymatic reactions carried out with ABTS. Color reactions were read on a Dynatech microELISA reader (Dynatech Laboratories, Inc., Alexandria, VA). Optical density readings were standardized to a known positive and negative serum included in each test run.

Virus neutralizing antibodies were measured by an infectivity inhibition assay. Subconfluent monolayers of Crandell feline kidney (Crk) cells were established in 96 well microtiter plates. One hundred tissue culture infectious doses 100% (100 TCID₅₀) of KT- or CT600-FeLV in 0.05 ml of tissue culture media was incubated for 1 hr at 37 C with an equal volume of a serial two-fold dilution of the test cat serum starting at 1:10. Specific pathogen free cat serum was used as a negative control and goat anti-FeLV serum as a positive control. After incubation the serum-virus mixture was overlaid onto the Crk cell monolayers and then incubated for 24 hours. At this time the media was completely changed. Tissue culture supernatants were harvested on day 7 and assayed for FeLV-p27 antigen. The titer of virus neutralizing antibody in a given serum was scored as the reciprocal of the highest dilution of serum that completely suppressed the expression of FeLV-p27.

The immunogenicity of purified FeLV-gp70 and -gp85 was tested by ELISA. Ninety-six well Imulon I plates were coated with 25 ng of FeLV-gp70 or 50 ng of FeLV-gp85 per well for 3 hours at 37 C and overnight at 4 C. The antigens were reacted with mouse monoclonal FeLV-gp70 antibodies (1:50 ascites fluid in 100 ul buffer) or goat anti-FeLV-gp70 serum (NIH 81S-210) (1:400 in 100 ul buffer) at 37 C for 1 hour. Seven different mouse monoclonal antibodies and a polyclonal goat antiserum were used for this study, each of which neutralized various subgroups of FeLV at titers of 1:250 or greater. Second antibodies consisted of goat anti-mouse IgG-HPASE (1:400), or rabbit anti-goat IgG-HPASE (1:400) (Cappel Laboratories, Cochranville, PA). Incubation was for 1 hour at 37 C. The ABTS substrate was allowed to react for 10 min. at room temperature. Optical density readings were scored as follows: 0.06 to 0.1 (+), 0.1 to 0.2 (+), 0.2 to 0.3 (++), 0.3 to 0.4 (+++), greater than 0.4 (++++).
Virus challenge systems

Two different basic challenge systems were used to test the efficacy of the various vaccine preparations. In the first system, cats were inoculated subcutaneously between the shoulder blades with $5 \times 10^6$ FF64, 90/ST-FeSV cells. This cell line was a primary feline fibroblast culture that had been productively transformed with ST-FeSV (Pedersen et al., 1984). Fibrosarcomas appeared at the site of inoculation within 6 days, concurrently with a systemic retrovirus infection. The systemic retroviremia has been shown to be due almost exclusively to the FeLV helper virus present in ST-FeSV transformed cells (deNoronha et al., 1983; Pedersen et al., 1984). Vaccine protection in this system is measured by 3 criteria, 1) reduction of maximum tumor size (Hunsmann et al., 1983), 2) a decrease in the proportion of cats that developed progressively growing and fatal fibrosarcomas, and 3) a decrease in the proportion of infected cats that developed a persistent FeLV helper viremia during the course of tumor growth or after tumor regression (Pedersen et al., 1984).

In the second challenge system, cats were inoculated oronasally with 40,000 focus forming units (ffu's) of CT600-FeLV every other day for 8 days, or similarly with 400,000 ffu's of R-FeLV. The CT600-FeLV was propagated in leopard cat testicle cells (Rasheed et al., 1981), and R-FeLV was propagated in fc9 feline fibroblast. Virus was titrated by the focus formation procedure of Fischinger and associates (1974). In this system, vaccine protection was measured by a decrease in the proportion of cats that were still viremic 12 to 16 weeks post challenge (Pedersen et al., 1977). Retroviremia was measured weekly using an ELISA procedure for the detection of FeLV-p27 (Lutz et al., 1983).

Statistical Methods

The effect of vaccines on the post challenge-exposure incidence of persistent viremia was evaluated using relative odds ratios \( R = \frac{ad}{bc} \), where \( a = \) no. vaccinated cats developing persistent viremia, \( b = \) no. of vaccinated cats with nondetectable or transient viremia, \( c = \) no. of nonvaccinated cats developing persistent viremia, and \( d = \) no. of nonvaccinated cats with nondetectable or transient viremia). Groups of cats receiving the same or equivalent treatments were combined \( (a = a_1 + a_2, \ldots), (b = b_1 + b_2, \ldots) \), etc.). \( R \)-values of 1.0 indicated that the vaccines had no effect on the observed incidence of persistent viremia. A \( R \)-value \( < 1 \) indicated that the vaccine had an inhibitory effect on persistent viremia, while values \( > 1 \) indicated enhancement of persistent viremia. The statistical significance of the odds ratios were confirmed with the chi-square \( (X^2) \) test (Glantz, 1981). With 1 degree of freedom, a \( X^2 \) of 3.84 or greater was considered to be significant at the 95% confidence level \( (P < 0.05) \).
RESULTS

Analysis of purified FeLV-gp70 and -gp85

FeLV-gp70 and -gp85 were analyzed for purity by immunoblotting according to the procedure of Towbin and associates (1979), except that the enzymatic reaction was developed using 0.5 mg/ml of 3,3-diaminobenzidine tetrahydrochloride monohydrate (Aldrich Chemicals, Milwaukee, WI) and 0.01% hydrogen peroxide in 0.1 M tris saline buffer, pH 7.4. A single band at a molecular weight of 70,000 daltons was identified in both preparations using the 25-5D mouse monoclonal FeLV-gp70 antibody (Fig. 3). FeLV-p27 was absent from both preparations, while only the FeLV-gp85 contained a 17,500 dalton band that reacted with the mouse monoclonal FeLV-p15E antibodies.

Figure 3 - Immunoblots of PAGE purified FeLV-gp85, detergent disrupted whole FeLV, FeLV-gp70, and FeLV-p27. Mouse monoclonal antibodies were against FeLV-gp70, -p27, and -p15E. FeLV-gp85, when run under reducing conditions, demonstrated a major band with the FeLV-gp70 monoclonal antibody at 70,000 daltons and a major band with the FeLV-p15E monoclonal antibody at 17,500 daltons. Immunoblots of whole FeLV, when reacted with the anti FeLV-gp70, -p27, and -p15E monoclonal reagents, demonstrated corresponding bands of reactivity at 70,000, 27,000 and 17,500 dalton regions respectively. FeLV-gp70 preparations were found to react only with FeLV-gp70 monoclonal antibody, and were free of FeLV-p27 and -p15E. As a specificity control, FeLV-p27 was also tested by immunoblotting. FeLV-p27 was only detected by the corresponding FeLV-p27 monoclonal antibody.

FeLV-gp70 and -gp85 were subjected to a number of physical and chemical insults during their purification. It was important, therefore, to deter-
mine whether major antigenic epitopes were still intact. The major antigenic epitope of FeLV-gp70 has been described by Nunberg and coworkers (1984). This epitope was contained in a 14 amino acid region in the amino-terminal half of -gp70, and was recognized by the 25-5D monoclonal antibody. To determine whether this epitope was still intact, the purified FeLV-gp70 and -gp85 were used as competitors for the binding of the peroxidase labeled 25-5D monoclonal antibody to whole FeLV in an ELISA assay. Detergent disrupted whole FeLV, FeLV-gp70, and FeLV-gp85 all competed with the labeled monoclonal antibody for binding to the whole FeLV (Fig. 4). The purified FeLV-gp70 and -gp85 were also reacted in an ELISA against a battery of 7 different mouse monoclonal FeLV-gp70 antibodies and a goat polyclonal anti-FeLV-gp70 serum (Table I). Purified FeLV-gp70 and -gp85 both reacted with each of these antibodies.

![Graph](image)

**Figure 4** - Inhibition of binding of in ELISA the 25-5D mouse monoclonal FeLV-gp70 antibody-HPASE to purified FeLV by detergent disrupted whole FeLV, FeLV-gp70 and FeLV-gp85. Disrupted whole virus and the subunit proteins equally inhibited the binding of the labeled monoclonal antibody, indicating that the major virus neutralizing epitope was still intact in those preparations.
The reactivity of purified FeLV-gp70 and-gp85 in ELISA with 7 different virus neutralizing mouse monoclonal FeLV-gp70 antibodies and with polyclonal goat anti-FeLV-gp70 serum (NIH 81S-210).

| Antibody        | FeLV-gp85 | FeLV-gp70 |
|-----------------|-----------|-----------|
| MC 18-1B        | +         | ±         |
| MC 18-1D        | +++       | +++       |
| MC 18-2A        | +         | +++       |
| MC 18-2B        | ++        | ++        |
| MC 25-4C        | ±         | +++       |
| MC 25-5D        | +         | ++        |
| MC 46-2F        | +         | ++        |
| goat anti-FeLV-gp70 | +++     | +++++     |

Efficacy of whole FeLV vaccines

A total of 96 kittens were vaccinated with inactivated whole FeLV vaccine and 87 kittens served as unvaccinated controls. These kittens were divided among 9 experimental groups (Tables II, III). The amount of virus contained in each dose of vaccine varied from 50 to 200 ml equivalents (approximately 150 to 600 ug/dose). Vaccinations were given at 3 week intervals starting when the kittens were 8 to 12 weeks of age and the total number of vaccinations per kitten varied from 2 to 3. Kittens were challenged subcutaneously with ST-FeSV (tumor cells) or oronasally with either R-FeLV or CT600-FeLV (tissue culture fluid) 1 week after the last vaccination.
| Group  | vaccine | adjuvant | doses | challenge | mean peak | regressing | progressing | undetectable or transient viremia | persistent viremia |
|--------|---------|----------|-------|-----------|-----------|------------|-------------|---------------------------------|------------------|
| **V43** a | Saline  | FICA     | 3     | ST-FeLV   | 169.0 g   | 9/12       | 3/12        | 9/12                            | 3/12             |
| b | 200 ml eq ST-FeLV | FICA | 3 | ST-FeLV | 38.0 g | 12/12 | 0/12 | 12/12 | 0/12 |
| **V44** a | Saline  | FICA     | 2     | ST-FeSV   | 659.0 g   | 4/9         | 5/9          | 4/9                             | 5/9              |
| b | 50 ml eq ST-FeLV | FICA | 2 | ST-FeSV | 295.0 g | 5/8 | 3/8 | 5/8 | 3/8 |
| c | 200 ml eq ST-FeLV | FICA | 2 | ST-FeSV | 173.0 g | 7/8 | 1/8 | 7/8 | 1/8 |
| **V49** a | Saline  | FICA     | 2     | ST-FeSV   | 223.0 g   | 4/5         | 1/5          | 4/5                             | 1/5              |
| b | 200 ml eq ST-FeLV | FICA | 2 | ST-FeSV | 35.0 g | 6/6 | 0/6 | 6/6 | 0/6 |
| **V45** a | Saline  | EMA      | 2     | ST-FeSV   | 220.0 g   | 10/12       | 2/12         | 10/12                           | 2/12             |
| b | 50 ml eq ST-FeLV | EMA | 2 | ST-FeSV | 110.0 g | 11/12 | 1/12 | 11/12 | 1/12 |
| **V54** a | Saline  | Alhydrogel | 2 | ST-FeSV | 137.0 g | 3/7 | 4/7 | 3/7 | 4/7 |
| b | 100 ml eq Alhydrogel ST-FeLV + Quil-A | 2 | ST-FeSV | 43.0 g | 10/11 | 1/11 | 10/11 | 1/11 |
| c | 100 ml eq Alhydrogel ST-FeSV | 2 | ST-FeSV | 20.0 g | 4/4 | 0/4 | 4/4 | 0/4 |
| **VSH** a | Saline  | EMA      | 3     | ST-FeSV   | 279.0 g   | 2/13        | 11/13        | 1/13                            | 12/13            |
| b | 200 ml eq ST-FeLV | EMA | 3 | ST-FeSV | 32.0 g | 5/5 | 0/5 | 5/5 | 0/5 |
Serum antibodies to whole FeLV and FeLV-gp70 were detected by ELISA in almost all of the vaccinated cats (Fig. 5). Virus neutralizing antibodies, however, were not detected following immunization in the serum of any vaccinated or unvaccinated animal (data not shown).

FIGURE 5 - ELISA antibody titers in the serum of cats vaccinated with inactivated whole FeLV vaccine. The antibody responses of this group of animals was considered representative of the responses seen in the group as a whole. The upper graph depicts serum antibody levels against purified FeLV-gp70, while the lower graph depicts the antibody levels against whole detergent disrupted FeLV. Control cats did not develop appreciable ELISA antibody titers and were therefore not included.
Cats immunized with inactivated whole ST-FeLV vaccine, regardless of the experimental group, resisted virulent virus challenge to a greater degree than the control nonimmunized animals (Table II). In the case of the 124 kittens challenged with ST-FeSV (Table II), vaccinated cats developed smaller tumors, fewer progressively growing tumors (6/66 vs 26/58), and a significantly lower incidence of persistent retroviremia (6/66 vs 27/58, R = 0.1, X^2 = 18.8, P < 0.01) than nonvaccinates. Similarly, ST-FeLV vaccinated cats challenged oronasally with virulent FeLV (R-FeLV or CT600-FeLV) developed a significantly lower rate of persistent retroviremia than nonvaccinated controls (1/30 vs 8/29, R = 0.1, X^2 = 4.96, P < 0.05) (Table III).

### Table III

| Group | Vaccine  | Adjuvant | Dose | Challenge | Outcome of Challenge |
|-------|----------|----------|------|-----------|----------------------|
|       |          |          |      |           | Undetectable or transient viremia | Persistent viremia |
| V46 a | saline   | EMA      | 3    | R-FeLV    | 12/14                | 2/12                 |
|       | b        | EMA      | 3    | R-FeLV    | 11/12                | 1/12                 |
| V50 a | saline   | FICA     | 2    | CT600-FeLV| 4/7                  | 3/7                  |
|       | b        | FICA     | 2    | CT600-FeLV| 8/8                  | 0/8                  |
| V51 a | saline   | EMA      | 2    | CT600-FeLV| 7/10                 | 3/10                 |
|       | b        | EMA      | 2    | CT600-FeLV| 10/10                | 0/10                 |

Although vaccinated cats fared far better than nonvaccinated cats as a group, kittens immunized with 100 to 200 ml equivalents of virus (300 to 600 µg/dose), and challenged with either FeLV or FeSV, were significantly more protected against persistent viremia than kittens given 50 ml equivalents (150 µg/dose) (1/49 vs 5/32) (Table IV). This indicated that 300 to 600 µg dose was at the upper part of the linear portion of the dose response curve.
Efficacy of different whole inactivated FeLV vaccines

| Vaccine                  | Persistent viremia |
|--------------------------|--------------------|
| Inactivated whole FeLV   |                    |
| Controls                 | 35/87 = 40%        |
| Vaccinates               |                    |
| 50 ml eq                 | 5/32 = 16%         |
| 100 to 200 ml eq         | 2/64 = 2%          |
| total (50 to 200 ml eq)  | 7/96 = 7%          |

Efficacy of FeLV-gp70 or -gp85 subunit vaccines

Forty 10 to 12 week-old kittens were vaccinated with purified FeLV envelope proteins (Table V, VI). Subunit proteins were either derived from KT-FeLV (V33,34,42), CT600-FeLV (V50), or ST-FeLV (V49). Challenge strains were either homologous in regards to the strains of virus from which the envelope proteins were derived (V33, 34, 42), or autologous (V49, 50). The subunit FeLV proteins were all given in saline or Freund's incomplete adjuvant in 2 to 4 doses ranging from 0.005 to 1.0 mg per dose. Thirty-seven kittens served as nonvaccinated controls.

Cats immunized with various FeLV envelope proteins produced good serum antibodies against whole FeLV and FeLV-gp70 (or FeLV-gp85) as detected by ELISA (Figs. 6,7). Virus neutralizing antibodies were not detected, however, in the serum of any of the vaccinated cats (data not shown).
### TABLE V

Results of experiments with purified native FeLV -gp70 and -gp85 vaccines and ST-FeSV challenge exposure.

| Group | Vaccine  | Adjuvant | Doses | Challenge | Mean Peak Tumor Size | Progressing Tumors | Undetectable or Transient Viremia | Persistent Viremia |
|-------|----------|----------|-------|-----------|----------------------|-------------------|-----------------------------------|--------------------|
| V33 a | saline   | None     | 3     | ST-FeSV   | 2.3 g                | 0/3               | 0/3                              | 0/3                |
| b     | saline   | FICA     | 3     | ST-FeSV   | 13.0 g               | 0/4               | 4/4                              | 0/4                |
| c     | 1.0 mg KT FeLV-gp85 | None | 3 | ST-FeSV | 1.3 g | 0/2 | 1/2 | 1/2 |
| d     | 1.0 mg KT FeLV-gp85 | FICA | 3 | ST-FeSV | 2.0 g | 0/4 | 2/4 | 2/4 |
| V49 a | saline   | FICA     | 2     | ST-FeSV   | 223.0 g              | 1/5               | 4/5                              | 1/5                |
| b     | 0.6 mg ST FeLV-gp70 | FICA | 2 | ST-FeSV | 81.0 g | 0/6 | 1/6 | 5/6 |
TABLE VI

Results of experiments with purified FeLV-gp70 and -gp85 vaccines and FeLV challenge exposure.

| Group | Vaccine  | Adjuvant | Doses | Challenge | Outcome of challenge |
|-------|----------|----------|-------|-----------|----------------------|
| V42 a | saline   | none     | 4     | R-FeLV    | undetectable or transient viremia 2/4 2/4 |
| b     | saline   | FICA     | 4     | R-FeLV    | persistent viremia 2/4 2/4 |
| c     | .005 mg  | FICA     | 4     | R-FeLV    | 1/4 3/4 |
|       | KT-FeLV  gp85 |        |       |           |                                     |
| d     | .05 mg   | FICA     | 4     | R-FeLV    | 4/4 0/4 |
|       | KT-FeLV  gp85 |        |       |           |                                     |
| e     | 0.5 mg   | FICA     | 4     | R-FeLV    | 1/4 3/4 |
|       | KT-FeLV  gp85 |        |       |           |                                     |
| V50 a | saline   | FICA     | 2     | C1600-FeLV| 4/7 3/7 |
| b     | 0.3 mg   | FICA     | 2     | C1600-FeLV| 1/8 7/8 |
FIGURE 6 - ELISA antibody titers in the serum of cats vaccinated with immunoaffinity column purified FeLV-gp70. The antibody response of this group of animals was considered representative of the responses seen in the group as a whole. The upper graph depicts serum antibody levels against purified FeLV-gp70, while the lower graph depicts the antibody levels against whole detergent disrupted FeLV. Control cats did not develop appreciable ELISA antibody titers and were therefore not included.

Two weeks after completing their courses of immunization, subunit vaccinated cats and control animals were challenged with either FeLV or FeSV. Following inoculation with FeSV, both vaccinates and nonvaccinates developed fibrosarcomas at the site of inoculation (Table IV). The mean peak tumor size was always smaller in cats vaccinated with FeLV-gp70 or -gp85. Surprisingly, the proportion of cats that remained persistently FeLV-helper viremic following FeSV inoculation did not mirror the decreased tumor growth. Vaccinates remained persistently retroviremic following
challenge-exposure at a higher rate than control animals (8/20 vs 2/22, \( R = 6.7, \chi^2 = 3.94, p < 0.05 \)) (Table V). Twenty FeLV -gp70 and -gp85 vaccinated and 15 nonvaccinated kittens were challenged oronasally with virulent FeLV (Table VI). Following challenge-exposure, a greater proportion of vaccinated cats became persistently viremic as compared to non-vaccinates (13/20 vs 7/15, \( R = 2.1, \chi^2 = 0.55, p > 0.5 \)), although the difference by chi-square was not statistically significant.

FIGURE 7 - ELISA antibody titers in the serum of cats vaccinated with purified FeLV-gp85 rosettes. The antibody response of this group of animals was representative of the responses seen in the entire group. The upper graph depicts the antibody levels against purified FeLV-gp85, while the lower graph depicts the antibody titers against detergent disrupted whole FeLV. Nonvaccinated control cats did not develop appreciable antibody titers and were therefore not included.
Overall, enhancement of persistent retroviremia by vaccination with purified FeLV envelope proteins was seen in 6/8 experimental groups (V33e,d; V42c,e; V49b; V50b). This was in contrast to the 11 groups of cats that were vaccinated with inactivated whole FeLV vaccines, all of which showed a lower incidence of persistent viremia than nonvaccinates within the same experiments.

**DISCUSSION**

Salerno and associates (1978) concluded that purified FeLV-gp70 was poorly immunogenic in cats because it induced antibodies detectable by radioimmunoassay but little or no virus neutralizing or cytotoxic antibodies. Our experiments confirmed their findings; none of the cats that we immunized with either FeLV-gp70 or -gp85 developed virus neutralizing antibodies, but serum antibodies detectable by ELISA were produced against both whole FeLV and FeLV-gp70.

The most surprising finding in our study was that cats vaccinated with FeLV-gp70 or -gp85 appeared to be more susceptible to infection with virulent FeLV or FeSV than nonvaccinated animals. This enhancement of infection was seen in 6/8 vaccine groups and was apparent whether cats were immunized with gp70 or with gp85. It also occurred regardless of whether the challenge viruses were autologous or homologous to the strain of FeLV from which the purified envelope proteins were extracted. These findings were opposed, therefore, to the immunoprotective effect of viral envelope protein vaccines demonstrated for murine leukemia virus infection (Hunemann et al., 1975, 1981), bovine leukemia virus infection of sheep (Onuma et al., 1984), and foot-and-mouth disease of cattle (Bittle et al., 1982).

The enhancing rather than protective effect of FeLV-gp70, -gp85, vaccines could have been due to several mechanisms. First, it was possible that the procedures used to purify the proteins altered their antigenicity in such a way that neutralizing epitopes were no longer present. This seemed unlikely, however, because purified FeLV-gp70 and -gp85 competed equally well with whole FeLV for binding to the 25-5D mouse monoclonal FeLV-gp70 antibody. This antibody recognizes the major neutralizing epitopes of the FeLV envelope protein (Numberg et al., 1984). In addition, purified FeLV-gp70 and -gp85 were immunoreactive in ELISA with 7 other virus neutralizing mouse monoclonal antibodies to FeLV-gp70 and with a highly specific goat polyclonal FeLV neutralizing antibody.

A second explanation for the possible enhancing effects of purified envelope protein vaccines was that they failed to induce the spectrum of immunity essential for protection, i.e., neutralizing antibodies as well as cellular immunity. Neutralizing antibodies might be generated only as a
result of exposure to larger envelope polyproteins (pre-cleavage products) present in immature particles and on the cell membrane. The role of cellular immunity in FeLV infection is largely unknown. Passive transfer of immune sera will greatly inhibit the retroviremia that follows experimental infection in cats (deNorohna et al., 1980), suggesting that humoral immunity is important in clearance of virus from the blood. Feline leukemia virus persists in the body as a latent infection, however, even in the face of strong humoral immunity (Pedersen et al., 1984). Cellular immunity has been implicated in the ultimate clearance of such latent infections in other virus systems, and such immunity is hard to evoke with killed or subunit proteins.

As a third explanation, non-neutralizing antibodies to the envelope proteins might have either interfered with immunity or had an enhancing effect on virus infectivity and spread. Non-neutralizing antibodies might have bound to virus and sterically blocked the binding of virus neutralizing antibodies that were induced as a result of virulent virus challenge-exposure (Massey and Schochetman, 1981). Non-neutralizing antibodies may have acted as opsonins and enhanced the uptake of antibody coated virus by phagocytic cells. FeLV replicates initially in phagocytic cells in lymph nodes regional to the site of entry and spreads from these organs to other target tissues via blood borne mononuclear cells (Rojko et al., 1978). Because FeLV grows very well in mononuclear cells, and mononuclear cells migrate throughout the body, increased virus uptake by phagocytic cells could lead to both increased virus infectivity and dissemination. This scheme is not without precedence. Dengue virus infection of man can be enhanced by pre-existing humoral immunity to homologous serotypes of dengue virus or related flavivirus infections (Porterfield, 1982). In a similar manner, feline infectious peritonitis (FIP) virus infection can also be potentiated by pre-existing homologous coronavirus antibodies (Pedersen et al., 1980). Vaccination against FIP virus also makes the experimentally induced disease worse rather than better (Pedersen et al., 1983).

A final possibility is that the envelope proteins acted as immunosuppressants rather than immunogens. FeLV-p15E has reportedly caused immunosuppression both in vitro and in vivo (Mathes et al., 1979). Cats that we immunized with FeLV-gp85 received both gp70 and p15E, and the p15E moiety may have enhanced the infectivity of the virulent FeLV challenge by virtue of its immunosuppressive effect. In support of this, it has been shown previously that FeLV-p15E, when given along with an FeLV vaccine, rendered cats more susceptible to FeSV challenge than if they had received the vaccine alone (Mathes et al., 1979). This phenomena does not, however,
explain our findings, because cats immunized with FeLV-gp70 were as hypersensitive to infection as cats immunized with FeLV-gp85.

The obvious question raised by these findings is whether immunity to FeLV can be induced with FeLV-gp70 or -gp85 vaccines. If the current findings and those of Salerno and associates (Salerno et al., 1978) are universally applicable, then the answer seems to be no. It is possible, however, that FeLV-gp70 or -gp85 would induce protective immunity if given in some other form, at some other dose, or with other adjuvants. These possibilities cannot be answered without a great deal of additional research. The point that should not be overlooked, however, is that many researchers have placed great hopes on subunit vaccines produced by genetically engineered bacteria or yeast, or on synthetic oligopeptides. These findings indicate that many problems and uncertainties exist with this approach, and that conventional inactivated whole virus vaccines should not be necessarily bypassed for the sake of more modern techniques.

Although our FeLV-gp70 and -gp85 vaccines enhanced persistent retroviremia, they did significantly reduce the growth rate of retrovirus induced fibrosarcomas. Why anti-FeLV-gp70 or -gp85 immunity would protect against tumors yet enhance retroviremia is not immediately understandable. It is known, however, that fibrosarcoma and retrovirus immunity are distinctly different phenomena (deNoronha et al., 1983; Pedersen et al., 1984). A dichotomy between tumor and antivirus immunity in FeLV-gp70 or -gp85 vaccinated cats is not, therefore, incompatible with what is known about FeSV immunity.

Although the current outlook for cheap and effective FeLV vaccines of recombinant or synthetic origin is somewhat clouded, it must be noted that conventional formalin inactivated whole virus vaccines worked very well. Adjuvanted and inactivated whole virus vaccines at a dose of 200 ml eq decreased the post-infection persistent viremia rate from 40 to 2% in our studies. This is comparable to the results of an earlier trial with a similar dose of antigen and natural contact challenge exposure (Pedersen et al., 1978).

The fact that whole inactivated FeLV is both immunogenic and induces protective immunity, while FeLV-gp70 and -gp85 are only immunogenic, indicates either that other proteins of the virus are responsible for protective immunity or that the envelope proteins are more favorably arrayed as immunogens on whole virus than as suspensions of pure protein. The results of a previous study indicated that antibody to proteins in addition to FeLV-gp70 were also correlated with protection (Lutz et al., 1982), thus supporting the first premise. In support of the latter possibility, FeLV virons are known to incorporate small amounts of membrane
proteins as they bud off of infected cells (Azocar et al., 1980). These
host proteins may enhance the immunogenicity of the viral proteins.

Another possibility was that the dose of FeLV-gp70 or -gp85 used in these
studies was lower than that present in 0.6 mg of whole FeLV. If about 13%
of the viral proteins in whole FeLV was gp70, than 0.6 mg of whole virus
would contain about 80 μg of gp70. We used 200 to 1000 mg of FeLV-gp70 or
-gp85 per dose of vaccine in most of our experiments. It does not seem,
therefore, that the difference in efficacy between whole virus and subunit
protein vaccines was due to differences in the relative amounts of envelope
protein in each.

Experiments that seemed at firsthand to be contradictory to our own
were recently reported by Osterhaus and coworkers (1985). They were able
to induce low levels of virus neutralizing antibodies in 6/8 young cats
immunized with 3 doses of immunostimulating complexes (iscoms) containing
FeLV-gp70-85. Moreover, these cats seemed to be much more resistant to
experimentally induced FeLV infection than nonvaccinated control animals.
Even the authors, however, were careful not to ascribe the protective
effect of the vaccine solely to its envelope protein content. Their iscoms
were made from disrupted whole virus and they induced antibodies to a wide
range of envelope and nonenvelope proteins. In effect, the studies of
Osterhaus and associates (1985) reconfirmed our studies with whole virus
vaccines. To prove that envelope proteins alone were responsible for the
protective immunity demonstrated by iscom preparations, cats should be
vaccinated with iscoms containing envelope proteins alone. If such pre-
parations should prove as protective as iscoms made from whole virus, then
we will have to concede that our experiences with envelope protein vaccines
were jaded by the adjuvants that we used.

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