STUDIES ON IMMUNITY TO FELINE LEUKAEMIA VIRUS

by

JAMAL SALIH MAHMOUD B.V.M.& S.

(Baghdad University - Iraq)

Thesis submitted for the degree of Doctor of Philosophy

in the Faculty of Veterinary Medicine

University of Glasgow

March 1984

Department of Veterinary Pathology

University of Glasgow

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To my Sons

Khalid and Doraid

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I wish to thank very much Professor W.F.H. Jarrett, head of the Department of Veterinary Pathology for the use of departmental facilities and Professor O. Jarrett, my supervisor, for his great support, encouragement and helpful discussions during my course of study.

I am also most grateful to Dr. D.E. Onions, Miss A. Keenan, Mrs J. Dunn. Mr M.C. Golder and Mr D. Hay for their assistance and support in the laboratory, and to Dr. M.F. Stewart and Miss J. Cole for help with the experimental animals. I thank Mrs E. Gault for her assistance in typing this thesis.

I am grateful for financial support from the Government of Iraq.

SUMMARY

The aim of the studies described in this thesis was to develop a vaccine against feline leukaemia (FeLV) based on inactivated virus. Previous attempts to produce an economical, inactivated virus vaccine which would protect kittens against FeLV infection had not been successful.

In the present study a vaccine was prepared which protected over 80% of weanling kittens against a viral challenge which induced persistent FeLV infection in 80-100% of unvaccinated kittens. Among the reasons for the comparative success of the vaccine described here were believed to be:

1. The use of FeLV of subgroup A produced in large quantities by the F422 line of feline lymphosarcoma cells.
2. Inactivation of the virus with a concentration of paraformaldehyde which preserved or enhanced the immunogenicity of the antigen.
3. The use of $Al(OH)_3$ and Freund's incomplete adjuvant in the vaccine.
4. A schedule for vaccination and challenge by FeLV which allowed the efficacy of a vaccine to be assessed clearly.

In this thesis, the General Introduction is a review of the biology of FeLV and of previous attempts to produce vaccines using FeLV antigens. Chapter 1 describes the general materials and methods used throughout the study.

In Chapter 2 the conditions for the preparation of FeLV for use in vaccination are described. It was found that the optimum conditions for producing virus from F422 cells were to seed the

cells at 1.2 X 10^6 cells per ml of L-M medium and harvest the fluid after 24 hours. In the second experiment of this chapter, the conclusion was that virus production was better in L-M medium than RPMI and the amount of virus harvested at 72 hours increased with increasing FCS concentration. In the last experiment, it was found that virus harvested at 24-48 hours after subculture was of optimum antigenicity.

Chapter 3 describes the inactivation of FeLV pseudotypes of murine sarcoma virus [MSV(FeLV)] with several chemicals. It was found that for complete inactivation of virus, paraformaldehyde (PF) should be used at 0.05%, a mixture of PF and glutaraldehyde at 0.004% (or less) and acetylethyleneimine (AEI) at 0.5%. An agent which stabilises disulphide bonds (2,2-dithiobis, 5-nitropyridine (DTNP)) was also found to inactivate virus, but not completely at the concentrations used. In this chapter, the adsorption of virus to $Al(OH)_3$ was examined. Approximately 75% of virus was absorbed under conditions employed.

Chapter 4 reports the first vaccination experiment in which FeLV preparations inactivated with PF, AEI or DTNP and PF were compared as vaccines. A vaccine prepared with PF-inactivated F422 cells was also used and $Al(OH)_3$ and saponin were used as adjuvants. The result was that the most effective vaccine appeared to be that inactivated with PF and the least effective was the F422 cellular vaccine. A scoring system was used to assess efficacy of the vaccines. Three further lessons were learned from this experiment and were incorporated into the second vaccine experiment (Chapter 7). First, the challenge dose of FeLV should be increased in order to produce viraemia in all

of the unvaccinated cats. Secondly, a larger number of cats per group should be used to increase the chance of demonstrating difference between vaccinated groups. Thirdly, the adjuvant saponin should be used at a lower concentration to avoid side-effects found during its use.

An innocuous dose of saponin was determined in Chapter 5. The dose of saponin which was harmless was 100 ug per cat, compared to 1500 ug which had been used previously in Chapter 4. Chapter 6 deals with the preparation and determination of an appropriate challenge dose of FeLV. The virus was prepared from feline embryo cells infected with FeLV-A/Glasgow-1 and was concentrated by ultracentrifugation. Cats were infected oronasally with a dose of 6.5×10^5 FIU and all of the cats became viraemic.

Chapter 7 contains the results of the second vaccination experiment, incorporating the improvements described above, in which a comparison of adjuvants was done, using PF-inactivated FeLV. In this chapter MSV(FeLV) was first inactivated with several concentrations of PF at 25° to obtain more information on the optimum concentration of PF to use. Secondly, FeLV was inactivated in the same way using the established optimum PF concentration. Traces of the inactivating agent were removed by ultrafiltration and the inactivated, washed antigen was used with two adjuvants, saponin and incomplete Freund's adjuvant. The results of this experiment indicated that the vaccine using the incomplete Freund's adjuvant was slightly superior to the vaccine with saponin.

Chapter 8 contains the result of the final vaccination experiment in which a comparison of antigens was done. The antigens and vaccines were prepared as in the previous experiment except that two viruses were used: F422 virus or FeLV-A/Glasgow-1, grown in feline embryo fibroblast cultures. A comparison of F422 virus using unconcentrated virus and virus concentrated tenfold was also made. It was found that the unconcentrated, PF-inactivated F422 virus protected 5/6 kittens from a challenge with FeLV which caused viraemia in 6/6 of unvaccinated kittens. The response to concentrated F422 virus was not better. The FeLV-A/Glasgow-1 virus vaccine did not significantly protect the cats. In this experiment it was also found that 4 of 5 vaccinated cats which resisted challenge had a latent FeLV infection in the bone marrow.

The results in Chapter 9 are from experiments to attempt to assess in vitro the antigenicity of FeLV exposed to four types of chemical inactivating agents (PF, Formaldehyde, AEI and betapropiolactone [BPL]). This was achieved by measuring the binding of monoclonal antibodies to FeLV treated with these agents, using an enzyme immunoassay. These results indicated that, as expected, the effect of formaldehdye and PF was similar but different to that of AEI or BPL. At the concentrations used, AEI and BPL had a less harmful effect on antibody binding capacity than formaldehyde or PF. These two latter agents markedly reduced antigenicity at high concentrations (1 or 2%) but enhanced antigenicity (by up to 65%) at concentrations similar to those used in the successful vaccines described in Chapters 7 and 8.

Persistence of FeLV neutralising antibodies in naturally infected cats was studied in Chapter 10. These results appeared to show that the titre of FeLV neutralising antibodies is maintained in cats for a period of over one year whether or not the cats are continuously exposed to cats excreting FeLV. These results indicate that following contact infection a cat becomes a low or a high responder.

Chapter 11 is a general discussion which reviews the results in this thesis and compares the results with those of previous experiments by others. Suggestions for future work are proposed.

ABBREVIATIONS

AEI	Acetylethyleneimine.
BDH	British Drug Houses
BPL	Betapropiolactone
CCC	Crandell cat kidney cell line
C81	Clone 81 cells
CFA	Complete Freund's adjuvant
DMSO	Dimethylsulphoxide
DTNP	2,2-dithiobis,5-nitropyridine
EFC	Eagle's medium with 10% foetal calf serum
EFC20	Eagle's medium with 20% foetal calf serum
EIA	Enzyme immune assay
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FEA	Feline Embryo A cell line
FeLV	Feline leukaemia virus
FeSV	Feline sarcoma virus
FFU	Focus forming units
FIU	Focus inducing units
FOCMA	Feline oncornavirus-associated cell membrane antigen
FOR	Formaldehyde
GA	Glutaraldehyde
ICFA	Incomplete Freund's adjuvant
IF	Immunofluorescence
IM	Intramuscular
IP	Intraperitoneal
L-M	Leibovitz L-15 + McCoys 5a Growth Medium

MCH	Multicat household
MLV	Murine leukaemia virus
MSV	Murine sarcoma virus
MSV(FeLV)	FeLV pseudotype of MSV
OD	Optical density
PB	Polybrene
PBS	Phosphate buffered solution
PF	Paraformaldehyde
PS	Penicillin-Streptomycin
SAS	Saturated Ammonium Sulphate
SC	Subcutaneous
SF	Superfos [$Al(OH)_3$]
SPF	Specific pathogen free
VC	Virus control
VI	Virus isolation
WRL	Wellcome Research Laboratories

GENERAL INTRODUCTION

Feline leukaemia virus (FeLV) was discovered in 1964 (Jarrett et al, 1964a) and has become the subject of much veterinary and comparative interest and research throughout the world. FeLV infection is common among cats in situations where contact is frequent and may be a major cause of death. The virus is responsible for diseases of the haemopoietic system including lymphoid and myeloid malignancies and degenerative diseases such as anaemia, marrow aplasia and immunosuppression. FeLV transmission is by contact (horizontal transmission) (Hardy et al, 1973; Jarrett et al, 1973) or congenitally from the mother to her kittens (vertical transmission) (Jarrett and Russell, 1978). Since some cats which are exposed to FeLV develop FeLV neutralising antibodies and become naturally resistant to FeLV infection, it is considered that it may be possible to develop an effective vaccine. Previous experiments led to the development of several prototype vaccines but none of these has yet been developed for field use. The aim of the present studies was to prepare a potent and safe FeLV vaccine in the light of previous experiments which would be simple and inexpensive to produce and would protect cats against FeLV infections and limit FeLV disease spread.

1. Discovery of FeLV

FeLV was first discovered in 1964 in association with a cluster of cats that had lymphosarcoma (Jarrett et al, 1964a; 1964b). The observed clustering of feline lymphosarcoma cases led to extensive research into the nature of FeLV and the means

by which it is transmitted from cat to cat.

2. FeLV related diseases

Experimental and epidemiological studies have revealed that FeLV is spread contagiously between cats living in their natural environment, as described below. The virus has the ability to transform haemopoietic cells into tumour cells and can also cause a number of fatal non-neoplastic diseases mainly involving the haemopoietic system.

FeLV-infected cats which are apparently healthy have an increased chance of developing FeLV-related diseases (Hardy et al, 1973). The incubation period for disease development after infection varies from weeks to years. The main diseases associated with FeLV infection are summarized in Table 1.

The most common tumour in cats is lymphosarcoma of which there are four main forms (Crighton, 1968). These are thymic lymphosarcoma, multicentric lymphosarcoma, alimentary lymphosarcoma and lymphatic leukaemia. The second type of malignant haemopoietic diseases involves the myeloid cells and includes myeloid leukaemia, erythroleukaemia and acute erythraemia (Jarrett and Mackey, 1974). Non-malignant haemopoietic diseases include two types of anaemia (haemolytic anaemia and erythroid hypoplasia) marrow aplasia and immunosuppression. The second type of non-malignant disease is infertility, mainly foetal resorption.

TABLE 1

Main Diseases Associated with FeLV Infection

Malignant haemopoietic diseases		
	Lymphoid tumours:	thymic lymphosarcoma
		multicentric "
		alimentary "
	Myeloid tumours:	myeloid leukaemia
		erythroleukaemia
		acute erythraemia
Non-malignant haemopoietic diseases		
	Anaemia:	haemolytic anaemia
		erythroid hypoplasia
	Immunosuppression:	thymic atrophy
	Marrow aplasia	
	Myelofibrosis and osteosclerosis	
Non-haemopoietic conditions		
	Reproductive failure:	foetal resorption
		abortion

Malignant haemopoietic diseases

Lymphosarcoma

Lymphosarcoma is the most common malignant tumour of the cat and accounts for approximately one third of all feline tumours (Hardy,1981a). The disease occurs with an annual incidence of about 45 per 100,000 cats at risk (Dorn et al, 1968). Lymphosarcoma does not have a predilection for any particular breed or sex, but often occurs in young cats less than 3 years old. Lymphosarcoma is classified according to the distribution of the major gross lesions in the following 4 groups (Mackey and Jarrett, 1972; Jarrett and Mackey, 1974):

a. Alimentary lymphosarcoma

This is the most common form of the tumour which is usually found either in one site in the intestinal wall or is associated with spread to the mesenteric lymph nodes and kidneys.

b. Thymic lymphosarcoma

This form is characterised by a large mass of malignant lymphoid cells infiltrating the site of the thymus gland in the anterior mediastinum. All thymic lymphosarcoma consist of T cells. Tumour cells are seldom found in the blood.

c. Multicentric lymphosarcoma

This is the third most common type of lymphosarcoma. Lymph nodes all over the body are usually involved, in addition to the liver and spleen. Multicentric lymphosarcoma mostly occurs in young cats (average age 4 years) and is characterised by gross enlargement of the lymph nodes due to infiltration of malignant lymphoid cells. At post-mortem examination or biopsy the affected lymph nodes are pale with loss of cortico-medullary

differentiation.

d. Lymphatic leukaemia

This form may be considered as lymphosarcoma which occurs in the bone marrow. Growth of malignant cells in the marrow results in impairment of haemopoiesis, so that anaemia and thrombocytopenia are often seen. The tumour cells spread to other organs by the blood, hence are found in the blood, liver and spleen. This kind of lymphosarcoma is often characterised by a great increase in the white blood cell count.

Myeloid leukaemias

a. Myeloid leukaemia

FeLV also causes a group of primary bone marrow neoplastic disorders involving myeloid cells. In myeloid leukaemia, malignant myeloid cells in various degrees of differentiation are found in the bone marrow, liver and spleen and relatively mature granulocytes may be frequently found in the blood.

b. Erythroleukaemia

FeLV infects and replicates in erythroid progenitor cells which still have nuclei and causes both degenerative and proliferative diseases. In erythroleukaemia both erythroid and myeloid cells are involved in the malignant process.

c. Acute erythraemia

In this type of leukaemia the major malignant cell type is an early erythroid cell.

Non-malignant haemopoietic diseases

I. Anaemias

a. **Haemolytic anaemia:** Anaemia in cats is more common than in other pet animals because of several factors, the most important of which is FeLV. FeLV-related anaemia may be a primary effect of FeLV on haemopoiesis or an indirect consequence of the effects of either lymphoid or myeloid leukaemias on haemopoiesis(Mackey et al, 1975).

The main types of FeLV-related anaemia which are recognised are erythroid hypoplasia and haemolytic anaemia. Often anaemias related to FeLV infection remain undiagnosed until the severe final stages of the condition. It has been suggested that one sixth of cats with a haematocrit of less than 0.20 will have this type of anaemia (Hardy, 1981b).

b. **Erythroid hypoplasia:** this anaemia is frequently diagnosed in cats and is analogous to pure red cell aplasia in man, in which only cells of the erythroid series are affected. The numbers of erythroblasts, normoblasts and reticulocytes in the bone marrow and the blood erythrocytes rapidly decrease, while the production of blood granulocytes and platelets is still normal (Hoover et al, 1974; Mackey et al, 1975; Onions et al, 1982). All these abnormal haematological processes happen due to a complete block of erythropoiesis. This type of anaemia is often termed "non-regenerative" anaemia.

II. Immunosuppression

FeLV is responsible for several secondary diseases which develop as a result of immunosuppression. FeLV causes both

specific (anti-viral) and non-specific (general) immunosuppression. The mechanism of immunosuppression is complex and not yet fully understood.

One contributing factor may be the effect of the virus on the thymus resulting in thymic atrophy and hence defects in the maturation of T lymphocytes (Anderson et al, 1971). Another suggestion for immunosuppression development is the effect of the viral envelope protein p15E which may act directly on lymphocytes preventing their participation in immune responses (Perryman et al, 1972; Olsen et al, 1980).

III. Marrow aplasia

This condition is characterised by hypoplasia of myeloid and erythroid cells in the bone marrow which leads to leukopaenia and anaemia. Following leukopaenia there may be rapid invasion of the intestinal epithelium by bacteria causing dysentery. This condition may be confused clinically with panleukopaenia but is often seen in cats vaccinated against feline panleukopaenia virus (Hardy, 1981b).

IV. Other diseases

These diseases include conditions of the reproduction system particularly resorption of foetuses and infertility in queens, and less frequently abortion. Another disease thought to be indirectly related to FeLV infection is glomerulonephritis (Anderson et al, 1971; Mackey, 1975) which may be caused by the accumulation of immune complexes containing FeLV antigens and complement in the glomeruli of some cats with persistent FeLV viraemia (Weksler et al, 1975).

Structure and biology of feline leukaemia virus

Viral structure

FeLV is a type C retrovirus, similar in structure to other retroviruses which cause leukaemia in chickens, cattle and mice (Jarrett et al, 1964b). The viral particle is approximately spherical and 110 nm in diameter. The viral genome is RNA and is enclosed in an internal protein core. Surrounding the core is a phospholipid envelope from which project small spikes (Laird et al, 1968a).

FeLV RNA is single-stranded and two identical molecules are present in the viral core (O. Jarrett et al, 1971). Bound to the RNA are several molecules of a reverse transcriptase enzyme which is a RNA-dependent DNA polymerase. The RNA contains three genes called gag, pol and env. The gag gene codes for the viral internal proteins (group-specific antigens). The pol gene codes for the reverse transcriptase, and the env gene codes for the proteins of the viral envelope.

The four internal proteins which are the products of the gag gene are termed p15, p12, p27 and p10 on account of their molecular weights which are 15,000, 12,000, 27,000 and 10,000 respectively (Green et al, 1973). These proteins are found at specific sites in the viral particle. Thus p15 is in the exterior of the viral core, p12 is located just inside the viral envelope, p27 is the major component of the viral core shell and p10 is a nucleoprotein which is associated with the viral RNA.

The envelope proteins are gp70 which is a glycoprotein with a molecular weight of about 70,000 and p15E which is a non-

glycosylated protein with a molecular weight of 17,500 (but 15,000 in SDS-polyacrylamide gel electrophoresis) (Neil, 1978). The p15E is very hydrophobic and is strongly associated with the envelope. This molecule acts to anchor the gp70 to the envelope by disulphide bonds. The gp70 constitutes the viral spike which projects from the viral surface. The spikes have three important functions: they contain the antigenic determinants which induce the production of virus neutralising antibodies; and they carry the sites which determine viral subgroup (A, B or C) and the ability to infect certain cells by binding to their specific surface receptors.

Subgroups of FeLV

Feline leukaemia viruses occur in 3 subgroups, A, B or C (Sarma and Log, 1973). All FeLV isolates made from cats with lymphosarcoma or clinically healthy carriers contain FeLV of subgroup A (FeLV-A) and in addition approximately half also contain virus of subgroup B (FeLV-B) (Jarrett et al, 1978; Sarma et al, 1973). Viruses of subgroup C (FeLV-C) are relatively rare, occurring in only 1-2% of isolates (Jarrett et al, 1978) but are quite common (28%) in cats with severe anaemia (Onions et al, 1982). Thus, the possible composition of FeLV isolates is FeLV-A, FeLV-AB, FeLV-AC or FeLV-ABC.

Several reasons have been put forward for the apparent dependence of FeLV-B and FeLV-C on FeLV-A. Sarma and Log (1973) showed that the reason was not because these viruses were defective and required FeLV-A as a helper virus since they could be separated and grown in cat cell cultures in the absence of

FeLV-A. Jarrett and Russell (1978) found that FeLV-B was not transmitted horizontally to tracer kittens which had been made viraemic by experimental inoculation with FeLV-B alone. However, both FeLV-A and FeLV-B were transmitted naturally from cats which were viraemic with FeLV-AB. These authors suggested that FeLV-B is only transmitted within phenotypic mixtures with FeLV-B because target cells for only FeLV-A are available in the oropharynx.

A different reason was suggested for the dependence of FeLV-C on FeLV-A by Russell and Jarrett (1978b). This was that FeLV-C viruses arise de novo in cats as recombinants of FeLV-A, acquired by exogenous infection, and endogenous FeLV-like DNA sequences in normal cat cell DNA (Okabe et al, 1976). Russell and Jarrett (1978b) considered it unlikely that FeLV-C viruses were actually transmitted between cats in nature.

The subgroups of FeLV are distinguished by interference tests (Fischinger and O'Connor, 1969; Sarma and Log, 1971). There are also differences in the host range of each subgroup. FeLV-A infects only cat cells while FeLV-B and C have a wider host range (Jarrett et al, 1973). Antigenically, by neutralisation tests, all FeLV-A viruses are monotypic but there is some antigenic variation between individual isolates of FeLV-B or FeLV-C and there is considerable cross-reactivity between subgroups (Russell and Jarrett, 1978a).

Replication of FeLV

Infection of the host cell by FeLV begins by the attachment of the spikes of the virus envelope surface to specific receptors

on the host cell membrane. The virus genome penetrates inside the cell by a method which is not completely understood. The reverse transcriptase enzyme uses the RNA as a template to make a single-stranded DNA copy. A double-stranded DNA molecule is then synthesised which integrates into cellular DNA to form a provirus. The provirus in the cell chromosome can be activated to produce virus.

The production of a progeny virus by the infected cells occurs by synthesis of mRNA which in turn produces the viral proteins in the cell cytoplasm. Complete infectious virus particles are assembled from the viral proteins and new RNA at the cell membrane and particles are made by budding through the membrane without causing damage, hence there is no cytopathic effect (Laird et al, 1968a). This occurs both in vivo and in infected cell cultures. While FeLV has the ability to transform infected feline cells in vivo it does not have this ability in vitro.

4. Transmission of FeLV

FeLV is transmitted contagiously among pet cats in two ways. The first means of transmission is horizontal (Hardy et al, 1973; Jarrett et al, 1973) by close contact via licking or biting between cats by means of contaminated saliva (Francis et al, 1977). This enables the virus to infect cats through the oral and nasal epithelium.

The second means of transmission is epigenetic or congenital transmission in which the virus is transmitted from the infected queen to the developing foetus across the placenta during

pregnancy (Jarrett and Russell, 1978).

5. Pathogenesis of FeLV

Persistently viraemic cats are the main source of FeLV infection. Subsequent to direct contact infection, the virus grows in the cells of the oropharynx and is transported to the bone marrow via the blood in mononuclear cells (Rojko et al, 1979). Bone marrow cells are very susceptible to the virus and large quantities of FeLV are released into the blood. The virus then may spread haematogenously to other tissues such as the upper respiratory tract epithelial cells and salivary glands and thus again to the mouth.

Although FeLV is distributed in this way all over the body of the infected cat, its pathogenic effects are mostly on the haemopoietic cells.

6. Outcome of FeLV infection

Cats exposed to FeLV, either experimentally or naturally, become persistently infected or recover (Hardy et al, 1973). Recovered cats either completely recover and eliminate thevirus or have a latent infection in the bone marrow (Rojko et al, 1982). There are two main factors which determine the outcome of FeLV infection. These are the age at which the cat is infected and the dose of the virus to which it is exposed. Thus, developing foetuses and newborn kittens are most susceptible and have a greater chance of becoming persistently infected than adult cats (Hoover et al, 1976). The dose of virus, under natural conditions, is dependent of the circumstances in which the cats live. As described below, among free range cats the

dose of virus transmitted is low while in multicat households the degree of contact is high and the dose of virus is large.

It is almost certain that these factors influence the immune response to FeLV infection and hence the ability of the cat to overcome the infection. Young kittens do not mount aneffective immune response and unless protected by maternal antibody (Hoover et al, 1977a; Jarrett et al, 1977) are immunosuppressed to FeLV. Older kittens seem to be more immunologically competent and the outcome depends on the balance between viral growth and rate of development of an immune response to the virus.

Cats exposed to FeLV have the following characteristics.

a. Persistently infected cats

Animals which are infected with FeLV and fail to mount an effective immune response develop a permanent infection with a persistent viraemia. These cats continuously excrete the virus from the mouth and are the main source of FeLV infection among the cat population. These viraemic cats have a high risk of developing FeLv-related diseases. The studies of McClelland et al (1980) showed the outcome in three different types of households during a 3.5 year period.

In the first households which were FeLV-free, 32 (17%) of 191 uninfected cats died of disease during the observation period. In contrast, 159 (83%) were still alive after the same period. The second households contained cats which were viraemic and those which were exposed but non-viraemic. The non-viraemic cats were isolated from the viraemic cats. Of 46 infected cats which were isolated from the 233 uninfected cats, 36 (83%) died

of disease during the observation period whereas only 29 (12%) of the 233 uninfected cats died of disease during the same period. In the third type of household which contained both infected and uninfected cats living together, of the 50 infected cats, 42 (89%) died of disease, in contrast to only 21 (24%) of the 88 uninfected cats which died of disease during the observation period. This study showed that there was a five-fold higher mortality rate among FeLV-infected cats than among uninfected cats in the households.

b. Recovered cats

Recovered cats are of two types. First, the cats may have completely eliminated FeLV and in these there is no viraemia, no virus may be isolated from the bone marrow cells, no infectious FeLV is excreted from the mouth and there is a low risk of FeLV-related diseases developing (Hardy et al, 1973). Secondly, a state of latent infection may develop in which there is no viraemia but virus can be isolated from bone marrow cells in culture (Rojko et al, 1982). In these cats no infectious FeLV is transmitted (Madewell and Jarrett, 1983). The risk of FeLV-related diseases in these cats is not yet known.

c. Transient viraemia

Some cats which recover from FeLV infection have a transient viraemia which may last from a day or two up to 8 weeks or longer (Jarrett et al,1982a; Pedersen et al, 1979). During this period the cat may also excrete the virus from the mouth and viral antigen and infectious virus may be found in the blood. Following experimental oronasal infection, the peak of transient

viraemia is at 3 weeks after infection and is usually followed by the development of high titres of neutralising antibodies (Jarrett et al, 1982a.). Under natural conditions of exposure the length of the transient viraemia is more variable (Pedersen et al, 1979).

7. Feline leukaemia virus immunity

The recovery of FeLV infected cats, whether they become latently infected or eliminate FeLV, appears to depend on both humoral and cell mediated immunity (Madewell and Jarrett, 1983; Rojko et al, 1982).

Serum of recovered cats contains two types of FeLV-specific antibodies: virus neutralising antibodies and antibodies to the feline oncornavirus-associated cell membrane antigen (FOCMA) (Essex et al, 1971). Virus neutralising antibodies are directed against FeLV gp70 and inactivate the virus in vitro and in vivo (Hoover et al, 1976; Jarrett et al, 1973; Russell and Jarrett, 1978a). Also, these antibodies are presumably responsible for the maintenance of the latent state of FeLV infection by their action on FeLV-infected cells in the bone marrow (Rojko et al, 1982). These antibodies protect the cats from FeLV infection and prevent viraemia in young kittens (Hoover et al, 1977a; Jarrett et al, 1977).

FOCMA was originally defined as an antigen on the surface of the FL74 cell-line of feline lymphosarcoma cells which reacted with antibodies in the sera of cats recovered from feline sarcoma virus infection (Essex et al, 1971) or exposed to FeLV (Riggs, 1971). Anti-FOCMA antibodies appear to protect cats from

lymphosarcoma (Essex et al, 1976) but not from FeLV infection and viraemia (Hardy et al, 1977).

The molecular nature of FOCMA or its genetic basis have not yet been resolved. Initially it was claimed that FOCMA was a tumour-specific antigen which was encoded in the genome of feline sarcoma virus and induced in tumour cells by FeLV (Stephenson et al, 1977). Sliski & Essex (1979) and Hardy et al (1977) suggested that FOCMA was not a FeLV structural antigen, mainly because purified FeLV or FeLV proteins could not absorb anti-FOCMA antibodies from viraemic cat sera. It should be noted, however, that the FeLV which was used in these experiments belonged to subgroups A and B only. Russell (1977) showed that in the sera of cats exposed to FeLV there was a strong correlation between antibody titres to FOCMA and neutralising antibody titres to FeLV-C but not to titres to FeLV-A or B. In the light of these results he suggested that there might be a relationship between FOCMA and FeLV-C envelope antigens, and indeed showed that the major antigen on Fl74 cells, which are the target cells in the FOCMA test, and released FeLV of subgroups A, B and C, was a FeLV-C antigen. Recently Vedbrat et al (1983) used both selected cat sera and mouse monoclonal antibodies to FeLV-C to show that the distribution of FOCMA could only be explained on the basis that it was an antigenic determinant related to FeLV-C.

Recent studies have found that cell mediated immunity may play an important role in recovery from FeLV infections. The cells involved are lymphocytes which play an important part in maintaining latent infection in the bone marrow and lyse cells

which produce virus in bone marrow cultures, derived from cats with latent infections (Rojko et al, 1982).

8. The epidemiology of feline leukaemia virus

Chronically (persistently) infected cats are the main source of infection in cat populations. FeLV is spread mainly by close contact between cats by means of licking or biting in which saliva containing virus is transferred. One millilitre of saliva may contain 5×10^3 to 2×10^6 infectious virus particles (Francis et al, 1977). In this way the virus infects the cat by way of the mouth and nose (horizontal transmission) (Hardy et al, 1973; Jarrett et al, 1973). The other route of spread of FeLV is across the placenta to the foetus.

The epidemiological pattern of FeLV infection is related to the housing situation in which cats are maintained of which there are two main types: open houses and closed houses. In the first type, cats are usually kept singly or in small numbers and range freely. Thus, contact between cats is frequent but intermittent and low doses of virus are transmitted from infected to healthy cats, hence the prevalence of exposure is high (50-70%) (Jarrett et al, 1973). By contrast, persistent infections are infrequent (1%) (Rogerson et al, 1975). In the second pattern, cats are generally kept in multicat households in which several cats are kept together and are isolated from the general cat population. Since they are in close contact with each other, the introduction of FeLV into these households by means of a carrier leads to widespread dissemination of the virus. In this way a high proportion of the cats (about 40%) develops persistent infections

(Hardy et al, 1973; Jarrett et al 1978). The main features in both types of cats are shown in Table 2.

9. Feline leukaemia virus control

In the light of epidemiological studies, it appeared that control of FeLV should be possible by removing the source of infection from the multicat households. Programmes of control to establish and maintain FeLV-free cat households have been developed (Hardy et al, 1976; Weijer & Daams, 1978). As described above, persistently viraemic cats are the main source of infection, hence isolation or destruction of these cats is the first important step to control the infection (Hardy et al, 1976).

Three diagnostic methods are currently used to detect viraemic cats.

a. An immunofluorescence (IF) method is simple, rapid and inexpensive (Hardy et al, 1973). FeLV antigen is detected in the cytoplasm of blood neutrophils in a fixed blood smear.

b. An enzyme-linked immunosorbent assay (ELISA) which is commercially available in kit form (Leukassay F) detects FeLV p27 antigen free in the plasma which is presumably released by virus-producing cells in the bone marrow.

c. Virus isolation (VI) is somewhat expensive and takes 6 days to carry out. However, it is very reliable since it detects infectious virus in the plasma (Jarrett et al, 1968; Jarrett et al, 1982b).

The next step in the control programme is to effectively disinfect the cat housing, litter, pans and feeding bowls. The

TABLE 2

FeLV epidemiological situations

Closed multicat households	
High exposure rate:	> 80% anti-FOCMA antibodies
High viraemia rate:	40% viraemia
High incidence of disease	
Free-range cats	
High exposure rate:	50% anti-FOCMA antibodies
Low viraemia rate:	1% viraemia
Low incidence of disease	

third step is to retest the cats at least 12 weeks after removal of the viraemic cats to establish whether any positive cats which have been retained are still positive and therefore have a permanent infection. More importantly, retesting the negative cats is done to ensure that they are still negative and were not incubating an infection at the initial test. Further testing periodically every 6 months ensures that the negative cats remain so, and detects if there are any new cases of infection. Also to protect against re-entry of FeLV, no new cats should enter the house before testing and satisfactory quarantine has been carried out.

The measures have been very successful in eradicating FeLV from many multicat households (Hardy et al, 1976; Weijer and Daams, 1978).

10. Anti-feline leukaemia virus vaccination

To complete a FeLV disease eradication programme an effective vaccine would be extremely useful. In a household from which virus had been eradicated, introductions, such as kittens born in the house or bought in from another source, could be protected against future exposure to FeLV at, for example, stud or cat shows. From epidemiological studies of FeLV it is clear that many cats form protective titres of virus neutralising antibodies as a consequence of a previous FeLV exposure and are known to be resistant to reinfection with virus suggesting the possibility of vaccination. Also we know that most FeLV transmission occurs horizontally, by close contact, so that the transmission cycle might be broken by vaccination.

Immunoprophylaxis is one of the major ways of preventing

infectious diseases in animals and man. The main means of achieving active immunity is the use of potent, safe and economical vaccines. The viral vaccines in current use are of two main types, attenuated (modified live) or inactivated (dead or killed). In addition subunit vaccines consisting of subviral antigens free of nucleic acid have been developed for a limited number of viruses (Bolognesi, 1976; Salerno et al, 1978; Balcarova et al, 1981). A fourth type of vaccine which is now being investigated comprises synthetic peptides which correspond to the relevant antigenic determinants of viruses (Bittle et al, 1982).

Modified live virus vaccines contain viruses which are non-pathogenic but retain the antigen(s) of the corresponding virulent virus which is(are) responsible for inducing a protective immune response. These attenuated viruses have been obtained in several ways, the most common of which are passage of virus for long periods in vitro, passage of virus in cells of a heterologous host or growth of a virus at low temperatures. In the cat, live vaccines are used for immunisation against feline panleukopaenia virus, feline calicivirus and feline herpesvirus. There are several potential advantages of live vaccines. Firstly, small doses of virus may stimulate an effective immune response because the virus replicates in the host. Secondly, the immune response to the vaccine may be similar in type to that produced following natural exposure to field virus. Thirdly, usually only one administration of vaccine is required. The major potential disadvantage of live virus vaccines is that the

virus may revert to virulence following growth in vaccinated animals and spread to other animals.

Inactivated virus vaccines are theoretically safer than attenuated vaccines since they cannot grow in the animal and therefore cannot cause any disease or revert to virulence. This assumes that the virus has been sufficiently well inactivated. Recently inadequate inactivation of a foot-and-mouth disease virus vaccine led to inoculated pigs being infected in France and the subsequent spread of live, virulent virus to the Channel Islands and the Isle of Wight in 1981 (Donaldson et al, 1982). In cats, inactivated viruses are used in a vaccine against feline panleukopaenia virus, feline calicivirus and feline herpesvirus (Vaxicat Plus: Tasman Vaccine Laboratories). In practice, the main disadvantages of inactivated virus vaccines compared to live virus vaccines are that several, usually two, inoculations of vaccine are required and a much larger amount of virus must be incorporated into the vaccine in order to provide sufficient antigenic mass for immunisation.

The choice of the type of vaccine which could be used in the field for immunisation against FeLV infections is restricted at present to inactivated preparations, for three main reasons. Firstly, attenuated FeLV strains have not yet been developed and are unlikely to be produced in the near future since testing FeLV for non-pathogenicity would require several years owing to the long incubation periods of most FeLV isolates (Mackey et al, 1972). Secondly, subunit vaccines derived from purified FeLV particles would be prohibitively expensive, since large amounts of purified virus would have to be produced. Thirdly, vaccines

based on synthetic peptides are still at the experimental stage. Another, non-scientific, reason is that it is very unlikely that product licensing authorities would permit the use of a live vaccine for a virus which is oncogenic in a species which lives closely with human beings.

W. Jarrett and his colleagues were the first to attempt to develop a FeLV vaccine (Jarrett et al, 1974). In an initial study they inoculated cats with live feline lymphoblastoid cells of the FL74 line and obtained high titres of anti-FOCMA antibodies. Some of these inoculated cats were subsequently challenged with live FeLV of a highly pathogenic strain and resisted the infection while another group of cats given the challenge virus became infected (Jarrett et al, 1975). In the same experiments, purified virus released from FL74 cells was inoculated into another group of cats. In addition cats were vaccinated with paraformaldehyde-inactivated FL74 cells. The results were that purified virus did not induce FOCMA antibodies in cats, while cats vaccinated with PF-inactivated FL74 cells developed FOCMA antibodies. No virus could be isolatd either from the inactivated vaccine or from the vaccinated cat tissues. The conclusion from these experiments was that FL74 cells were immunogenic but released low infectivity virus and might be of value in devising vaccines against FeLV.

Another FeLV vaccine preparation was made by Pedersen and his co-workers (Pedersen et al, 1979) who vaccinated cats with living virus released from FL74 cells, formaldehyde-inactivated virus from the same source or formaldehyde-inactivated FL74 cells

and challenged the cats with virulent FeLV by contact. Their results were that neither inactivated vaccine produced a measurable anti-FOCMA or virus neutralising antibody response nor completely prevented natural infection with virulent FeLV. However, the live virus vaccine dramatically decreased the proportion of infected kittens. The cell vaccine did not protect against challenge.

A third attempt to develop a FeLV vaccine was made by Salerno and his co-workers (Salerno et al, 1978). They apparently pursued the development of a subunit vaccine against FeLV as a guideline to preparing similar vaccines for human beings in the event that retroviruses were found to be causes of leukaemia in man. A subunit vaccine was chosen to avoid the use of a live oncogenic virus or the oncogenic viral RNA. The antigen selected for subunit vaccine preparation was the viral envelope glycoprotein, gp70. The glycoprotein was purified from FeLV-AB virus and was given to cats and guinea pigs with and without adjuvant. An aqueous vaccine induced neutralizing antibody in only 50% of guinea pigs while an adjuvanted vaccine induced higher levels. In contrast to guinea pigs, cats immunised with twice the guinea pig dose produced little or no response with the aqueous vaccine and even less of an immune response than guinea pigs when vaccinated with an $Al(OH)_3$ adjuvanted vaccine. At the same time this glycoprotein was inoculated into goats and induced a high titre of antibody which specifically neutralised FeLV.

Finally, Olsen and his co-workers have prepared several FeLV vaccines. First, they immunised cats with thermally inactivated

FL74 cells and these cats produced high titres of antibody to FOCMA and were protected from oncogenic feline sarcoma virus challenge. However, the vaccine did not induce virus neutralising antibody, nor did it prevent FeLV viraemia following challenge (Olsen et al, 1976). A second attempt to prepare a FeLV vaccine was made by giving the cats a dual vaccine composed of killed FL74 cells and killed FeLV (formalin-treated or ultraviolet light inactivated) in an attempt to prevent both viraemia and ensuing neoplastic disease. The unexpected result of using this vaccine was that the cats became more susceptible to FeSV challenge than the unvaccinated control cats. It was considered that the combination of both FeLV and tumour cell vaccine abrogated protective tumour immunity. Their explanation of this result was that a small molecular protein (p15E) from FeLV which was in the vaccine was immunotoxic and led to suppression of the cats' lymphocyte functions rendering the vaccinated cats more susceptible than the controls. At the same time these workers vaccinated cats with killed FeLV or killed FL74 cells with the result that virus neutralising antibodies were induced in adult cats, but not in young kittens at first vaccination. Protective immunity against the subsequent neoplastic disease was evident, but the vaccine did not prevent FeLV viraemia (Olsen et al, 1980a).

In the light of these experiments, Olsen and his co-workers subsequently developed a soluble tumour cell vaccine containing viral proteins and "FOCMA", which was prepared from concentrated spent media of FL74 cells grown in serum-free medium. This

material was non-infectious and was used with complete or incomplete Freund's adjuvant, or $Al(OH)_3$ to vaccinate cats. The vaccination results were that potent anti-FOCMA antibodies were induced and a high proportion (80%) of the cats were protected from FeLV viraemia and related diseases after challenge with the Rickard strain of FeLV. In contrast all control cats developed FeLV viraemia after challenge (Olsen et al, 1980a). The vaccinated cats made antibody to FeLV gp70, FeLV p27 and FeLV p15, as shown by immunoprecipitation of radiolabelled viral proteins.

The present study

The primary aim of the studies described in this thesis was to develop a vaccine against FeLV infection based on chemically inactivated whole virus.

In the thesis, Chapter 1 deals with the general materials and methods used in these studies while specific materials and methods are described in the individual chapters. Chapter 2 is an account of studies to find the optimum conditions for producing cells and virus for use in the preparation of vaccines. Chapter 3 describes the results of studies on the inactivation of FeLV pseudotypes of mouse sarcoma virus to find the conditions to inactivate FeLV. Using these results, several FeLV vaccines were prepared with these agents and compared for efficacy in an experiment described in Chapter 4. Some problems involving side-effects of the adjuvant and the challenge dose of virus were encountered in this experiment, and Chapters 5 and 6 present the results of experiments to overcome these problems.

Chapter 7 and 8 describe two further vaccination experiments. In the first, a comparison was made of two different adjuvants, saponin and incomplete Freund's adjuvant. In the second, Freund's adjuvant was used and FeLV produced from two sources, suspension cultures or monolayercultures, was compared as an antigen. Chapter 9 is an account of experiments in which attempts were made to develop an *in vitro* test of antigenicity of FeLV antigen treated with chemical inactivating agents. Finally, in Chapter 10 an experiment is described which examined the persistence of FeLV antibodies in cats which had recovered from natural FeLV infection.

CHAPTER 1

MATERIALS AND METHODS

Materials

1. Media

The Glasgow (BHK21) modification of Eagle's Minimal Essential Medium (MEM) was supplied by the Institute of Virology, University of Glasgow. Growth medium (EFC) was Eagle's MEM with 10% foetal calf serum (FCS). Occasionally medium with 20% FCS was used (EFC20). FCS was purchased from Gibco-Europe Ltd and batches were selected for their capacity to support the growth of FeLV pseudotypes of murine sarcoma virus (MSV(FeLV)).

Leibovitz L-15 medium was purchased from Gibco-Europe Ltd. This medium is independent of CO_2 for buffering and for this reason was used to make dilutions of viruses and sera during assays.

McCoy's 5a medium (Gibco-Europe Ltd) was used with an equal volume of Leibovitz L-15 medium and 15% FCS to grow suspension cell cultures. This growth medium was termed L-M medium. Penicillin (100 units/ml) and streptomycin (100 units/ml) were used in all media.

2. Cell cultures

The following cells were used.

a. FEA cells: These feline embryonic fibroblast cells were used between passage 15 and 35 (Jarrett et al, 1973). The cells were routinely grown in 250 ml glass bottles with 12 ml of EFC medium and one quarter of the total cells were sub-cultured twice a week.

b. C81 cells: The clone 81 cells were originally derived from the Crandell feline kidney fibroblast cell line (CCC) and contain the genome of Moloney mouse sarcoma virus (MSV) (Fischinger et al, 1974). These cells were grown and sub-cultured as above.

c. FL74 cells: These are neoplastic lymphoid cells derived from a lymphosarcoma tumour of the kidney of a cat infected with FeLV (Theilen et al, 1969). The cells were grown in suspension and were sub-cultured with L-M medium at 1 X 10^6 per ml density and were sub-cultured twice a week. The cells continuously release FeLV-ABC/KT strain in large quantities.

d. F422 cells: This cell line was established from a thymic lymphosarcoma of a kitten inoculated with the second passage of the Rickard strain of FeLV (Rickard et al, 1969). The cells grow in suspension culture with L-M medium and release FeLV of sub-group A.

3. Feline leukaemia viruses

The following feline leukaemia viruses were used.

a. FeLV-A/Glasgow-1 was originally isolated from a cat with alimentary lymphosarcoma. The virus was released from chronically infected FEA cell cultures (Jarrett et al, 1973).

b. FeLV-A/F422 was released from F422 suspension cultures.

c. FeLV-ABC/FL74 was produced from FL74 cells.

4. FeLV pseudotypes of mouse sarcoma virus

A FeLV-A/Glasgow-1 pseudotype of Moloney mouse sarcoma virus was used (MSV (FeLV-A/Glasgow-1). The Moloney mouse sarcoma virus was rescued from C81 cells by FeLV-A/Glasgow-1 (Russell and Jarrett, 1976).

5. Experimental cats

The experimental kittens were obtained from the specific pathogen free cat colony of the Veterinary School of Glasgow University. Generally these kittens were 8 weeks old at the beginning of the experiments.

Methods

1. Cell growth and sub-cultures

All cells growing in suspension culture were at a cell density of 1 X 10^6/ml in 8 ounce glass bottles or 25 cm^2 plastic flasks (Nunc) with 12 ml L-M medium and were sub-cultured every 3-4 days. Seeding of these cells was restricted to living cells which were indicated by using Trypan blue (1%) to stain the dead cells. In some cases where there was a high percentage of dead cells, a Ficoll-Hypaque solution was used to remove these cells from the culture. A volume of 15 ml of suspension culture was layered gently on top of 20 ml of Ficoll-Hypaque (Pharmacia) in a 50 ml centrifuge tube which was spun at 2000 rpm for 10 minutes. The live cells which collected in a band at the interface were removed, resuspended in 20 ml L-M medium and spun again at 1000 rpm for 10 minutes. The cells were finally resuspended in L-M medium at the appropriate density and were incubated at 37^{o}.

To make sub-cultures of FEA cells growing as monolayers in 8 ounce glass bottles, the old medium was removed, the monolayer was washed with 5 ml of trypsin-versene prepared by adding 5 ml 0.01% trypsin to 20 ml 0.02% versene (EDTA). The washing was repeated and then 1 ml of the trypsin-versene solution was added

to the bottle which was incubated at 37 ^{o} for 5 minutes. Then the detached cells were suspended in medium, were carefully dispersed and one-fourth of the cells was seeded in 12 ml EFC medium in 8 ounce glass bottles. A mixture of 5% CO_2 in air was added and the culture was incubated at 37^{o}. Sub-cultures were made twice a week.

2. Preparation of FeLV

Two FeLV stocks were prepared by different methods. The first method was to seed FeLV-A/Glasgow-1 infected FEA cells in 9 cm plastic plates at a cell density of 6 X 10^5 per plate with 10 ml EFC20 medium and to harvest the culture fluid 3 days later. The fluid was spun at 2000 rpm for 10 minutes and the supernatant fluid was stored at -70^{o} in volumes of 5 ml or 1 ml. The stock prepared in this way had a titre of 1.8 X 10^5 focus inducing units (FIU) per ml.

The second method was similar except that McCoy's 5a medium with 10% FCS was used and the culture fluid was harvested 2 days after seeding. The titre of this virus was 9.4 X 10^6 FIU/ml afterconcentration by ultrafiltration as described in detail in Chapter 7.

3. Assay of FeLV

a. One-stage assay

Five cm plates were seeded with 3 X 10^5 cells of FEA/C81 mixture at a 10:1 ratio in 4 ml EFC. Twenty four hours later each plate was inoculated with 1.0 ml of a virus dilution in EFC with 4 ug/ml polybrene after removing the old medium. The cells

were incubated at 37° for two hours and the inoculum was replaced with 4 ml EFC. Three days after inoculation the medium was replaced with 4 ml fresh EFC and the results were scored four days later. Virus transformed cell foci were counted microscopically.

b. Two-stage assay

Clone 81 cells were seeded in 4 ml EFC containing 8 X 10^4 cells and were incubated at 37° in 5% CO_2 in air. After 24 hours dilutions of virus were made in EFC containing 4 ug/ml polybrene (PB). The old culture medium was removed and 1 ml of diluted virus was added to each plate. The plates were incubated at 37° for 90 minutes. The inoculum was then replaced with 4 ml EFC and the plates were reincubated. Three days later the medium was replaced with 4 ml EFC containing 1 X 10^6 FEA cells and the plates were reincubated at 37°. Foci were counted microscopically after a further 4 days. The number of foci in a plate multiplied by the dilution factor gave the titre in FIU per ml. The titre was usually calculated from counts of plates containing about 20-30 foci.

4. Preparation of MSV (FeLV)

Five cm plastic plates were seeded with 8 X 10^4 C81 cells per plate in 4 ml EFC and were incubated at 37° in 5% CO_2 in air. On the next day the plates were inoculated with 1 ml of FeLV diluted in EFC containing 4 ug/ml PB. The plates were incubated for 90 minutes at 37°. The inoculum was then replaced with 4 ml EFC and the plates were reincubated.

Three days later the medium was removed and replaced with

1 X 10^6 of FEA cells in 4 ml EFC. The plates were reincubated at 37°. Two days later the fluid was harvested, centrifuged at 2000 rpm for 10 minutes and the fluid was stored in glass vials in 0.2 ml volumes at -70°.

The titre of virus prepared by this procedure was 4 X 10^6 focus forming units (FFU)/ml.

5. Assay of MSV (FeLV)

The method of Russell and Jarrett (1976) was followed. In the assay of MSV (FeLV-A) 3 X 10^4 cells chronically infected with FeLV-B were seeded in 4 ml EFC in 5 cm plastic plates and were incubated at 37°. After 24 hours tenfold dilutions of MSV (FeLV) were made in EFC20 with 4 ug/ml PB. The old medium was removed and 2 ml of each dilution was inoculated on to each culture plate which was incubated overnight at 37°. Next day the medium was removed and each infected culture was seeded with 1-1.2 X 10^6 FEA cells in 4 ml EFC20 and the plates were reincubated. After a further 3 days the plates were stained with 0.5% crystal violet in 5% methanol and 10% formalin for 15 minutes. The plates were then washed in water, dried and the foci were counted macroscopically. The titre was expressed in focus forming units (FFU) per ml.

6. Isolation of FeLV from plasma

FeLV was isolated from heparinised plasma as described by Jarrett et al (1982b). Cells were seeded at 4 X 10^3 cells per well in 24 well plastic cluster plates (Nunc) in 0.5 ml EFC with 4 ug/ml PB. The plates were incubated at 37° for 24 hours. A volume of 0.1 ml plasma was then added to each well and incubated

for 2 hours. The old medium with the inoculum was then replaced with a fresh 1 ml of EFC medium. Three days later the medium was removed and the inoculated culture was seeded with 1 X 10^5 FEA cells in 1 ml fresh EFC per well and reincubated. After 4 days the wells were examined microscopically for cell transformation and were scored as positive or negative for FeLV.

7. FeLV neutralisation test

To establish the presence of virus neutralising antibodies to FeLV-A in the sera of the cats used in the experiments, the following assay was used which was a modification of the method of Russell and Jarrett (1978a). FEA cells infected with FeLV-B were seeded at 4 X 10^3 cells per well in 0.5 ml EFC20 with 4 ug/ml PB in 24-well plastic plates and incubated at 37^o. Next day a volume of 50 ul serum diluted in L-15 containing 10% FCS medium was mixed with an equal volume of MSV (FeLV-A) diluted with the same medium in 96 well U-bottomed plastic plates. The mixture was incubated at 37^o for 90 minutes and 25 ul of each reaction was then inoculated into one well of the FEA/FeLV-B cells. Next day the infected culture was seeded with 1 ml EFC20 containing 1 X 10^5 FEA cells per well after removing the old medium. The plate was reincubated under the same conditions. Three days later the plates were stained with 0.5% crystal violet as above, washed with tap water, dried and the foci were counted. Positive neutralisation was scored when the number of foci was reduced to less than 25% of the number in the virus control without serum. Approximately 30 foci were present in the virus control.

CHAPTER 2

CONDITIONS FOR PREPARATION OF FeLV FOR VACCINES

Introduction

To begin to prepare an effective vaccine an appropriate antigen should be identified and methods for its production established. Previous research workers used either live whole FeLV, inactivated whole FeLV, FeLV gp70 or inactivated FeLV-infected cells (Jarrett et al, 1974; Olsen & Lewis, (1981); Pedersen et al 1979; Salerno et al, 1978). In each case the source of antigen was FL74 lymphoblastoid cells or the FeLV of subgroups A, B and C which is released by these cells. Some of the results were not completely satisfactory for the reasons which were described above and are discussed further in Chapter 11.

For the present series of experiments it was decided to concentrate on FeLV of subgroup A as the antigen. There were two main reasons for this choice. First, it appeared that FeLV-A viruses are monotypic based on neutralization tests involving 19 individual isolates (Russell and Jarrett, 1978a) so that an immune response induced by one isolate might be expected to be effective against any other FeLV-A. Secondly, since the horizontal transmission of FeLV-B and the appearance of FeLV-C are dependent on FeLV-A (Jarrett and Russell, 1978; Russell and Jarrett, 1978b) immunity to FeLV-A should also protect against contact infection by viruses of the other two subgroups.

The sources of FeLV-A which were considered were the lymphosarcoma suspension cell line, F422 (Rickard et al, 1969) and FEA monolayer cells chronically infected with FeLV-A/Glasgow-

1 (Jarrett et al, 1973). The advantage of F422 cells is that large quantities of viral particles are released which may be useful in providing sufficient antigenic mass for vaccine preparation. About 5 mg of viral protein is recovered from 1 litre of culture fluid (Neil, 1978). A possible disadvantage of using these cells is that the virus which is released has a low specific infectivity, compared to virus grown in monolayer cultures. This may indicate a defect in F422 viral gp70 which may not be efficient as an immunogen. In contrast, using infected FEA cells has the advantage that FeLV released from these cells is more infectious and perhaps more immunogenic, but at the same time has the disadvantage that there is a low viral mass (about 500 ug of viral protein in 1 litre of culture fluid).

The present chapter describes 3 experiments designed to establish the optimum conditions for the growth of these cells and viruses before they were used in the preparation of vaccines.

Results

Experiment I: Optimum conditions for the production of F422 cells and virus

F422 cells were grown in plastic flasks (25 cm^2) in 5 ml of L-M medium in each flask. Five different cell densities were used with 2 flasks for each density. The densities were 3×10^5, 6×10^5, 9×10^5, 12×10^5 and 24×10^5 per ml. The cultures were incubated at 37^o and culture samples were collected at 24, 48, 72, 96 and 120 hours after seeding. The fluid was centrifuged at 2000 rpm for 10 minutes and the supernatant was

stored at -70^{0} for FeLV assay later. At the same time a viable cell count was made. The results of cell counts, virus titration and ratio of infectivity per cell are shown in Table 2.1 and Fig. 2.1.

From these results it is seen that the highest virus titres were inthe samples collected from the cultures seeded at a cell density of 12 X 10^5/ml, 24 and 72 hours after seeding (5 X 10^4FIU/ml). The highest cell count was in the sample collected from the culture at 24 X 10^5/ml, 120 hours after seeding (1.0 X10^7/ml). The highest ratio of virus to cell was obtained from the cell culture seeded at 12 X 10^5/ml, 24 hours after seeding (34 X 10^{-3}).

Experiment 2: Growth of F422 cells in different media and FCS concentrations

The F422 cells were grown in 25 cm^2 plastic flasks with 10 ml of growth medium supplemented with FCS at 5%, 10% or 15%. Two media were compared: L-M and RPMI 1640. Three 25 cm^2 plastic flasks were seeded with F422 cells at a density of 7 X 10^5/ml in 10ml L-M medium. Each flask contained 5%, 10% or 15% FCS. Another 3 flasks were set up in the same manner using RPMI 1640 medium, but in this case all flasks were gassed with 5% CO_2 in air before incubation. Subsequently, 24, 48, 72 and 96 hours later, the cell concentration in each culture was determined by taking two 0.5 ml volumes from each flask, dispersing the cells and mixing 0.1 ml with an equal volume of 1% trypan blue for cell counting. Culture fluids were collected from all the flasks 72 hours after seeding, centrifuged at 2000 rpm for 10 minutes and

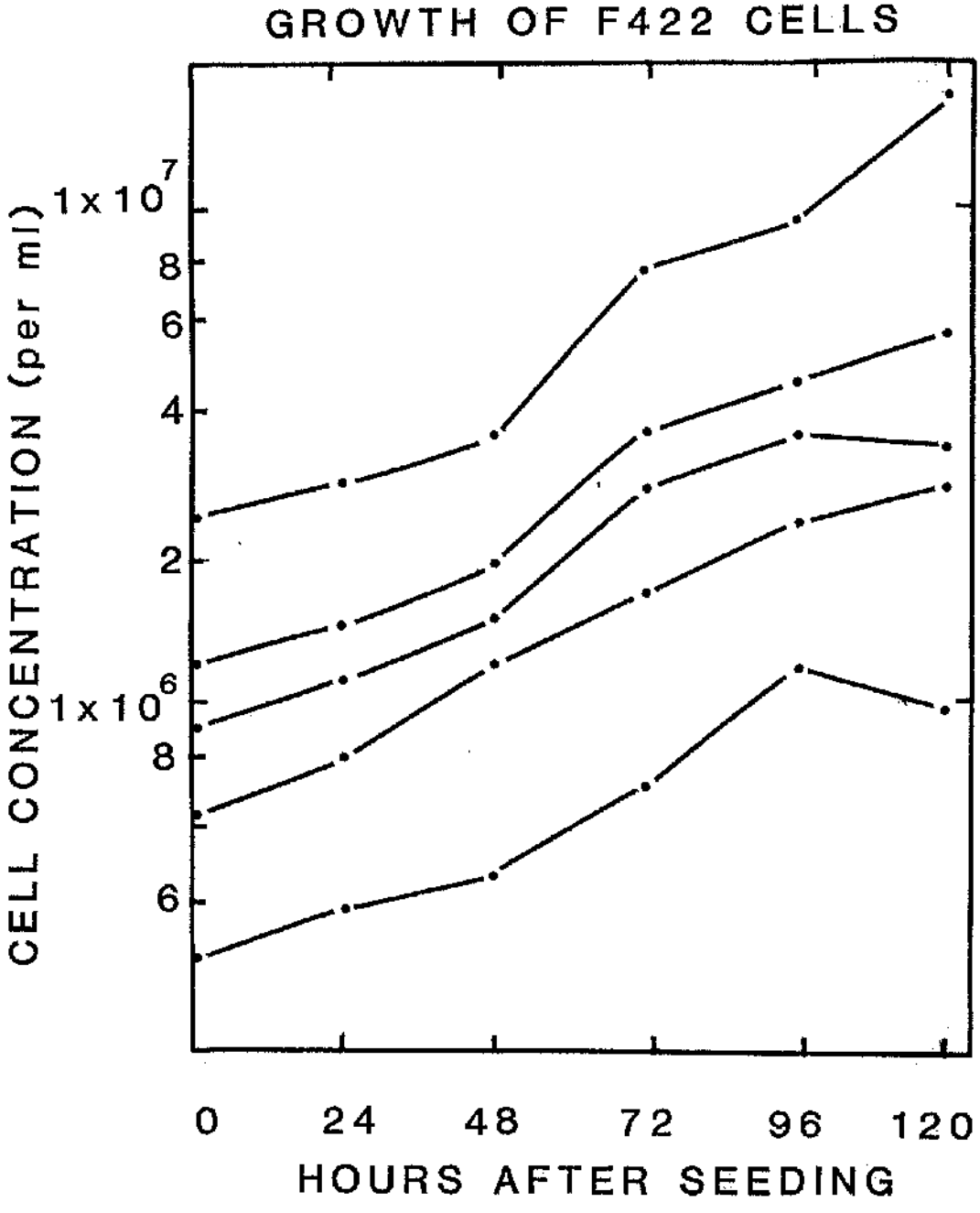
TABLE 2.1

Optimum conditions for the growth of F422 cells and virus

Starting cell concentration		Time after seeding (hours) 24	48	72	96	120
3×10^5	cells	3.8	4.4	6.6	10.2	9.8
	virus	0.2	0.6	0.8	0.3	0.2
	v:c	6	14	12	3	2
6×10^5	cells	7.8	10.2	16.8	22.7	27.3
	virus	2.5	1.9	2.5	2.0	1.3
	v:c	32	18	14	9	5
9×10^5	cells	11.0	14.8	27.2	35.2	32.6
	virus	3.0	3.0	2.5	2.0	1.0
	v:c	29	20	9	6	3
1.2×10^6	cells	14.6	19.2	36.3	45.3	57.2
	virus	5.0	4.5	5.0	2.0	2.5
	v:c	34	23	14	4	4
2.4×10^6	cells	28.0	35.2	77.1	97.8	107.8
	virus	4.5	3.8	4.5	4.3	3.8
	v:c	15	11	6	4	4

Cells: $\times 10^5$ cells per ml
Virus: $\times 10^4$ FIU per ml
Virus/cell ratio : $\times 10^{-3}$

FIG 2.1


GROWTH OF F422 CELLS
CELL CONCENTRATION (per ml)
1x10^7
8
6
4
2
1x10^6
8
6
0
24
48
72
96
120
HOURS AFTER SEEDING

the supernatant fluid was stored at -70^{o} before being assayed for FeLV.

The results of cell counts and virus titrations for all 6 cultures are shown in Table 2.2.

The results obtained from this experiment indicated that L-M medium with 15% FCS was better than RPMI 1640 medium for both cell growth and virus production. The highest cell count was in the sample collected from the L-M culture with 15% FCS, 96hours after seeding and the highest virus titre was obtained from the same sample.

Experiment 3: Conditions for the optimum growth of FEA/FeLV-A cells and virus

Eight 5 cm plastic plates were seeded with FeLV-A infected FEA cells at 3×10^{5} cells per plate in 4 ml EFC medium and were incubated at 37^{o} in 5% CO_2 in air. After 24 hours culture fluid samples were collected and cells counted from the first 2 plates. These samples were centrifuged at 2000 rpm for 10 minutes and stored at -70^{o}. This process was repeated after 48, 72 and 96 hours using 2 plates each time. The stored culture fluid samples were assayed for FeLV.

The second part of the experiment was done in the same way except that media for all plates was changed daily. In the third part of the experiment cell counts and collection of culture fluid were done 72 and 96 hours after seeding and the medium was changed every other day.

Cell counts and results of virus titrations of all 3 parts of the experiment are shown in Fig. 2.2 and Table 2.3. From

TABLE 2.2

Growth of F422 cells and virus in different media

Medium	FCS (%)	Cell count at (hours)[a]: 24	48	72	96	Virus[b] (FIU/ml)
LM	5	11.0	20.0	29.0	30.8	1.2×10^5
	10	12.4	20.8	30.5	42.1	2.3×10^5
	15	14.1	20.2	37.2	46.0	2.2×10^5
RPMI	5	13.1	20.7	21.8	25.5	0.7×10^5
	10	14.0	17.3	23.4	29.2	1.7×10^5
	15	13.5	23.1	28,5	32.5	2.0×10^5

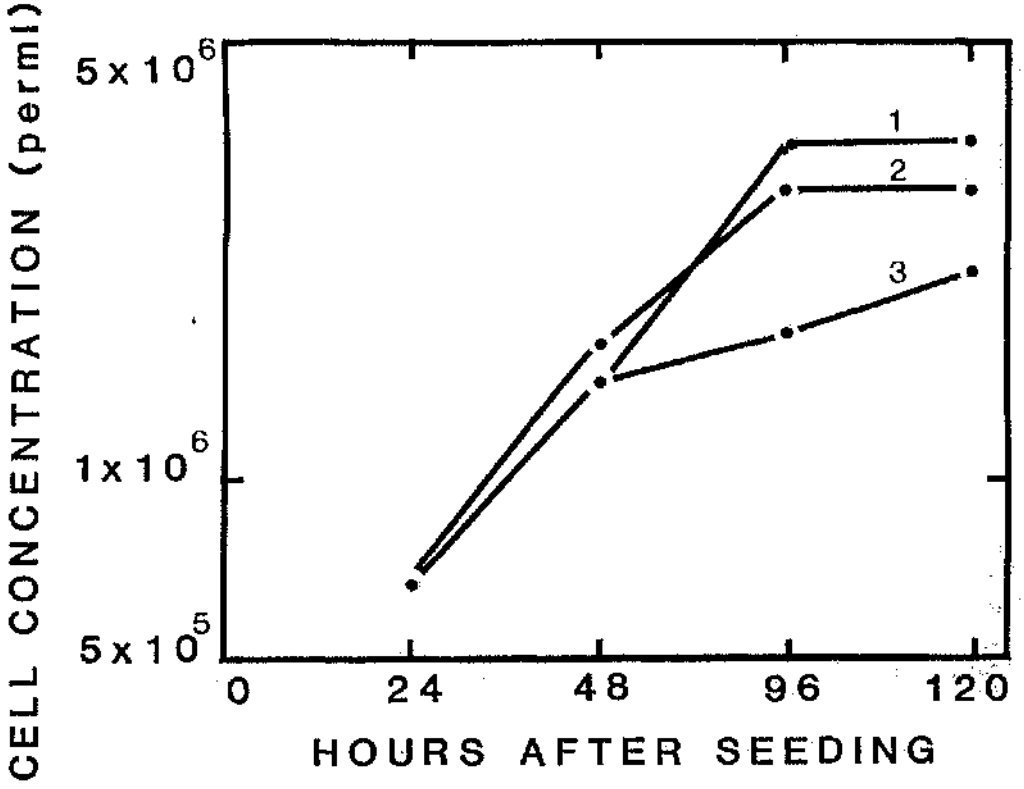
a Cells $\times 10^5$

b Virus sampled at 72 hours.

Fig 2.2

GROWTH OF FEA CELLS

INFECTED WITH FeLV-A



(1) MEDIUM CHANGED AT 48 HOURS

(2) MEDIUM CHANGED DAILY

(3) CONTINUOUS MEDIUM

TABLE 2.3

Growth of FEA/FeLV-A cells and virus

Conditions of medium		Time after seeding (hours) 24	48	72	96
Continuous	cells	6.8	14.3	16.9	21.7
	virus	4.5	5.1	4.1	3.0
	V:C	65	36	24	14
Changed	cells	-	16.6	29,3	29,6
daily	virus	-	5.8	7.1	8.6
	V:C	-	34	29	29
Changed	cells	-	-	35.7	34.9
every other	virus	-	-	6.4	5.1
day	V:C	-	-	18	15

CELLS: X 10^5

VIRUS: X 10^4 FIU per ml

VIRUS:CELLS: X10^{-3}

these results, the highest cell count and virus titre were found in samples collected 96 hours after seeding in the cultures in which medium was changed daily (8.6×10^4 FIU/ml) and the highest cell count was in the 72 hour culture in medium changed every 2 days (3.6×10^6). The greatest ratio of infectious virus per cell was in the sample collected 24 hours after seeding (65×10^{-3}).

Discussion

Three types of experiments were done to select the best way of obtaining good quantity and quality of cells and virus which might be used for FeLV vaccine production. The quantity of virus in a cell culture is obviously important for vaccine preparation because an inactivated vaccine must contain sufficient antigenic mass to immunise. However, the quality of the virus may be equally important. With retroviruses, the antigenic determinants of the gp70 are crucial in immunity. Although it is not known if the antigenicity, or immunogenicity, of the gp70 of FeLV declines after the virus is produced from the surface of the cell, there is evidence from electron microscopy that the envelope spikes which are composed of gp70, are prominent when the virus is first produced but become less obvious as the viral particle matures (Laird and Jarrett, personal communication). The infectivity of the virus also declines. This may indicate also a loss of antigenic structure. Therefore, in these experiments it was considered that the amount of infectious virus produced by the cells might be an important indicator of its immunogenicity, and

that the ratio of cells to virus in a culture might give a guide to the efficiency of the cell culture.

In the first experiment it was found that F422 cells grew at the same rate for each starting concentration (Fig. 2.1) and that in the range of starting concentrations between 3×10^5 and 1.2×10^6 cells per ml there was a reasonable correlation at all sampling times between the concentration of cells and the amount of infectious virus in the culture fluid. However, with increasing time the amount of virus per cell declined which probably reflects a decrease in the production of virus with time as well as increasing activation of virus due, perhaps, to the action of proteases and nucleases released from the cells. This experiment also showed that in all cultures within the range 6×10^5 to 2.4×10^6, the highest ratio of virus to cells was at 24 hours after seeding. This probably is because a higher proportion of the cells are in mitosis during this intitial period compared to later times and it is known that FeLV is produced by the cells principally during mitosis (Toth, 1980). Thus the main conclusion from these results was that the optimum conditions for producing virus from F422 cells were to seed the cells at 1.2×10^6 cells per ml and harvest the fluid after 24 hours. This would appear to be the most economical way of producing a large amount of virus, since the virus is of relatively high infectivity and it is, therefore, most likely to be antigenically active.

In the second experiment the aim was to find if RPMI-1640 medium was adequate for growing F422 cells compared to L-M

medium. RPMI-1640 is widely used for the growth of suspension cultures of leukaemic cells (Creemers et al, 1978) and is less expensive than L-M medium which is a major consideration in vaccine production. The first conclusion of this experiment was that, with each medium, increasing FCS concentration gave better growth presumably by supplying greater quantities of essential growth factors. Secondly, it was found that F422 cells grew reasonably well in RPMI-1640 but not as well as in L-M medium. For example a concentration of 15% FCS was required with RPMI-1640 to give the same growth rate as L-M with 5% serum. Thirdly, in general, virus production was better in L-M than in RPMI and the amount of virus harvested at 72 hours increased with increasing FCS concentration. The main conclusion was that L-M with 10 or 15% FCS should be used for F422 virus production.

In the third experiment involving a monolayer of FEA cells infected with FeLV-A the results of the first part in which the cells were grown without medium change indicated that the initial increase in cell growth slowed after 48 hours, perhaps due to exhaustion of nutrients and growth factors in the medium, since cells in cultures in which the medium was changed daily continued to increase in number. In this case the cell numbers reached a plateau after 72 hours. The continued stimulus of medium changes appeared to lead to the production of large amounts of virus and the virus:cell ratio did not alter much from 48-96 hours. It appeared, therefore, from this experiment that in continuous medium, the rate of inactivation of virus was balanced by rate of production. In cultures in which the medium was changed daily, there was a higher rate of virus production, or lower rate of

inactivation, perhaps due to removal of proteases and nucleases.

Finally in the third part of this experiment in which the medium was changed two days after seeding, the cell counts and infectious virus as well as the virus:cell ratio were essentially the same at 72 and 96 hours. In spite of the rapid increase in cell numbers between 48 and 72 hours in these cultures, the cells quickly became confluent and there was not a corresponding increase in the production of virus. Also the ratio of cells to infectious virus remained rather low compared to cultures in which there was continuing growth of cells.

The conclusion of this experiment was that, as for F422 cells, the highest virus:cell ratio is at 24-48 hours. Thus it is likely that virus harvested at 24-48 hours after subculture is of good antigenicity.

CHAPTER 3

INACTIVATION OF FeLV PSEUDOTYPES OF MURINE SARCOMA VIRUS

Introduction

The aims of this study were to gain information on the inactivation of FeLV by several chemical agents, and to examine the binding of virus to the adjuvant, $Al(OH)_3$ gel.

The initial choice of virus for studies on inactivation of FeLV was not FeLV itself but MSV (FeLV). These pseudotype viruses have been widely used in studies on FeLV because they can be obtained with particular FeLV envelope antigens (Fischinger and O'Connor, 1969) and so can be used in FeLV subgrouping using interference tests (Sarma and Log, 1973), in determining the host range of FeLV strains (Fischinger and O'Connor, 1970; Jarrett *et al*, 1973) and in detecting and titrating virus neutralising antibodies (Sarma & Log, 1973; Russell and Jarrett, 1978a). This wide range of uses of MSV (FeLV) is mainly because the virus can be assayed more easily and with greater precision than FeLV (Russell and Jarrett, 1976). In this study, inactivation of MSV (FeLV) rather than FeLV was used for these reasons and also because the MSV (FeLV) assay can be read macroscopically after 4 days (Russell and Jarrett, 1976) while the FeLV assay takes 6-7 days and has to be read microscopically (Jarrett *et al*,1982b).

It is very likely, however, that the inactivation kinetics of MSV (FeLV) and FeLV are very similar since the envelope of the pseudotype virus is identical to that of FeLV and should react in the same way with proteotropic inactivating agents. Also, the RNA genomes of the two viruses are similar in size and would be

expected to have similar responses to nucleotropic inactivating agents.

The reasons for the choice of paraformaldehyde (PF), PF and glutaraldehyde (GA) together (PF + GA), acetylethyleneimine (AEI), and 2, 2-dithiobis, 5-nitropyridine (DTNP) as inactivating agents were as follows. PF was used previously by Jarrett et al (1975) with success in inactivating FL74 cells for use as a cellular vaccine against FeLV. PF + GA is widely used as a fixative in electron microscopy and gives good morphological preservation of the envelope spikes of FeLV (Laird et al, 1967), so it might be expected also to preserve antigenicity. The action of DTNP is to stabilise disulphide bonds between proteins and in particular has been shown to cross-link retroviral gp70 and p15E (Pinter & Fleissner, 1971. This action might have advantages in the preparation of a FeLV vaccine in two ways. First, it might preserve the structure of gp70 on the viral envelope since it is anchored by the p15E into the lipid of the envelope (Bolognesi et al, 1978). Secondly, it might prevent the breakdown of the envelope and release of p15E which may be immunosuppressive (Olsen et al, 1980) and act against effective immunisation. AEI has been used successfully for foot-and-mouth disease virus vaccines (Brown et al, 1963b; Graves and Arlinghaus, 1967; Wild and Brown, 1968; Doel and Baccarini, 1981). AEI and the other imines mainly react with the viral nucleic acid.

The second aim of the experiments described in this chapter was to determine the adsorption of MSV (FeLV) to $Al(OH)_3$ gel. It was decided to use this substance as an adjuvant because of

its capacity to delay the release of the antigen from the site of injection. This adjuvant has been used previously in FeLV vaccines by Jarrett et al (1974, 1975) and by Pedersen et al (1979).

Materials and Methods

I. Preparation of MSV (FeLV-A/Glasgow-1)

A FeLV pseudotype of MSV was obtained by rescue of MSV from C81 cells after superinfection with FeLV. In the initial experiment, eight 5 cm plastic plates were seeded with C81 cells at 8×10^4 cells in 4 ml EFC per plate and were incubated at 37° in 5% CO_2 in air. Twenty four hours later the cells were inoculated with 1 ml of FeLV in EFC containing 4 ug/ml PB and incubated for 90 minutes. The inoculum was then replaced with 4 ml EFC medium and the plates were reincubated at 37°. Samples of culture fluid were collected 24, 48, 72 and 96 hours after inoculation and stored at -70°. These samples were titrated as described above and the highest MSV (FeLV) titre obtained was found to be in the sample collected 72 hours after inoculation (1.30×10^5 FFU/ml) as shown in Table 3.1. For the preparation of a stock of virus, C81 cells were infected with FeLV as above and after 72 hours when MSV (FeLV) production was maximal, the medium was replaced with 4 ml EFC containing 1×10^6 FEA cells per plate and the culture fluid was harvested 2 days later when transformation involved all the cells. In this case the titre obtained was 4×10^6 FFU/ml.

TABLE 3.1

Release of MSV (FeLV) from FeLV-infected C81 cells

Time after infection (hours)	MSV (FeLV) titre (FFU/ml)
24	6.0×10^1
48	1.0×10^4
72	1.3×10^5
96	5.5×10^2

2. MSV (FeLV) inactivation.

(a) Inactivation of MSV (FeLV) with PF

A stock solution of 4 % PF was made as follows. Two grammes of paraformaldehyde powder (BDH) was suspended in 20 ml of distilled water and heated to 60° with stirring. The temperature was maintained at 60° and 0.1 N NaOH solution was added drop by drop with stirring until the solution became clear. The solution was then removed from the heat and 30 ml of 0.25M phosphate buffer (pH 7.2) and 425 mg of NaCl was added. When this was dissolved the solution was filtered, cooled and stored at 4°.

In the inactivation experiment MSV (FeLV) was treated with 0.1, 0.05 or 0.01% PF by adding 100 ul of either 1%, 0.5% or 0.1% PF, made from the stock solution, to 1 ml MSV (FeLV) in glass bottles. A volume of 100 ul of L-15 medium was added to 1 ml MSV (FeLV) as a virus control. The experiment was incubated at room temperature for 24 hours. Samples of 150 ul were collected from all reactions immediately after addition of PF and at intervals of 3, 6, 12 and 24 hours later. These samples, including the virus control, were treated with 15 ul of 20% sodium thiosulphate for 30 minutes at room temperature to neutralize the action of PF and were stored at -70°. The residual infectivity of these samples was assayed as described above.

(b) Inactivation of MSV (FeLV) with PF and Glutaraldehyde

A mixture of paraformaldehyde (PF) and glutaraldehyde (GA) prepared as 1.3% PF and 1.6% GA in 0.1 M sodium phosphate buffer, pH 7.2, was used in different concentrations (0.5%, 0.1%, 0.02%

or 0.004%) to inactivate the virus. MSV (FeLV) inactivation was carried out by adding 0.1 ml of a 10 X concentrated solution to 1 ml of virus and incubating the mixture at room temperature for 24 hours. Samples of 150 ul of each reaction at 0, 3, 6 and 24 hours were collected and kept at -70° until assayed later for viral infectivity.

(c) Inactivation of MSV (FeLV) with AEI

In this case the inactivation procedure was similar to that used in the first experiments except AEI concentrations of 0.5, 0.1, 0.02 or 0.004% were used and after each sample was collected it was neutralised by the addition of a one-tenth volume of 20% sodium thiosulphate and incubation at room temperature for 30 minutes. The samples were then stored at -70° and assayed as before.

(d) Treatment of MSV (FeLV) with DTNP in DMSO

One hundred ml of MSV (FeLV) was concentrated tenfold by ammonium sulphate precipitation and ultracentrifugation as described in Chapter 1. The virus was pelletted in the SW50.1 rotor at 30,000 rpm for 60 minutes, resuspended in L-15 medium and divided into 3 volumes of 150 ul. To the first aliquot 15 ul of DMSO was added. To the second aliquot 15 ul of 0.2% DTNP in DMSO was added, incubated at room temperature for 15 minutes and stored at -70° for assay. The third volume of concentrated MSV (FeLV) was also stored at -70° after 15 minutes at room temperature as a virus control.

3. Adsorption of MSV (FeLV) to $Al(OH)_3$

Two types of $Al(OH)_3$ were available: the first was obtained by courtesy of Wellcome Research Laboratories (WRL) and the second was purchased from Superfos (SF). Each contained $Al(OH)_3$ at a concentration of 2%. To compare the absorption ability of each for MSV (FeLV) the following experiment was carried out.

First, the pH of the $Al(OH)_3$ alone and diluted 1:10 in L-M medium was determined to ensure that it was within the physiological range. The pH of each was found to within the range 7.4-7.6 which is similar to that of L-M medium. In the absorption experiment, 0.6 ml containing 2.4×10^6 FFU MSV (FeLV) was diluted with 5.4 ml of L-M medium. Then 0.2 ml of each type of $Al(OH)_3$ was added to 2 ml of diluted virus. A volume of 0.2 ml PBS was added to 2 ml of the diluted virus as a virus control. The mixtures were incubated at 4^o for 16 hours and were then spun at 6000 rpm for 20 minutes. Samples of 0.5 ml were taken from the supernatant fluid and assayed for MSV (FeLV) residual infectivity.

Results

Kinetics of inactivation of MSV (FeLV)

a. PF: The results of inactivation of MSV (FeLV) with PF are shown in Table 3.2. There was complete inactivation with PF concentrations of 0.1% and 0.05% at all times, while the virus control titres in the same period dropped only slightly from 1.5×10^5 FFU/ml to 7.7×10^4 FFU/ml. The virus titre of MSV (FeLV) inactivated with 0.01% PF in the same interval decreased from 6.4×10^4 FFU/ml to 4.3×10^3 FFU/ml.

b. PF + GA: In this case there was complete inactivation in all

TABLE 3.2

Inactivation of MSV (FeLV) with paraformaldehyde

Concentration (%)	Virus Titre (FFU/ml) at hours: 0	3	6	12	24
0.1	0	0	0	0	0
0.05	0	0	0	0	0
0.01	6.4×10^4	2.6×10^4	8.6×10^3	4.3×10^3	4.3×10^3
None	1.5×10^5	1.0×10^5	8.2×10^4	9.0×10^4	7.7×10^4

samples at each time point as shown in Table 3.3. In contrast the decrease in the titre of each sample of the virus control was from 5.1×10^3 FIU/ml to 2.4×10^2 FIU/ml in 24 hours.

c. AEI: The results of AEI inactivation are shown in Table 3.4. There was complete inactivation by 0.5% AEI at all intervals and using 0.1% AEI for 24 hours.

d. DTNP + DMSO: As shown in Table 3.5, there was incomplete inactivation of MSV (FeLV) either by using DMSO alone or DTNP (0.2%) in DMSO. There was, however, an obvious difference in titre between the virus exposed to DMSO alone (1.0×10^4 FFU/ml) and the virus inactivated with DTNP in DMSO (6.4×10^2 FFU/ml) compared to the virus control titre (6.1×10^4 FFU/ml), thus a slight inactivating action of DTNP was indicated.

e. Adsorption of MSV (FeLV) to $AlOH_3$: The results shown in Table 3.6 indicated that the absorptive capacity of both $Al(OH)_3$ types were approximately similar. There was an obvious difference in MSV (FeLV) adsorbed to $Al(OH)_3$ compared with PBS as control. The titre of residual MSV (FeLV) after exposure to $Al(OH)_3$ was 4×10^2 FFU/ml in contrast with the virus control titre which was 1.6×10^3 FFU/ml, indicating that the absorption capacity of the adjuvant was approximately 75%.

Discussion

As shown in Table 3.2 the degree of inactivation of untreated MSV (FeLV) over a 24 hour period was relatively small, decreasing to about 50% of the original titre. By contrast, inactivation of 5 logs of MSV (FeLV) was complete at PF

TABLE 3.3

Inactivation of MSV(FeLV) with paraformaldehyde and glutaraldehyde

Concentration (%)	Virus titre (FFU/ml) at hours 0	3	6	24
0.5	0	0	0	0
0.1	0	0	0	0
0.02	0	0	0	0
0.004	0	0	0	0
None	5.1×10^3	3.5×10^3	3.8×10^3	2.4×10^3

TABLE 3.4

Inactivation of MSV(FeLV) with acetylethyleneimine

Concentration (%)	Virus titre (FFU/ml) at hours; 0	3	6	24
0.5	0	0	0	0
0.1	3.2×10^2	3.2×10^2	3.2×10^2	0
0.02	7.3×10^2	9.6×10^2	6.4×10^2	2.4×10^2
0.004	8.8×10^2	6.4×10^2	3.2×10^2	5.6×10^2
None	9.6×10^2	6.4×10^2	3.2×10^2	3.2×10^2

TABLE 3.5

Inactivation of MSV(FeLV) with DTNP

Treatment	Virus Titre (FFU/ml)
DTNP + DMSO	6.4×10^2
DMSO alone	1.0×10^4
None	6.2×10^4

TABLE 3.6

Adsorption of MSV(FeLV) to $Al(OH)_3$

$Al(OH)_3$ type	Unabsorbed virus (FFU/ml)
WRL	4.0×10^2
Superfos	4.0×10^2
None (PBS)	1.6×10^3

concentrations of 0.1% and 0.05%. There was partial inactivation by PF at 0.01% to one-quarter of the starting titre. In this case there was an initial reduction of infectivity over 6 hours but the rate of inactivation subsequently decreased.

The second experiment, the results of which are in Table 3.3, showed that glutaraldehyde had an enhancing effect on PF in inactivating MSV (FeLV) since even at very low concentrations (0.004%) and at the starting time, no infectious virus was recovered from the reaction.

By contrast, the third inactivation experiment where four different concentrations of AEI were used, the result was that only 0.5% AEI had the ability to completely inactivate virus at all intervals. At 0.1% there was only slight inactivation initially and 3 logs of virus were inactivated by 24 hours. In this case, the minimum AEI concentration which should be used in virus activation is 0.5%.

In the last experiment of inactivation in which DTNP and DMSO were used for a short period, the result was that there was incomplete inactivation of MSV (FeLV). However, there was an obvious difference in the virus titres between inactivation with DMSO + DTNP and with DMSO alone, which indicated an inactivating action of DTNP on the virus (Table 3.5). The reason for inactivation of virus by DTNP is not clear but may be due to the effect of the agent in stabilising the structure of the viral envelope so that the entry of the virus into susceptible cells is inhibited. For use in the vaccine experiments described in Chapter 4 it was considered that additional inactivation with PF was required to ensure the safety of the vaccine.

The last experiment described in this chapter was to determine the absorptive capacity of two types of an $Al(OH)_3$ adjuvant. The results of this experiment indicated that there was similar absorption of MSV (FeLV) by both types of $Al(OH)_3$ (Table 3.6). It should be noted that only unabsorbed virus remaining in the supernatant fluid was assayed and not the virus presumably attached to the $Al(OH)_3$.

In the formulation of commercial vaccines it would be of interest to find if a greater concentration of $Al(OH)_3$ absorbed more virus and whether virus could be recovered from the gel for assay.

The general conclusions from these experiments were that to inactivate MSV (FeLV) under the conditions used, PF should be used at 0.05%, PF and GA at 0.04% (or less) and AEI at 0.5%. DTNP treatment should be supplemented with treatment of virus with an inactivating agent.

There seemed to be little difference in the absorptive capacities of the two types of $Al(OH)_3$.

CHAPTER 4

VACCINATION EXPERIMENT 1: COMPARISON OF INACTIVATING AGENTS

INTRODUCTION

For reasons discussed in the Introduction and in Chapter 2 it was proposed to attempt to use inactivated FeLV preparations as vaccines. Chemical inactivating agents were chosen because in general these have been most successful in previous inactivated viral vaccines. The chemical agents used in the experiments described here were paraformaldehyde as a representative of the aldehydes which react mainly with both nucleic acids and proteins of viruses and produce cross-linking within the nucleic acid and between the nucleic acid and any adjacent protein, and AEI which is a highly reactive nucleotropic chemical. In addition, DTNP together with PF was used. The inactivation of MSV (FeLV) by these agents is described in Chapter 3 and as far as possible the conditions which proved sufficient to inactivate MSV (FeLV) at the minimum concentration of the chemical were used here to inactivate FeLV.

The FeLV-A virus produced by F422 was used as a source of antigen. Two concentrations of antigen were used which corresponded to unconcentrated virus from the culture harvests, and virus concentrated tenfold.

The adjuvants which were used were $Al(OH)_3$ together with saponin. In retrospect, the choice of saponin was not completely satisfactory. Experiments described in Chapter 7 showed that saponin was probably not as efficient as an oil adjuvant. However, at the time when this study began, saponin was being

used experimentally with some success in vaccines against foot-and-mouth disease virus (Dalsgaard et al, 1977) and it was believed that it might be useful in a vaccine for cats since it is soluble in water, would be easy to incorporate into a vaccine and would be easy to administer to cats.

Two doses of vaccine were inoculated with an interval of 3 weeks between doses. Cats were monitored for FeLV viraemia before each vaccination, before challenge and at intervals after challenge. Because of the age resistance to FeLV which develops in cats by about 16 weeks of age (Hoover et al, 1976) it was considered necessary to complete vaccination and challenge within this period. Hence cats were vaccinated when 8 and 11 weeks old and were challenged when 13 weeks of age. While it was believed that this protocol would produce a situation in which all of the unvaccinated cats could be made persistently viraemic following an appropriate challenge dose, it was recognised that vaccination of such young kittens might not give an optimum immune response. In practice, in natural conditions, vaccination might be delayed up to 12 weeks of age. Vaccination at this age would also almost certainly avoid interference by maternal antibodies (Hoover et al, 1977; Jarrett et al, 1977).

Challenge was by administration of FeLV into the nose and mouth. This route of administration was used in order to simulate natural transmission. Hoover et al (1977a) showed that kittens could be successfully infected with FeLV by intranasal instillation and subsequently developed lymphosarcoma. More recently Jarrett et al (1982a) used oronasal infection and

produced persistent infections in all of the 8-week old kittens infected, but only in about half of the 16-week old animals.

In the vaccination experiments described in this and later chapters, the kittens were monitored after challenge for FeLV viraemia and FeLV neutralising antibodies. Samples were taken 3 weeks after challenge since it has been shown that this is the time when a transient viraemia would be most likely to be detected (Jarrett et al, 1982a). Further samples were taken 6 and 9 weeks after challenge to test for the development of a persistent infection.

MATERIALS AND METHODS

Seven types of inactivated FeLV vaccine were produced using 3 different inactivating agents either alone or in combination. A control vaccine preparation was also made.

Vaccine A1: PF (1)

One hundred ml of FeLV was collected from F422 cell cultures 48 hours after seeding with 1.2 X 10^6 cells per ml in L-M medium. The fluid was spun at 2000 rpm for 20 minutes and the supernatant was stored at -70°. When required the medium was thawed and inactivated with 0.5% PF at room temperature for 24 hours. A one-tenth volume of 20% sodium thiosulphate was added for 30 minutes at room temperature. Then two 0.5 ml samples were taken and stored at -70° for assays of viral infectivity. Ten ml of this preparation was taken and mixed with 1 ml of $Al(OH)_3$, kept at 4° overnight and spun at 3000 rpm for 30 minutes. The sediment was resuspended in 4 ml PBS and 4 ml of saponin

containing 12 mg of the active ingredient was added to give a final volume of 8 ml. Four ml was used immediately while the other 4 ml was kept at 4° until used as the second dose of vaccine.

Vaccine A2: PF (10)

The remainder of the FeLV which was inactivated in the first step of the preparation of vaccine A1 was precipitated with SAS and concentrated as described above. Then 0.9 ml of $Al(OH)_3$ was added to 9 ml of the concentrated antigen, incubated at 4° overnight, spun at 3000 rpm for 30 minutes and the sediment resuspended in 4 ml PBS. Four ml of saponin containing 12 mg was added and 4 ml of this vaccine was used as vaccine, while another 4 ml was kept at 4° until used for a boosting dose in the same cats, 3 weeks later.

Vaccine A3: AEI (1)

Fifty ml of FeLV which had been collected from F422 cell culture 48 hours after seeding at 12 X 10^5/ml in L-M medium was spun at 2000 rpm for 20 minutes and the supernatant was stored at -70°. When required, the fluid was thawed and inactivated with 0.2% AEI for 24 hours at room temperature. The AEI was then neutralised by the addition of a one-tenth volume of 20% sodium thiosulphate and incubation for 30 minutes at room temperature. The virus was then concentrated by adding an equal volume of SAS.

The suspension was incubated for 30 minutes at 4° and was then spun at 3000 rpm for 30 minutes. The sediment was resuspended in 10 ml PBS and was layered into an SW41 centrifuge

tube containing 1 ml of 50% sucrose and 2 ml 20% sucrose. The tube was centrifuged at 40,000 rpm for 1 hour and the virus band which formed between the sucrose layers was recovered in 0.5 ml by puncturing the bottom of the tube. Sucrose was removed by passing the virus through a Pharmacia PD10 column containing Sephadex G25. The final volume was 2 ml in PBS.

To prepare the vaccine, 0.2 ml of this final preparation was added to 1.8 ml PBS. A volume of 0.2 ml of $Al(OH)_3$ was added and the mixture was incubated overnight at 4°. Two ml of saponin containing 6 mg of active ingredient was then added, so that the final volume was 4 ml.

Vaccine A4: AEI (10)

The remainder of the inactivated and concentrated FeLV in the first experiment (1.8 ml) was used to prepare the AEI X 10 vaccine by adding 0.2 ml of $Al(OH)_3$, incubating at 4° overnight and then adding 2 ml of saponin. The booster doses for both vaccines A3 and A4 were prepared in the same way as the first doses, but on a separate occasion.

Vaccine A5: DTNP/PF (1)

A volume of 100 ml FeLV which had been prepared and stored as in the previous experiments was thawed and precipitated with SAS and concentrated as described above. The virus was recovered in 2 ml PBS. Of this,100 ul was added to 0.9 ml L-M medium and was stored at -70° as two 0.5 ml volumes. In addition, two 0.5 ml samples were collected before SAS precipitation and stored at -70°. Inactivation was done by adding 0.2 ml of 0.2% DTNP in DMSO to the concentrated antigen (2 ml) and incubation at room

temperature for 30 minutes. Then 100 ul of inactivated virus was added to 0.9 ml L-M medium and stored at -70° as two 0.5 ml volumes for assay of residual infectivity. The remainder of the DTNP treated virus was diluted to 10 ml with PBS and was inactivated with 0.5% PF by adding 1.25 ml of 4% PF and incubating at room temperature for 24 hours. Then 1.1 ml of 20% sodium thiosulphate was added for 30 minutes at room temperature. A volume of 0.8 ml of the final material (12 ml) was added to 7.2 ml PBS to make 8 ml of X1 antigen concentration, while the remaining volume (11.2 ml) was used as the X10 antigen. To prepare vaccine A5, 0.8 ml of $Al(OH)_3$ was added to the 8 ml of the X1 and incubated at 4° overnight. Then 1 ml of saponin containing 12 mg was added to form the final formulation of the vaccine as 9 ml which was used to vaccinate and boost the cats.

Vaccine A6: DTNP/PF (10)

This vaccine was prepared by addding 0.8 ml of $Al(OH)_3$ to 8 ml of X10 inactivated antigen, incubating at 4° overnight and then adding 1 ml of saponin containing 12 mg of the active ingredient. The final vaccine (A6) was used to vaccinate and boost the cats. One ml of X10 inactivated antigen was added to 1 ml of L-M medium before addition of the adjuvants and was stored at -70° as two 0.5 ml lots to assay the viral residual infectivity.

Vaccine A7: F422 cells

F422 cells were grown at 12 X 10^5/ml in 100 ml L-M medium and collected 48 hours after seeding by spinning the culture

fluid at 1000 rpm for 10 minutes. The cells were washed twice with PBS by repeating the spinning and resuspension at room temperature and were finally resuspended in 50 ml of cold PBS. An equal volume of 0.1% PF was added drop by drop with constant stirring at 4^{o}. The cells were incubated for 24 hours, washed 3 times with PBS at 4^{o}, counted and resuspended in 2 ml of PBS. Six ul of this cell suspension was taken as a sample for assaying of FeLV infectivity and 0.2 ml of $Al(OH)_3$ was added to the remaining cell suspension and incubated at 4^{o} for 3 hours. Two ml of saponin containing 6 mg of the active ingredient was added to prepare the final vaccine. Each ml contained 3.5×10^{6} cells. The booster dose was prepared in the same way. The infectivity of the inactivated cells sample was checked as follows. The cell sample was washed once with EFC, resuspended in 2 ml of EFC medium and 1 ml was inoculated into two of twelve, 5 cm plastic plates which had been seeded 24 hours previously with FEA/C81 cells at a ratio of 10:1 in 4 ml EFC medium. Of the other 10 plates, 2 were used for a cell control while 8 were used to inoculate 4, tenfold dilutions of freshly prepared F422 cells in L-15 with 10% FCS with 2 plates for each dilution. Three days later the medium was changed and after a further 4 days the cells were checked microscopically for transformation. There was no cell transformation in the cell control plates or in the plates inoculated with the inactivated F422 cells. In contrast, cell transformation occurred in all plates inoculated with freshly prepared F422 cells which indicated that PF treated F422 cells were completely inactivated.

Control Vaccine A8

This "vaccine" was prepared by treating 10 ml L-M medium with 0.2% AEI for 24 hours at room temperature. The AEI was then neutralised by adding one-tenth of the volume of 20% sodium thiosulphate. The mixture was incubated for 30 minutes. A volume of 1 ml of $Al(OH)_3$ was added and incubated at 4^o overnight. The mixture was then spun at 6000 rpm for 20 minutes and the sediment was resuspended in 4 ml PBS. Four ml of saponin, containing 12 mg of the active ingredient was added. Part of this preparation was used to inoculate cats and the remainder (4 ml) was kept at 4^o until used for a second inoculation, 3 weeks later.

Animal Experiments

Thirty-two SPF cats were obtained when 8 weeks of age. These cats were randomised and arranged in 8 groups of 4 cats. Each group was inoculated with one of the seven kinds of vaccine or the control vaccine. Vaccine was given by subcutaneous injection of 1 ml of vaccine in the interscapular region of the neck. The booster doses were given in the same way, 3 weeks later. The FeLV challenge was done by administration of 1 ml of FeLV containing 8.5×10^4 infectious particles oronasally to all cats including control vaccine cats 2 weeks after the second dose of the vaccines. A volume of 0.25 was given into each nostril and the remainder given by mouth. Blood samples (2 ml: 1 ml clotted blood for serum and 1 ml in lithium heparin) were collected from the jugular vein of all cats before giving the vaccines, before challenge and at 3, 6 and 9 weeks after

challenge. These blood samples were processed and used in virus isolation and serum neutralising antibody tests.

RESULTS

Response to Vaccination

The response of cats to vaccination with the 7 vaccines and to challenge with FeLV are shown in Table 4.1. The response was measured in two ways: first, whether or not a cat was viraemic and secondly, whether or not a cat had virus neutralising antibodies.

Following vaccination but before challenge, none of the cats had virus in the blood. Only one cat in group A6 appeared to have antibodies before challenge. After challenge, over two-thirds of the cats responded either by becoming viraemic or developing antibodies. It was, however, difficult to compare the response of the vaccinated cats with that of the unvaccinated controls since not all of the control cats became viraemic. Three of these four cats were viraemic at 3 or 6 weeks after challenge but only one was still viraemic at 9 weeks. Apart from the cats in group A7 which were inoculated with PF-inactivated cells of which 3 or 4 were viraemic at 9 weeks, the response of the other vaccinated groups were a little better than the controls. For example, in group A1 none of the cats became viraemic. In each of the groups A4, A5 and A6 only one cat was transiently viraemic. At 9 weeks in group A2 one cat was viraemic and in group A3 two cats were viraemic.

Virus neutralising antibody was found in the sera of cats

TABLE 4.1 Results of Vaccination Experiment A

Group No. vaccine	Time (weeks) relative to challenge *					
	-5 VA	-2 VA	0 VA	3 VA	6 VA	9 VA
A1 PF (1)	-0	-0	-0	-8	-8	-0
	-0	-0	-0	-0	-8	-32
	-0	-0	-0	-0	-8	-128
	-0	-0	-0	-0	-0	-0
A2 PF (10)	-0	-0	-0	-8	-8	-0
	-0	-0	-0	-8	-8	-0
	-0	-0	-0	-0	-8	-0
	-0	-0	-0	-0	-0	+0
A3 AEI (1)	-0	-0	-0	+0	+0	+0
	-0	-0	-0	-0	-0	-0
	-0	-0	-0	-0	-0	+0
	-0	-0	-0	-0	-0	-0
A4 AEI (10)	-0	-0	-0	-0	-0	-8
	-0	-0	-0	+0	+0	-0
	-0	-0	-0	-8	-8	-32
	-0	-0	-0	-0	-8	-32
A5 DTNP/PF (1)	-0	-0	-0	-0	-0	-0
	-0	-0	-0	-8	-0	-0
	-0	-0	-0	-0	-0	-0
	-0	-0	-0	+0	-0	-0
A6 DTNP/PF (10)	-0	-0	-0	-0	-0	-0
	-0	-0	-8	-8	-8	-0
	-0	-0	-0	-0	-0	-0
	-0	-0	-0	-0	+0	-0
A7 F422 cells	-0	-0	-0	+0	+0	+0
	-0	-0	-0	-0	+0	+0
	-0	-0	-0	-0	-0	-0
	-0	-0	-0	+0	+0	+0
A8 Unvaccinated			-0	-0	+0	+0
			-0	-0	-0	-0
			-0	-0	+0	-0
			-0	+0	+0	-0

* v = virus isolated from plasma
A = titre of virus neutralising antibody
0 = <8

only in the vaccinated groups. Antibody was detected only after challenge except in one cat in group A6 which had a titre of 8 at the time of challenge, two weeks after the booster inoculation.

The major aim of this initial experiment was to have a guide as to which inactivating agent might be most effective in producing a viral vaccine. From the individual responses to challenge it was difficult to assess which group had responded best. As an aid to the evaluation of the efficacy of each vaccine a scoring system was used which compared the response of each vaccinated group of cats and the unvaccinated group. Two aspects of the response were included. These were first, the proportion of cats which became viraemic and secondly, the proportion which developed virus neutralising antibodies. In the case of viraemia, for each sampling time after challenge the proportion of viraemic cats in a group, expressed as a percentage, was multiplied by a factor to give a virus score. This factor was 1 for 3 weeks, 2 for 6 weeks and 3 for 9 weeks. It was reasoned that if a cat was viraemic 9 weeks after challenge it was likely to be permanently infected and therefore should have a higher factor than a cat viraemic after 3 weeks which might only have a transient infection. By similar reasoning it was considered that cats with antibody 3 weeks after challenge had responded better than the cats which had antibody 6 or 9 weeks after challenge. The corollary of this was that cats with no antibodies at 3 weeks were poor responders and should have a higher score than cats with no antibodies at 6 or 9 weeks. Therefore, for each sampling time the percentage of cats with no antibody was multiplied by a factor of 3 for 3 weeks, 2 for 6

weeks and 1 for 9 weeks.

As an example of the scoring system, in group A2 at 9 weeks after challenge, 1 of the 4 cats (25%) was viraemic. Therefore, a virus score of 75 (25 X 3) was given to group A2 at that time. At 3 and 6 weeks the scores were 0 (0 X 3) and 0 (0 X 2) respectively, making a total virus score of 75. Likewise at 9 weeks all (100%) of the same group had no antibodies and so the antibody score was 100 (100 X 1). At 3 and 6 weeks the scores were 150 (50 X 3) and 50 (25 X 2) respectively so that the total antibody score was 300. The total score for group A2 was, therefore, 75 + 300 (375). A total score was computed for all of the groups in this way. The rank of each group was then established according to this score, the lowest score being ranked 1 and the highest, 8. A low score indicated a better response to challenge than a high score. The scores and ranking are shown in Table 4.2.

Using this system of scoring, the two paraformaldehyde-inactivated vaccines had the lowest scores and the F422 cell vaccine had the highest score.

Side-effects of Vaccination

Following inoculation of the first dose of vaccine, it was observed that many of the cats suffered some discomfort. Therefore, 24 hours after the second inoculation of vaccine, the cats were examined more carefully for swelling at the site of inoculation and for pain on handling the site. The rectal temperature was also taken from most of the cats. The results, shown in Table 4.3, indicated that the vaccines produced a

TABLE 4.2

EFFICACY SCORES IN VACCINATION EXPERIMENT A

Vaccine Group		Virus Score *				Antibody Score				Total	Rank
No.	Vaccine	A	B	C	Total	A	B	C	Total	Score	
A1	PF(1)	0	0	0	0	225	50	50	325	325	1
A2	PF(10)	0	0	75	75	150	50	100	300	375	2
A3	AEI(1)	25	50	150	225	300	200	100	600	825	6=
A4	AEI(10)	25	50	0	75	225	100	25	350	425	3
A5	DTNP/PF(1)	25	0	0	25	225	200	100	525	550	5
A6	DTNP/PF(10)	0	50	0	50	225	150	100	475	525	4
A7	F422 cells	50	150	225	425	300	200	100	600	1025	8
A8	UNvaccinated	50	100	75	225	300	200	100	600	825	6=

* A = 3 weeks
B = 6 weeks
C = 9 weeks

TABLE 4.3

Side-effects of saponin-adjuvanted vaccines

Vaccine Group	Cat No.	Response * P	S	T (°C)	Vaccine Group	Cat No.	Response * P	S	T (°C)
A1	1	-	-	38.4	A5	1	-	-	39.5
	2	-	-	38.9		2	-	-	40.3
	3	-	-	38.9		3	+	+	40.3
	4	+	+	40.6		4	+	+	40.3
A2	1	-	-	38.9	A6	1	+	+	40.3
	2	+	+	40.3		2	+	+	40.6
	3	+	+	40.6		3	+	+	40.6
	4	+	+	40.6		4	+	+	40.6
A3	1	-	+	ND	A7	1	+	+	40.6
	2	-	+	ND		2	+	+	40.6
	3	-	+	ND		3	+	+	40.6
	4	-	+	ND		4	+	+	40.6
A4	1	-	+	ND	A8	1	+	+	ND
	2	-	+	ND		2	-	-	ND
	3	-	+	ND		3	+	+	ND
	4	-	+	ND		4	-	+	ND

* P = pain on handling site
S = swelling at site
T = rectal temperature
ND = Not Done.

harmful reaction at the site of inoculation in many cats. In addition most cats which developed side effects also had slight pyrexia.

To find whether saponin or $Al(OH)_3$ might be responsible for these effects, members of a group of 5.8-week old kittens were inoculated subcutaneously with 1 ml of PBS containing 1.5 mg of saponin. A further group of similar kittens were inoculated with 1 ml of PBS containing 0.1 ml of $Al(OH)_3$ suspension. These concentrations were the same as used in the vaccines. The kittens were examined 24 and 48 hours after injection for pain and swelling at the inoculation site and their rectal temperature was obtained. The results are shown in Table 4.4.

From the results in Table 4.3 it is clear that all of the vaccines produced side-effects in at least some of the inoculated cats. In general, cats which showed pain and swelling also had a mild pyrexia. The saponin adjuvant in the vaccine appeared to be partly or wholly responsible for these effects since, as shown in Table 4.4., kittens inoculated with a similar amount of saponin as was used in the vaccines, developed side-effects. Kittens inoculated with $Al(OH)_3$ remained clinically normal with only a minor rise in temperature 24 hours after the injection.

Discussion

It is usual for the efficacy of viral vaccines to be evaluated by the immune response of the host to vaccination, which is often measured by the production of serum antibodies to the antigen, and by the resistance of vaccinated animals to

TABLE 4.4

Effect of inoculation of saponin or $Al(OH)_3$

Treatment	Cat No	Response* (24 hours) P	S	T (°C)	Response (48 hours) P	S	T(°C)
Saponin	1	+	-	40.3	-	-	40.3
	2	+	-	40.6	-	-	40.3
	3	+	-	40.0	-	+	40.3
	4	+	-	41.1	-	+	40.6
	5	+	-	40.6	-	+	40.3
$Al(OH)_3$	1	-	-	40.3	-	-	38.9
	2	-	-	39.5	-	-	38.9
	3	-	-	39.5	-	-	38.9
	4	-	-	39.5	-	-	38.5
	5	-	-	39.5	-	-	38.9

*

P = pain on handling site

S = swelling at site

T = rectal temperature

challenge with a dose of virus which would cause an infection or disease in all or most of unvaccinated control animals. In the experiment described in this chapter, it was difficult to assess the efficacy of the various vaccines which were used. The main reason for this was that not all of the unvaccinated control cats became viraemic. The dose of virus which was administered oronasally was 8.5×10^4 FIU and was believed at the beginning of these experiments to be sufficient to cause permanent viraemia in most cats. This proved not to be the case and although 2 of 4 control cats had a transient viraemia, only 1 had a viraemia 9 weeks after challenge.

A more marked difference between some of the vaccinated cats and the unvaccinated cats was seen by the appearance of virus neutralising antibodies. None of the control cats developed antibodies. Only one cat in the group vaccinated with the DTNP/PF vaccine had antibodies following vaccination but before challenge. Curiously, these antibodies persisted for a period of 6 weeks but were not detected at 9 weeks after challenge. The reason of this disappearance is not known. A similar situation was observed after challenge in some other groups and rising titres of antibodies were seen only in the groups given the AEI(10) and PF(1) vaccines. It appears from these results that the vaccine had primed the immune response so that following infection with live virus a secondary response occurred. It was clear that cats which had virus neutralising antibodies were not viraemic which is similar to the situation in the field when cats are infected naturally (Russell and Jarrett, 1978b) and it is assumed, but not proven, that cats with antibody would be

resistant to further reinfection with FeLV.

The main aim of this initial experiment was to gain information about the inactivating agent which would produce the most effective FeLV viral vaccine. To try to compare the responses of the vaccinated groups, a scoring system was used which took into account the proportion of cats in a group which became viraemic and the proportion which developed antibodies. Using this system, the most effective vaccines appeared to be those inactivated with PF and the least effective was the F422 cellular vaccine. There was little difference in the score between unconcentrated and concentrated PF vaccines. It was decided on the basis of these results to concentrate future work on PF vaccines.

Three further lessons were learned from this experiment and were incorporated into the second vaccine experiment which is described in Chapter 7. The first challenge dose of FeLV should be increased in order to produced viraemia and if possible a persistent viraemia in all of the unvaccinated cats. Therefore, a stock of FeLV-A/Glasgow-1 was produced with a high titre and was tested in cats before being used in a vaccine experiment, as described in Chapter 6. Secondly, a larger number of cats per group should be used to increase the chance of demonstrating differences between groups. Thirdly, the adjuvant saponin should be used at a lower concentration which had fewer side-effects than those observed in the present experiment. Chapter 5 describes an experiment to find an appropriate dose level of saponin. A fourth improvement would be to monitor the cats for a

longer period after challenge to ensure that any observed effect of the vaccine was permanent. It was not possible to hold the cats for longer than 9 weeks in the initial experiment but in further experiments the cats were maintained for longer periods.

CHAPTER 5

DETERMINATION OF AN INNOCUOUS DOSE OF SAPONIN

Introduction

In vaccination with inactivated vaccines, enhancement of the immune response is considered desirable and is often necessary. Materials used for this purpose are called adjuvants, many of which have been used over the years. Their mode of action is still in many cases unclear, however, but one adjuvant function is that of retarding the release of antigen into the body. It is possible to slow antigen elimination rate by forming a depot which consists of a combination of antigen and the insoluble salt such as those of aluminium. Aluminium hydroxide gels were used in the present vaccination experiments and no side effects were noted when inoculated into kittens.

A second action of adjuvants is to enhance co-operation between cells involved in immune responses, especially macrophages and lymphocytes. In the first vaccination experiment described in the previous chapter, saponin was used for this purpose. However, it was noted that in some cats this substance caused pain and swelling at the site of injection and a rise in body temperature. In the experiments described in this section, the dose of saponin which was innocuous for cats wasdetermined. In the first vaccination experiment, saponin was used at a dose of 1.5 mg which is similar to that recommended for guinea-pigs in studies on foot-and-mouth disease virus (A.J.M. Garland - personal communication).

In the present experiment, the same 4 cats were given graded

doses of saponin, allowing clinical recovery between each dose, to establish a dose which gave no side effects.

Materials and Methods

Four healthy 4 month old specific pathogen free cats were obtained from the colony and housed in the animal house in the Veterinary School. Three concentrations of saponin in PBS were prepared: 500, 250 and 100 ug/ml. The pH of the solution in PBS was 7.3. Four cats were inoculated subcutaneously in the interscapular region with 1 ml of saponin at a concentration of 500 ug/ml and the clinical signs and rectal temperatures were monitored daily for 4 days. The cats returned to normal and 3 days later were given a dose of 250 ug of saponin and observed as before. One week later the same cats were given 100 ug. Finally after 1 week the cats were again given 500 ug to determine if they had become tolerant to saponin.

Results

The results of these experiments are shown in Table 5.1. After inoculation of 500 ug the rectal temperature rose in all 4 cats 24 hours after inoculation and did not return to within the normal range until 72 hours after inoculation. Pain on handling was obvious in three of the four cats on the day after inoculation and did not reduce in 2 of the cats until the fourth day of the experiment. Swelling appeared in 3 of the cats 24 hours after injection, continued after 48 hours and was reduced in 2 cats after 72 hours.

TABLE 5.1

Reaction of cats to different doses of saponin

		Days after inoculation											
		1			2			3			4		
Dose (ug)	Cat No.	T	P	S	T	P	S	T	P	S	T	P	S
500	1	38.7	-	-	40.0	-	-	38.8	-	-	38.2	-	-
	2	39.0	+	-	40.0	+	+	39.0	-	+	38.3	+	+
	3	38.7	+	-	40.0	+	+	39.1	+	+	38.6	+	+
	4	38.9	+	-	40.0	-	-	38.9	-	-	38.6	-	-
250	1	40.3	-	-	39.8	+	+	39.3	-	-	39.5	-	-
	2	39.8	-	-	39.0	-	-	39.2	-	-	39.8	-	-
	3	40.0	-	-	40.3	+	+	39.8	-	-	39.5	-	-
	4	40.1	-	-	39.3	+	-	38.8	-	-	38.9	-	-
100	1	38.4	-	-	38.3	-	-	38.1	-	-	38.2	-	-
	2	38.1	-	-	38.2	-	-	38.1	-	-	38.1	-	-
	3	38.5	-	-	38.5	-	-	38.9	-	-	38.6	-	-
	4	39.7	-	-	39.1	-	-	38.4	-	-	38.2	-	-
500	1	38.9	-	-	39.2	+	-	38.8	-	-	38.6	-	-
	2	38.8	+	-	40.2	+	+	39.2	+	+	38.4	-	+
	3	39.8	-	-	40.5	-	-	39.3	-	-	39.3	-	-
	4	39.3	-	-	39.4	+	-	38.8	-	-	38.7	-	-

T = Temperature (^{o}C)

P = Pain

S = Swelling

Dose = ug saponin/ml PBS

When the cats were again clinically normal, they were given a dose of 250 ug saponin. In this case there was a slight increase in rectal temperature in one of the cats (3) in the first and second days after inoculation while that of the others was normal. Pain was evident in 3 cats on the second day and swelling appeared in 2 cats on the second day of inoculation and disappeared later.

The result of the third inoculation with 100 ug of saponin were that no rise in rectal temperature, pain or swelling at the site of injection were noted. Finally, when the cats were again given a dose of 500 ug a similar response to that in the first inoculation was observed, with pyrexia in 2 cats, pain in 3 and swelling in 1.

Discussion

A dose of saponin of 500 ug, which was three-fold less than that given with the first vaccine (Chapter 4) was found to produce toxic side-effects. By decreasing the dosage in the same cats, a dose of 100 ug was found to be harmless and this dose was used subsequently in experiments described later in Chapter 7.

That this result was not due to the cats becoming tolerant to saponin was suggested by the fact that when the cats were inoculated again with 500 ug, the same effects as in the first administration were observed. It is clear from this experiment that the response of animals to saponin varies between species and must be ascertained for each animal prior to the use of this material as an adjuvant.

CHAPTER 6

DETERMINATION OF AN APPROPRIATE CHALLENGE DOSE OF FeLV

Introduction

A successful vaccine should have the essential properties of innocuity, potency and stability. Potency is the ability of the vaccine to introduce a minimum level of immunity which is capable of affording protection against the maximum dose of virulent field virus. Hence, in the development of vaccines, an appropriate challenge dose of virus should be established which is sufficient to provoke a response in essentially all unvaccinated animals. In the experiment described in the previous chapter the dose of challenge FeLV which was selected was considered to be appropriate based on past experience (Jarrett et al, 1977). It was observed, however, that this dose was insufficient to induce a persistent viraemia in all of the unvaccinated kittens which made it difficult to assess the efficiency of the vaccines being tested. In this chapter an experiment is described in which a higher dose of virus was used, prepared from concentrated virus. In this way a dose which gave a consistent response was achieved.

Materials and Methods

Preparation of Challenge FeLV

This virus was prepared in the following way. Each of seven 9 cm plastic plates was seeded with 6 X 10^5 FEA cells chronically infected with FeLV-A in 10 ml EFC20 and incubated at 37° in 5% CO_2 in air. Seventy-two hours later the culture fluids were

harvested and centrifuged at 2000 rpm for 10 minutes. The fluid was removed and centrifuged in a SW 27 rotor at 10,000 rpm for 10 minutes. The supernatant fluid was then spun at 24,000 rpm for 90 minutes in a SW 27 rotor and the resulting virus pellet was resuspended in 5 ml L-15 medium with 1% FCS and was stored at -70° in 4 one ml aliquots and two 0.5 ml volumes as assay samples. This virus was titrated using the two-stage method with 5-fold dilutions. The titre was 6.5×10^5 FIU/ml.

Infection of cats

Four 12 week old SPF cats were infected oronasally with concentrated FeLV-A/Glasgow-1 as a single dose of 1 ml containing 6.5×10^5 FIU per cat as described in Chapter 4. These cats were monitored for FeLV viraemia and virus neutralising antibodies over a 14 week period. Two ml of blood was obtained from the jugular vein at 2, 11 and 14 weeks after FeLV infection. The sera and plasma samples were assayed for neutralising antibodies and virus as described in Chapter I.

Results

The results are shown in Table 6.1. It is seen that 3 of the 4 cats became viraemic 2 weeks after FeLV administration and that all 4 were viraemic after 14 weeks. None of the cats developed neutralising antibodies.

TABLE 6.1

FeLV Isolated from Challenged Cats

Cat No.	Virus isolation at weeks:		
	2	11	14
1	+	+	+
2	+	+	+
3	+	-	+
4	-	-	+

Discussion

The results of this experiment indicated that a dose of 6.5×10^5 FIU of FeLV-A given oronasally was sufficient to produce a long-lasting, presumably permanent, viraemia in most, if not all cats.

Hoover *et al* (1976) showed that the response of cats to FeLV is related to age and that cats over 16 weeks of age are relatively resistant to infection. As discussed above, it was necessary to challenge the cats in these experiments at an age when they were still fully susceptible to FeLV. This age was around 12-13 weeks. It might be expected, however, that at this age the kittens are in a transitional period between full susceptibility and resistance and that a dose of FeLV which induced persistent viraemia when administered to 8 week old kittens might be insufficient to permanently infect 12 or 13 week old animals. While it is desirable to challenge cats in vaccine experiments with a dose of virus which will infect all of the unvaccinated control animals, care must be taken that an unnaturally large dose of virus is not administered that would overwhelm immunised animals which would normally resist challenge by the natural route of infection. The dose of virus used in the first vaccine experiment described in Chapter 4 was obviously insufficient. The results of the present experiment indicated that a dose of 6.5×10^5 FIU given oranasally might be more satisfactory and this or slightly higher doses, were used in subsequent experiments.

CHAPTER 7

VACCINATION EXPERIMENT 2: COMPARISON OF ADJUVANTS

Introduction

The results of the vaccination experiments described in Chapter 4 were inconclusive. In the light of these results further experiments were planned to obtain more precise information on the efficiency of inactivated FeLV as an antigen. The following points were considered.

1. Paraformaldehyde was chosen as the sole inactivating agent since PF-inactivated virus appeared to be most effective in the first experiment.
2. More precise details of the inactivation of both MSV (FeLV) and FeLV itself with PF were obtained in order to employ the minimum effective concentration of the agent in vaccine preparations.
3. A comparison of adjuvants was undertaken, namely saponin and incomplete Freund's adjuvant (ICFA). Saponin was used at the lower, non-toxic dose as determined in the experiment reported in Chapter 5.
4. The challenge dose of FeLV-A was increased to 1×10^6 FIU in order to obtain the establishment of persistent viraemia in all of the unvaccinated control cats as described in Chapter 6. In this way differences between the response of vaccinated and unvaccinated cats might be more obvious.
5. The number of cats in each group was increased to 6, again with the object of increasing the probability of observing differences between the effects of the various vaccines.

This chapter describes first, experiments on the inactivation of MSV (FeLV) and FeLV by paraformaldehyde and attempts to detect infectious virus in inactivated viral preparations. Secondly, a vaccination experiment is described in which PF-inactivated FeLV was used in vaccines, and saponin and ICFA were compared as adjuvants.

Materials and Methods

Inactivation of MSV (FeLV)

The kinetics of inactivation of MSV (FeLV-A/Glasgow-1) by several concentrations of PF at 25° were studied over a period of 24 hours. Inactivation was done with PF concentrations of 0.01, 0.02, 0.04 and 0.06% in a water bath. To four 5 ml glass bottles, each containing 1 ml of MSV (FeLV) (3.5×10^6 FFU/ml) at 25°, 100 ul of PF at a concentration of 0.1, 0.2, 0.4 or 0.6% was added. A further bottle containing virus had no PF added. The bottles were shaken and 150 ul of fluid drawn from each was immediately collected. A volume of 15 ul of 20% sodium thiosulphate was added and the mixture was incubated at 4° for 30 minutes and was then stored at -70°. This represented the samples at time 0. Further samples were taken at 3, 6, 12 and 24 hours after addition of PF. The samples were subsequently tested for residual infectivity. At the same time, 4 bottles containing only 1 ml EFC received PF as above. These were then incubated at 25° for 24 hours and a one-tenth volume of sodium thiosulphate was added. The mixture was incubated at 4° as before and then stored at -70°.

Inactivation of FeLV

FeLV was collected from the fluid of F422 cells seeded at 1.2 X 10^6/ml and harvested 48 hours later. The fluid was clarified by spinning at 2000 rpm for 10 minutes and then was treated with 0.01, 0.02, 0.04 and 0.06% PF in a 25° water bath for 24 hours. The inactivation procedure was carried out by addition of 1.5 ml of 0.1,0.2, 0.4 or 0.6% PF and 1.5 ml of L-15 medium to 5 universal bottles each containing 15 ml of freshly prepared FeLV. The mixture was shaken well and 3 ml of each reaction was collected, neutralised with 0.3 ml of 20% sodium thiosulphate at 4° for 30 minutes and stored at -70° as 0 time samples. The reaction was immediately immersed in a 25° water bath and sampled in the same manner 3, 6, 12, and 24 hours later. These samples were treated as above and stored at -70°.

Infectivity assay of PF-treated FeLV

The residual infectivity of FeLV following PF treatment was assayed in 4 ways: by the one-stage or two-stage quantitative assays using FEA and C81 cells; by the qualitative interference test; and by a modification of the two-stage FEA/C81 assay. In each case five-fold dilutions of the virus preparation were used, beginning with neat virus.

The quantitative tests were carried out as described in Chapter 1. The interference test was done as follows:
Ten 5 cm plastic plates were seeded with 1 X 10^6 FEA cells in 4 ml EFC and incubated at 37° in 5% CO_2 in air. Twenty four hours later, 2 plates were used to inoculate each dilution of the PF-treated virus. Polybrene was added to a concentration of 8

ug/ml. The neat sample was in 1 ml and subsequent dilutions in 2 ml. The cells were incubated for 90 minutes at 37°, washed twice with Eagle's medium and 4 ml EFC was added to each plate. After 72 hours of infection and when the culture became confluent, it was subcultured. One fourth of the cells of each plate were seeded into a new plate. This subculturing was repeated for 3 weeks when the cells were tested for the presence of FeLV by an interference test.

The fourth assay was a modification of the quantitative two-stage assay and was carried out by using C81 cells in higher density than previously at 1×10^5 in 4 ml EFC per 5 cm plate. After 24 hours incubation the cells were inoculated with neat virus samples or samples diluted 1:5 and were incubated at 37° in 5% CO_2 in air for 3 hours. The inoculum was then removed and the cells were subcultured so that one-third was seeded into each of 3 plates (3×10^4 cells each). The assay was then continued in the same way as the two-stage FEA/C81 assay.

Removal of inactivating agents by ultrafiltration

As described in the Results section of this Chapter, it was not possible to determine whether or not there was infectious virus in some of the low dilutions of PF-treated viral samples owing to the toxicity of the PF for the cells. The following procedure was used to remove the residual PF.

Two 50 ml lots of F422 virus freshly prepared as before were inactivated separately with 0.01 and 0.02% PF in a 25° water bath for 24 hours and were concentrated using an Amicon stirred cell with a Nuclepore membrane (1×10^6 MW cut-off) at 4° for 6

hours until the original volume was reduced to 5 ml. This was thenincreased to 50 ml with L-15 medium and filtered again to 4 ml. Thus the virus concentration was 10 times that of the original and the PF concentration was reduced by a factor of 1:120. The concentrated virus (4 ml) was added to 21 mlL-15 medium so that the concentration of the virus was twice the original and 1 ml of it was stored as two 0.5 ml lots at -70° as samples for viral residual infectivity assay.

The remainder (24 ml) was stored at -70° in 4 lots of 6 ml each. These stored samples were assayed using the two-stage procedure described in Chapter I with 5-fold dilutions.

Concentration of FeLV by ultrafiltration

Ten, 9 cm plastic plates were seeded with 6 X 10^5 FeLV-A infected FEA cells in 10 ml McCoy's 5a medium with 10% FCS in each plate. The plates were incubated at 37° in 5% CO_2 in air. Forty-eight hours later the culture fluids were harvested and centrifuged at 2000 rpm for 10 minutes. The supernatent fluid was removed and two 0.5 ml volumes were taken and stored at -70°. The remaining fluid was filtered through a Nalgene disposable filter unit with a pore size of 0.45 um and samples of the filtrate were collected and stored as before. The filtrate was then placed in an Amicon stirred cell with a 43 mm Nuclepore filter of 10^6 molecular weight cut-off. Using nitrogen gas at a pressure of 10 pounds per square inch, the fluid was ultrafiltrated at 4° for 6 hours until the volume was reduced to 6.5 ml. Samples of the filtrate (80 ml) and concentrated virus were taken as before and stored at -70°. All of the samples were

subsequently titrated using the two-stage method. The concentrated virus was stored at -70° as thirteen 0.5 ml aliquots, and was used later to challenge the vaccinated and control cats in the second vaccine experiment, described in this chapter.

FeLV vaccine preparation

In the light of the results of the last infectivity assay experiment 0.02% PF-inactivated FeLV which had been stored at -70° as 6 ml and found to contain no residual infectivity, was used to prepare vaccines B1 and B2. A volume of 0.6 ml of $Al(OH)_3$ was added to 6 ml of the stored inactivated antigen, mixed and incubated at 4°overnight. Then 6 ml of a dilution of 1:1250 saponin was added and mixed. This was vaccine B2.

For vaccine B1 the same procedure was used to prepare the vaccine except instead of saponin, incomplete Freund's adjuvant (ICFA) (Miles Laboratories) was used. Two volumes of ICFA were added to the virus preparation and the mixture was emulsified in a Sylverson homogeniser. The emulsion was dispensed in 1 ml volumes in syringes and were kept at 4° until used. The emulsion remained stable over a period of 3 weeks.

Vaccination experiment

Three groups of 6 8-week old kittens were used in the experiment. One group was inoculated with vaccine B1, one with vaccine B2 and the other was untreated and served as a challenge control.

The kittens were inoculated with 1 ml of vaccine IM in the

left hind leg. Three weeks later a second booster dose was given in the right hind leg. The animals were challenged one week later by the oronasal installation of 1 ml of FeLV-A/G-1, containing 1 X 10^6 FIU, 0.1 ml into each nostril and 0.8 ml into the mouth.

Sampling of all experimental cats was by collection of clotted and heparinised blood samples before each vaccination, at the challenge and at 3, 9, 21 and 28 weeks after challenge. Two ml was taken from the jugular vein, 1 ml for serum and the other for plasma. The serum and plasma were stored at -70° until assayed using virus isolation techniques for viraemia and neutralisation test for antibody.

Results

Kinetics of inactivation of MSV (FeLV) by paraformaldehyde

The results of exposure of MSV (FeLV) to various concentrations of PF are shown in Table 7.1.

MSV (FeLV) was exposed to four different concentrations of PF in a 25° water bath for 24 hours and samples were collected at 0, 3, 6, 12 and 24 hour intervals, stored at -70° and assayed later. There was an immediate fall in the titre of MSV (FeLV) inactivated by these 4 concentrations of PF at 0 time. The titre ranged between 8.9 X 10^4 to 6.4 X 10^3 FFU/ml compared to the titre of the virus control which was 2.6 X 10^5 FFU/ml. Subsequently, the titre of virus decreased according to the PF concentration. At the second interval (3 hours), the titre of the virus for the first three PF concentrations was between 3.2 X 10^3 and 5 X 10^1 FFU/ml and no virus was detected in the

TABLE 7.1

Inactivation of MSV (FeLV) by paraformaldehyde

Paraformaldehyde Concentration (%)	Virus titre (FFU/ml) Time (hours) 0	3	6	12	24
0.01	8.9×10^4	3.2×10^3	0	0	0
0.02	7.7×10^4	4.0×10^2	0	0	0
0.04	3.8×10^4	5.0×10^1	0	0	0
0.06	6.4×10^3	0	0	0	0
None	2.6×10^5	3.9×10^4	4.3×10^4	4.0×10^4	3.2×10^4

sample exposed to 0.06% PF. The virus control titre was 3.84×10^4 FFU/ml. At the remaining intervals the virus was completely inactivated at all PF concentrations, while the virus control titre was 4.3×10^4 at 6 hours and 3.2×10^4 FFU/ml at 24 hours interval.

FeLV inactivation

The results of the assay of the PF-inactivated FeLV were as follows. FeLV samples were exposed to 0.01, 0.02, 0.04 and 0.06% PF for 24 hours at 25° and were assayed for residual infectivity, using the one-stage method. The cells detached after inoculation with the undiluted virus samples from all the PF concentrations used and also with the inoculation of the samples diluted 1:5 from virus inactivated with 0.06% PF.

The cells appeared unhealthy in cultures inoculated with the 1:5 and 1:25 dilutions of FeLV samples inactivated with 0.01 and 0.04% PF. The cultures inoculated with these same dilutions of the virus inactivated with 0.02% PF were contaminated with bacteria. At all other dilutions (1:125, 1:625 and 1:3125) the cells were healthy and there was no evidence of FeLV. In the FeLV sample inactivated with 0.06% PF, the result was that there were unhealthy cells in the cultures inoculated with 1:25 dilution and healthy cells in other cultures and there was no evidence of leukaemia virus. In contrast the culture cells which were inoculated with all dilutions of the virus control sample were healthy and there were 4 foci in the last dilution (1:3125) so that the virus titre was 1.3×10^4 FIU/ml.

In using the two-stage assay method, no results were

obtained for the samples taken at 0 time, owing to bacterial contamination. In cultures inoculated with samples collected at the 3 hour interval, the cells detached after the cultures were inoculated with undiluted samples of the virus inactivated with all four PF concentrations. The same happened in cultures inoculated with the 1:5 dilution of samples which were inactivated with 0.02, 0.04 or 0.06% PF and also with the 1:25 dilution of the samples inactivated with 0.06% PF. At the other dilutions of the sample inactivated with 0.01% PF, the cells were healthy and there were 4 foci in the 1:625 dilution so that the titre was 2.5×10^3 FIU/ml. All other cultures which were inoculated with the remaining dilutions of the FeLV samples inactivated with 0.02, 0.04 or 0.06% PF were healthy and there was no evidence of infectious FeLV. In the same experiment the results of the assay of samples collected at 6 hours were similar to the results of the assays of samples collected at 3 hours except that no infectious virus was detected in the 0.01% PF inactivated sample at any dilution indicating that there was complete inactivation of the virus except, perhaps, in the undiluted sample or the sample diluted 1:5 which could not be tested due to toxicity of the inoculum.

The interference test results were that infectious virus appeared in the neat dilution of the sample inactivated with 0.01% PF for 24 hours. In this test only neat and a 1:5 dilution of the FeLV inactivated with the four PF concentrations for 24 hours was used. The side-effect of the PF on the cells was that the cells were unhealthy in the cultures inoculated with neat

samples inactivated with 0.01 or 0.02% PF and the cells detached in neat samples inactivated with 0.04 or 0.06% PF. Also, the cells were unhealthy in 1:5 dilutions of the same samples.

The last way of assaying the PF inactivated samples of FeLV was by using a higher initial density of C81 cells with subsequent re-plating and addition of further FEA cells. The results were that all cells detached after inoculation with neat and 1:5 dilutions of the samples so that no results were obtained with this test.

Removal of inactivating agent by ultrafiltration

From the results described above it was clear that the residual inactivating agent in the virus samples was toxic for the cells in the infectivity assays when undiluted samples were tested. Consequently, it was considered necessary to remove the PF from the samples in order to ensure that there was no virus in the vaccines. This was achieved by "washing" the PF-treated virus by ultrafiltration.

In the light of the results of inactivating MSV (FeLV) described in this Chapter, two concentrations of PF were used to treat FeLV at 25° for 24 hours. These were 0.01% and 0.02%.

After ultrafiltration the samples were tested for infectious virus by using the two-stage assay. A sample of inactivated virus was also titrated. The results were that there was no toxic effect of PF-treated virus on the cells. A few foci were found in the neat, 1:5 and 1:25 dilutions of the virus treated with 0.01% PF, but none were found in the virus treated with 0.02%. The titre of the uninactivated virus control was

6.3×10^2 which was rather low. The results indicated, therefore, that at least 3 logs of virus were inactivated by 0.02% PF since the PF-treated preparations were concentrated two-fold.

Concentration of FeLV by ultrafiltration

The results of titrating the samples obtained during Amicon ultrafiltration of FeLV-A/Glasgow-1 are shown in Table 7.2. In this process the virus was concentrated by a factor of 13.3 and 62% of the original infectivity was recovered. No virus was detected in the ultrafiltrate indicating that all of the virus was retained within the Amicon cell. The final concentrate had a titre of 4.4×10^6 FIU/ml and was used diluted in L-15 medium to 1×10^6 FIU/ml as the challenge virus in the vaccination experiment.

Vaccine preparation

From the results described above, the vaccine which was used consisted of the FeLV preparation inactivated with 0.02% PF and detoxified by ultrafiltration, in which no infectious virus was detected. This vaccine was used to vaccinate two groups of six 8-week old kittens. Two adjuvants were compared: saponin and incomplete Freund's adjuvant. A third group was left unvaccinated as a virus control. The second dose of vaccine was given 3 weeks later and the challenge was made one week after the second vaccination.

The results of the vaccination experiment are shown in Table 7.3. All the unvaccinated control cats were viraemic 3, 9 and 21 weeks after challenge and did not develop virus neutralising

TABLE 7.2

Concentration of FeLV by Amicon Ultrafiltration

	FIU/ml	Volume (ml)	Total FIU
Culture supernatant fluid	6.0×10^5	86.5	5.2×10^7
0.45 um filtrate	6.5×10^5	86.0	5.6×10^7
Amicon Ultrafiltrate	10	80.0	-
Amicon concentrate	4.9×10^6	6.5	3.2×10^7

TABLE 7.3 Results of Vaccination Experiment B

Group		Time (weeks) relative to challenge *													
		-4		-1		0		3		9		21		28	
No.	Vaccine	V	A	V	A	V	A	V	A	V	A	V	A	V	A
B1	PF (ICFA)	-	0	-	-	-	0	-	0	-	0	-	16	-	32
		-	0	-	-	-	0	+	0	+	0	+	0	+	0
		-	0	-	-	-	0	-	0	+	0	+	0	+	0
		-	0	-	-	-	0	+	0	-	0	-	0	-	0
		-	0	-	-	-	0	+	0	+	0	-	8	-	0
		-	0	-	-	-	0	+	0	-	0	-	16	-	128
B2	PF (Saponin)	-	0	-	-	-	0	-	0	-	0	+	0	+	0
		-	0	-	-	-	0	-	0	-	0	+	0	+	0
		-	0	-	-	-	0	+	0	+	0	+	0	+	0
		-	0	-	-	-	0	+	0	+	0	+	0	+	0
		-	0	-	-	-	0	-	0	-	0	-	32	-	64
		-	0	-	-	-	0	-	0	-	0	-	8	-	0
B3	Unvaccinated					-	0	+	0	+	0	+	0		
						-	0	+	0	+	0	+	0		
						-	0	+	0	+	0	+	0		
						-	0	+	0	+	0	+	0		
						-	0	+	0	+	0	+	0		
						-	0	+	0	+	0	+	0		

* V = Virus isolated from plasma

A = Titre of virus neutralising antibody

0 = < 8

antibodies. These cats were assumed to have persistant FeLV infections. In the group of cats vaccinated using saponin (B2) the results were that none of the cats was viraemic before challenge, but 2 of them were viraemic 3 and 9 weeks after challenge and those cats together with another 2 cats were viraemic 21 and 28 weeks after challenge. None of the 6 cats had virus neutralising antibodies before challenge. None of the 4 viraemic cats developed antibodies after challenge and only one of the non-viraemic cats had antibodies at 21 and 28 weeks.

The results of vaccination using ICFA as adjuvant in the second group of vaccinated cats were that again none of the cats were viraemic before challenge, but 4 of them became viraemic 3 weeks after challenge. One of these was persistently viraemic at 9, 21 and 28 weeks and one other was viraemic at 21 and 28 weeks. The other 3 cats which were viraemic at 3 weeks appeared to recover from the infection. The sixth cat was not viraemic at any time. As in the two other groups, none of the viraemic cats had neutralising antibodies. Only 2 of the 4 non-viraemic cats had antibodies at 28 weeks after challenge.

Since neither of the vaccines protected 100% of the kittens from challenge, the scoring system described in Chapter 4 to compare the vaccines was used and the results are shown in Table 7.4. These show that there was little difference in efficacy between the vaccines using saponin or incomplete Freund's adjuvant although the latter appeared to be slightly more effective.

TABLE 7.4

Efficacy Scores in Vaccination Experiment B

Vaccine Group		Virus Score *				Antibody score				Total	Rank
No.	Vaccine	A	B	C	Total	A	B	C	Total	Score	
B1	PF (ICFA)	67	66	99	232	300	200	50	550	782	1
B2	PF (Saponin)	33	66	201	300	300	200	67	567	867	2
B3	Unvaccinated	100	200	300	600	300	200	100	600	1200	3

* A = 3 weeks
B = 9 weeks
C = 21 weeks for Group B3
28 weeks for Groups B1 and B2

Discussion

In this chapter, the inactivation of MSV (FeLV) by PF was studied more thoroughly than in the experiment described in Chapter 4. These results gave an indication of the minimum concentration (0.02%) of PF which might be used to inactivate FeLV. FeLV was then treated with PF at concentrations which completely inactivated 4 logs of MSV (FeLV). However, great difficulty was encountered in testing whether or not there was residual infectious virus in the PF-treated FeLV preparations because these were toxic for the cells in the usual assay systems when inoculated neat or diluted 1:5. Four different types of assay were tried but none were successful. The first two methods were the standard one-stage and two-stage assays which used C81 and FEA cells. Each of these assays uses cells which are initially seeded at low density and it was considered that these cells might be particularly sensitive to PF. Therefore, two other methods were tried in which the initial cells were almost confluent: an interference test using FEA cells, and a modified two-stage C81/FEA assay in which the C81 initiator cells were confluent when infected. However, neither of these tests produced a result when undiluted virus was used as the inoculum. By contrast, assays of MSV (FeLV) which was inactivated with the same concentrations of PF were successful. The reason for this difference may be that the initiator FEA cells infected with FeLV-B which were used in the sarcoma virus assay were less sensitive to the effects of the residual inactivating agent in the PF-treated MSV (FeLV) preparations than the uninfected FEA

and C81 cells used in the FeLV assays. The reason for this difference is not known.

Comparing these results with the results of Pedersen et al (1979), these authors did not mention problems in the assay of formaldehyde-inactivated FeLV, presumably because they started their assays with a 1:10 dilution of the inactivated virus. It is not clear, therefore, if Pedersen's vaccine was completely inactivated. To ensure as far as possible that the virus used in the present experiment was completely inactivated it was finally necessary to wash the PF-treated FeLV by ultrafiltration which should have removed molecules of less than 1×10^6 molecular weight. There was no detectable infectious virus in this sample.

In the light of these results, it was decided then to use the washed virus as vaccine. The reasons were that as well as being non-toxic, such a preparation might also have the advantage that p15E might have been removed. This molecule is supposed to be immunosuppressive (Olsen et al, 1980b) and its absence from a vaccine might produce a better immune response. On the other hand, free gp70 and FOCMA might also be removed (Olsen et al, 1980b) so that some useful antigens might be lost. The results of concentrating live FeLV-A/Glasgow-1 by Amicon ultrafiltration which are shown in Table 7.2, indicated that 62% of the infectious virus was retained in the process. Since this procedure was carried out over a period of 6 hours, it is likely that at least some of the loss of infectivity was due to the regular inactivation process. Therefore, it would be expected that greater than 62% of PF-inactivated virus would be included in the vaccine. Although in the production of the PF-treated

FeLV only 3 logs of virus were shown to be inactivated, further evidence that there was no infectious virus in the vaccine was that vaccinated cats did not develop viraemia or produce virus neutralising antibodies.

The results of vaccination were better than in the previous experiment described in Chapter 4. All of the unvaccinated cats became persistently viraemic as expected from the results of Chapter 6, so that the protective action of vaccination was more obvious. In addition, none of the unvaccinated cats made virus neutralising antibodies.

In the vaccinated group, only two of the six cats given vaccine with saponin as adjuvant resisted the challenge after 28 weeks. One of these cats developed virus neutralising antibodies. There was a slightly better response in the group which was given vacccine with the oil adjuvant. Four of six of these cats resisted the challenge and were not viraemic after 28 weeks. However, three of these cats were transiently viraemic, 3 weeks after challenge. These results are similar to those of Pedersen et al (1979) who showed that formaldehyde-inactivated vaccine did not prevent transient viraemia but did prevent persistent viraemia in a proportion of cats, following natural exposure to FeLV-excreting cats.

The reason why half of the six vaccinated cats which resisted challenge, including two which had a transient viraemia, did not develop virus neutralising antibodies is not clear. It is known, however, from epidemiological studies that there is a small but significant proportion of cats naturally exposed to

FeLV which are non-viraemic and which do not have neutralising antibodies (Hardy et al, 1976).

The scoring system indicated that the vaccine using the incomplete Freund's adjuvant was slightly superior to the vaccine with saponin. Thus in the final vaccination experiment which is described in the next chapter, the oil adjuvant was used.

CHAPTER 8

VACCINATION EXPERIMENT 3: COMPARISON OF ANTIGENS

INTRODUCTION

The results of the two vaccination experiments reported in Chapters 4 and 7 indicated that a considerable degree of protection against experimental FeLV challenge was obtained using paraformaldehyde-inactivated FeLV derived from the F422 cell line. However, protection was not complete and it was considered that there might be at least two reasons for this. First, it might be that the quantity of viral antigen used in the vaccines was insufficient although in the first experiment described in Chapter 4 no difference was observed in the response of cats immunised with antigen equivalent to unconcentrated or X10 concentrated F422 cell culture fluid. Nevertheless, since the results of the second experiment were more encouraging, it was decided to compare again an unconcentrated and a X10 concentrated FeLV-A/F422 vaccine.

A second possibility might be that the quality of the F422 virus was poor and, in particular, that the viral envelope gp70 was not present in such high concentrations as in FeLV grown in fibroblast cultures. Electron microscopy of F422 cells has shown that budding virus has prominent spikes on the envelope but that the virus which is present around the cells following release from the cells does not have obvious spikes (Toth, 1980). This is in contrast to FeLV produced in feline embryo fibroblast cultures which has prominent spikes when budding and maintains its spikes for 24-48 hours afterwards (Laird, H.M. and Jarrett,

O., personal communication). In the light of these observations it was decided to compare FeLV obtained from F422 cells and from fibroblast cultures as a source of vaccine. It was assumed that a virus with well-preserved spikes might be more antigenic than one without spikes. It should be emphasised again that the amount of FeLV produced by F422 cells is approximately ten times that produced by FEA cells. Therefore FeLV grown in fibroblasts was concentrated tenfold, to make it equivalent to unconcentrated F422 virus.

While these experiments were in progress it was found that some cats which appear to recover following exposure to FeLV, maintain a latent FeLV infection in bone marrow cells (Post and Warren, 1980; Rojko et al, 1982; Madewell and Jarrett, 1983). It was considered important to determine whether vaccinated cats which resisted challenge with FeLV had eliminated the virus or had a latent infection. Therefore, bone marrow cultures were made from protected cats and examined for the presence of latent virus.

Materials and Methods

Experimental groups

Five groups of cats were used in this experiment. These were designated as follows:

C1: Vaccinated with FeLV-A/F422, X1

C2: Vaccinated with FeLV-A/F422, X10

C3: Vaccinated with FeLV-A/Glasgow-1, X10

C4: Inoculated with control "vaccine", FEA culture fluid X10

C5: Control group, unvaccinated

Vaccines C1 and C2

Two hundred ml of F422 culture fluid harvested from a culture seeded 24 hours before at 1.2 X 10^6/ml cells per ml in L-M medium was clarified by spinning at 2000 rpm for 20 minutes. The virus was inactivated with 0.02% PF in a 25° water bath for 24 hours and a one-tenth volume of 20% sodium thiosulphate was added for 30 minutes at 4°. The inactivated virus was concentrated at 4° by ultrafiltration in an Amicon stirred cell using a 43 mm Nuclepore membrane with a 1 X 10^6 molecular weight cut-off at a nitrogen pressure of 20 lb per in^2. A volume of 175 ml was reduced to 75 ml. An additional 100 ml of L-15 medium was added and this was further concentrated to a volume of 55 ml. Samples of 5 ml were stored at -70°. The virus was assayed using the two-stage assay which indicated complete inactivation, while the infectivity of uninactivated FeLV was 3.5 X 10^4 FIU/ml. After the test for residual infectivity the inactivated antigen was thawed and spun in a Beckman SW41 rotor at 40,000 rpm for 60 minutes and the pellet was resuspended in 6.6 ml L-15 medium which represents a concentration of 30-fold. Then 0.6 ml of the 30X antigen was diluted to 6 ml in L-15 medium to make a 3X concentrate. Each of these antigens was mixed with a one-tenth volume of $Al(OH)_3$, incubated at 4° overnight and then two volumes of ICFA was added. The mixtures were emulsified in a Sylverson homogeniser and used to vaccinate 8 week old cats as 1 ml IM in the hind leg. The remainder of the vaccines were kept at 4° until used in the boosting dose.

Vaccine C3

This vaccine was prepared in a similar manner to those above. About 200 ml of FeLV-A/Glasgow-1 culture fluid was harvested from two chronically FeLV-infected FEA monolayer cell cultures in 2.5 litre roller bottles 48 hours after seeding with 1×10^7 cells in 100 ml McCoy's medium with 10% FCS. The fluid was clarified by spinning at 2000 rpm for 20 minutes, inactivated with 0.02% PF in a 25° water bath for 24 hours, neutralised with a one-tenth volume of 20% sodium thiosulphate at 4° for 30 minutes and concentrated by Amicon ultrafiltration as above to a final volume of 40 ml.

Samples were taken for virus assay before inactivation and after inactivation and concentration. No virus was observed in the inactivated sample while the virus titre of the uninactivated sample was 1.2×10^4 FIU/ml. To prepare the vaccine the inactivated and concentrated FeLV was thawed, spun at 40,000 rpm for 60 minutes in the SW41 rotor and the pellet was resuspended in 6.6 ml L-15 medium, so that the final concentration of the inactivated antigen was X30. A volume of 0.66 ml of $Al(OH)_3$ was added and kept at 4° overnight, when two volumes of ICFA were added and the mixture was emulsified.

Control vaccine C4

This vaccine was prepared in the same way as the C3 vaccine except that instead of using FeLV-infected FEA cell culture fluid, the fluid of uninfected FEA cells was used. This was treated with PF, concentrated, sampled and assayed as before. The $Al(OH)_3$ and ICFA were added as for the other preparations.

Vaccination experiment

All 30 kittens were vaccinated with the different types of vaccine as described in Chapter 7. These kittens, together with the six kittens which comprised the unvaccinated group were then challenged at 12 weeks of age (one week after the second vaccination) by giving 0.8 ml of Amicon concentrated FeLV-A (6.25 x 10^5 FIU) by dropping 0.1 ml in each nostril and 0.6 ml in the mouth of each kitten. All the experimental kittens were bled before each vaccination and challenge, and at 3, 6, 12 and 18 weeks after challenge. Samples of 1 ml clotted blood for serum and 1 ml with lithium heparin for plasma were taken, processed and stored at -70^{o} until assayed for FeLV viraemia by virus isolation, or for neutralising antibodies.

Establishment of bone marrow cultures

Bone marrow cultures were established by a modification of the method of Madewell and Jarrett (1983). Cells were obtained by aspiration of femoral marrow using a Rosenthal pattern needle (18 gauge, 1 inch). This procedure was carried out by Prof. O. Jarrett. The cells were immediately dispersed in cold medium consisting of Alpha medium (Gibco) supplemented with 10% FCS, 10^{-6} M hydrocortisone sodium succinate (Sigma) and 10 units/ml of preservative-free heparin sodium BP (Pabyrn).

Bone marrow cell cultures were prepared in duplicate in 25 cm^2 flasks with 10 ml of growth medium (as above but without heparin) as follows. Five ml of the aspirated cell suspension was centrifuged at 1000 rpm for 5 minutes and the pellet was resuspended in 5 ml of cold NH_4Cl and incubated at 4^{o} on ice for

5 minutes to lyse contaminating red blood cells. The cells were then pelleted by centrifugation and resuspended in growth medium. The nucleated cells were counted in a haemocytometer and the cell suspension was adjusted to 2 X 10^6 cells per ml in growth medium. Two flasks were seeded each with 10 ml of cell suspension, gassed with 5% CO_2 in air and incubated at 37°. These cultures were designated NA or NB.

The remaining 5 ml of the original marrow cell suspension was centrifuged and the pelleted cells were resuspended to 2 X 10^6 per ml in growth medium based on the cell count of the cells treated with NH_4Cl. Two cultures were prepared as above and designated A or B.

Half of the medium from each culture was replaced every 7 days with fresh medium. The medium which was removed was centrifuged at 2000 rpm for 10 minutes and the supernatant fluid was stored at -70° before being tested for FeLV.

Results

As described in the other two previous vaccination experiments in Chapter 4 and 7, in this final experiment a comparison of antigens was made in which F422 and FeLV-A/Glasgow-1 viruses were used. At the same time culture fluids of uninfected FEA cells were used as a control vaccine to establish if there might be any response in the cats to the serum proteins in the medium or to cellular antigens shed into the culture fluid.

Results of vaccination in this experiment are shown in Table

8.1. These results were assessed by comparing the proportion of viraemic cats in each vaccinated group with the proportion in the unvaccinated group at challenge and 3, 6 and 18 weeks after challenge. There were no antibodies in any group of the cats, vaccinated or unvaccinated at challenge. None were viraemic at this time.

The cats in the unvaccinated group were viraemic 3, 6 and 18 weeks after challenge. None of these cats produced virus neutralising antibodies. Also the cats in the control vaccine group all became viraemic within three weeks and were persistently viraemic. None produced virus neutralising antibodies at any time. Two of the cats in this group died, six weeks after challenge.

Of the vaccinated cats, the majority of these given inactivated F422 virus resisted challenge with FeLV. By contrast, only one of six cats vaccinated with the inactivated FeLV-A/Glasgow-1 virus resisted challenge. There was little difference in the results of the groups given F422 (X1) or F422 (X10) vaccine. In the first group (C1) one of the six cats became persistently viraemic following challenge. None of the other cats in this group became either transiently or persistently viraemic. While none of the cats had neutralising antibodies following vaccination but before challenge, all developed antibodies after challenge.

In the second vaccination group (C2) two of the cats became permanently viraemic after challenge. One of these viraemic cats (cat 6) died 16 weeks after challenge. One other cat (cat 4) had

TABLE 8.1 Results of Vaccination Experiment C

Group		Time (weeks) relative to challenge *													
		-4		-1		0		3		6		12		18	
No.	Vaccine	V	A	V	A	V	A	V	A	V	A	V	A	V	A
C1	F422 (1)	-	0	-	0	-	0	-	0	-	8	-	32	-	128
		-	0	-	0	-	0	-	0	-	8	-	16	-	32
		-	0	-	0	-	0	-	0	-	16	-	32	-	32
		-	0	-	0	-	0	+	0	-	0	+	0	+	0
		-	0	-	0	-	0	-	8	-	8	-	8	-	16
		-	0	-	0	-	0	-	8	-	0	-	8	-	16
C2	F422 (10)	-	0	-	0	-	0	-	0	-	0	-	8	-	8
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	-	8	-	8	-	8	-	8
		-	0	-	0	-	0	-	8	+	0	-	16	-	64
		-	0	-	0	-	0	-	8	-	16	-	16	-	64
		-	0	-	0	-	0	-	0	-	16	+	0	D	D
C3	FeLV-A (10)	-	0	-	0	-	0	+	0	-	0	+	0	+	0
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	-	0	-	128	-	64	-	64
		-	0	-	0	-	0	-	0	-	0	+	0	+	0
		-	0	-	0	-	0	+	0	-	0	+	0	+	0
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
C4	Control	-	0	-	0	-	0	+	0	+	0	+	0	+	0
	Fluid	-	0	-	0	-	0	+	0	D	D	D	D	D	D
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	+	0	-	0	+	0	+	0
		-	0	-	0	-	0	+	0	D	D	D	D	D	D
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
C5	Unvaccinated	-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	+	0	+	0	+	0	+	0
		-	0	-	0	-	0	+	0	+	0	+	0	+	0

* V = virus isolated from plasma
A = Titre of virus neutralising antibody
0 = <8

a transient viraemia which was detected six weeks after challenge but not at 12 or 18 weeks. As in the first group, all of the non-viraemic cats developed virus neutralising antibodies.

The response of the third vaccinated group (C3) to challenge was much poorer. Five of the six cats became persistently viraemic. Only one (cat 3) resisted the challenge and produced virus neutralising antibodies.

Three cats developed disease during this experiment and had to be destroyed. These were cat 6 in group C2 and cats 2 and 5 in group C4. All of these cats were viraemic at the time of death. All were suffering from a haemorrhagic colitis with haemorrhagic mesenteric lymph nodes. Large numbers of B-haemolytic E. coli were isolated from the colonic contents.

The results of using the scoring system as in Chapters 4 and 7 are shown in Table 8.2, from which it is clear that the F422 (X1) vaccine ranked first in efficacy of all the vaccines tried in this experiment. The cats given the control FEA culture fluids vaccine gave essentially the same response as the unvaccinated cats, and the response of the FeLV-A/Glasgow-1 vaccinated cats was only marginally better.

Establishment of bone marrow cultures

The latency condition of FeLV in the first group (C1) of this vaccination experiment was determined. The fluids of the cultures established from bone marrow aspirates were tested for infectious FeLV using the two-stage virus isolation procedure. The results are shown in Table 8.3. Only cat 4 was positive for virus in the fluid of the aspirated bone marrow samples while the

TABLE 8.2

Efficacy scores in vaccination experiment C

Vaccine Group No.	Vaccine	Virus Score* A	B	C	Total	Antibody Score* A	B	C	Total	Total Score	Rank
C1	F422 (1)	16	0 0	48	64	201	66	16	283	347	1
C2	F422 (10)	16	66	60	142	150	100	20	270	412	2
C3	FeLV-A (10)	67	66	249	382	300	166	83	549	931	3
C4	Control fluid	100	150	300	550	300	200	100	600	1150	4
C5	Unvaccinated	100	200	300	600	300	200	100	600	1200	5

* A = 3 weeks

B = 6 weeks

C = 18 weeks

TABLE 8.3

Latent FeLV in bone marrow cultures of vaccinated cats

Cat No.	Viraemia	FeLV in BMSF *	Marrow culture	FeLV isolated (weeks) 1	2	3
1	-	-	A	+	+	+
			B	+	+	+
			NA	+	+	+
			NB	+	+	+
2	-	-	A	+	+	+
			B	+	+	+
			NA	+	+	+
			NB	+	+	+
3	-	-	A	-	-	-
			NA	-	-	-
4	+	+	A	+	+	+
			NA	+	+	+
5	-	-	A	+	+	+
			B	-	-	-
			NA	+	+	+
			NB	-	+	+
6	-	-	A	+	+	+
			B	+	+	+
			NA	+	+	+
			NB	+	+	+

* BMSF = Bone marrow supernatant fluid. The medium into which the bone marrow aspirate was taken was tested for infectious FeLV.

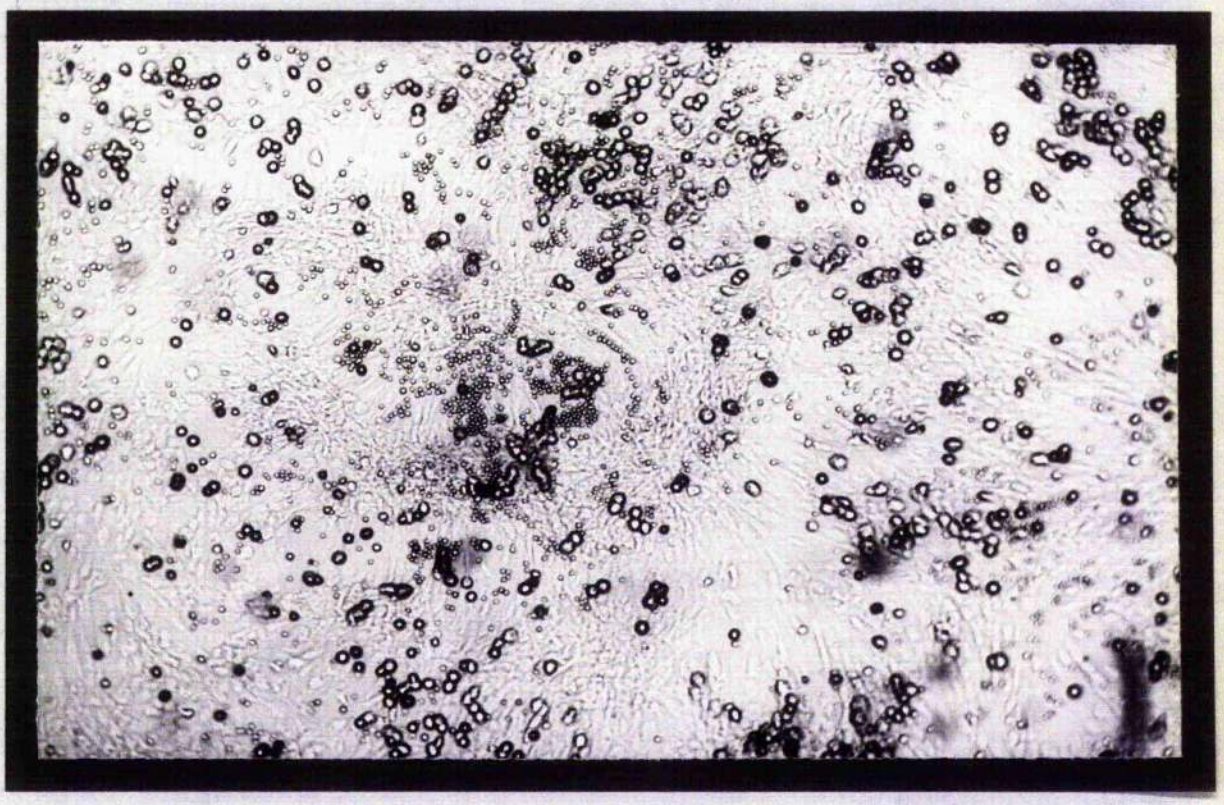
other five cats of the group were negative. These results were expected from the results of virus isolation from the plasma samples collected 18 weeks after challenge. The other results of FeLV isolation from the cell culture fluids collected one week after seeding of the bone marrow were that cat 3 was negative for the virus while the others were positive. Samples of fluids were tested 2 and 3 weeks after establishment of the cultures and the same results were obtained.

There was no difference in the results whether or not the bone marrow cells had been treated with NH_4Cl to remove red blood cells. A typical bone marrow culture, 3 weeks after seeding, is shown in Fig. 8.1.

Discussion

The plan of this experiment on FeLV vaccination was decided upon in the light of the results of the two previous experiments. There were two main aims. The first aim was to find whether or not a greater degree of protection was achieved by using a greater antigenic mass of F422 virus. A vaccine containing 10 times concentrated virus was compared with one prepared from the equivalent of unconcentrated virus. The results obtained were that there was a good response to vaccination with either vaccine. Five of six cats vaccinated with the unconcentrated virus vaccine were non-viraemic 12 and 18 weeks after challenge. Approximately similar results were obtained following vaccination with the concentrated virus vaccine. In addition, good virus neutralising antibody titres developed in these cats, ranging from 8 to 32 and from 16 to 128 at 12 and 18 weeks respectively

Fig. 8.1. Feline bone marrow culture



This culture was established 3 weeks previously and contains fibroblasts, large macrophages and small round myeloid cells (X 125)

after challenge. It appeared that there was little difference between the results of vaccination. with F422 (X1) or (X10) vaccines. A similar result was found in the experiment described in Chapter 4. The reason why increasing the antigenic mass of the vaccine had no effect on the immunising capacity of the inactivated virus is not clear. In other systems, the immune response to vaccines is dependent on the quantity of antigen in the vaccine. Thus, in potency tests of rabies vaccine, Romanova (1981) showed that by reducing the antigen content of vaccine by five-fold, there was a corresponding decrease in the antibody response of inoculated mice and in the protection to challenge with virulent virus. An example of the effect of vaccine concentration on the extent of the immune response to a retrovirus vaccine was the experiment of Charney et al (1976) in which mice were vaccinated with serial doses of formalin-inactivated murine mammary tumour virus (MMTV) in either complete or incomplete Freund's adjuvant. The result was that there was significant protection against challenge with live MMTV in mice which received 1 ug of viral antigen, less in mice given 0.1 ug and no protection in mice vaccinated with 0.01 ug or less.

It would be interesting to know if by decreasing the antigenic mass of the present FeLV vaccine, the potency of the vaccine would be decreased or increased. If a plateau had been reached in the relationship between antigenic mass of FeLV and the immune response, one would expect that a reduction in antigen quantity would lead to a reduction in protection. However, if there was an inhibitory factor in the concentrated vaccine (e.g.

p15E), it might be expected that dilution of the vaccine might give increased protection.

The level of protection in the present vaccination experiment was 83%, while in the experiment of Pedersen et al (1979) it was 100% (0/6) with ICFA and 92% (1/13) with $Al(OH)_3$. It should be noted, however, that in Pedersen's vaccination experiments the cats were vaccinated when 16 weeks old and challenged when 20 weeks old by exposure to persistently viraemic carrier cats. Only 10/25 (40%) of the unvaccinated control cats became viraemic following challenge so it is difficult to compare those results with the results in the present study. The level of protection in the present study was similar to that achieved by Olsen et al(1980a) which was more than 80%. A second aim in this study was to compare the protection achieved by F422 virus and FeLV-A/Glasgow-1 virus from FEA cells.

The FeLV-A/Glasgow-1 vaccine was not successful. It had been expected that the gp70 antigen in FeLV-A might be more immunogenic than the F422 virus. In this experiment an attempt was made to equalise the amount of virus in each preparation but since neither virus was purified, it was not possible to measure the protein content of each virus to ensure that each contained the same quantity. It was assumed, based on previous experience, that the F422 virus would contain about ten times the amount of viral protein of FeLV-A/Glasgow-1. The reasons for failure of FeLV-A/Glasgow-1 as an immunogen might be due to a loss of antigen during concentration or to a more deleterious effect of paraformaldehyde on the viral antigen, compared to F422 virus. The vaccine made from FEA culture fluid did not protect cats.

This result indicated that it is not a non-specific effect of serum proteins or cellular antigens which protects vaccinated cats against FeLV challenge.

An additional study in this experiment was to determine whether or not cats which had been vaccinated and resisted challenge eliminated the challenge virus or whether the virus was latent in their bone marrow cells. It was shown recently that cats recovered from natural or experimental FeLV exposure may be latently infected (Post and Warren, 1980; Rojko et al, 1982; Madewell and Jarrett, 1983). The cats vaccinated with the F422 (X1) vaccine were tested for latent virus and it was found that of the five cats which resisted challenge, four had bone marrow cells which released FeLV after 7 days in culture. This result indicates that in most cats the challenge virus was not prevented by vaccination from reaching the bone marrow and setting up an infection. However, since no virus was found in the blood of the five cats which resisted challenge, there was a rapid development of an immune response which prevented further spread of the virus and restricted the virus to the latent state in the bone marrow.

CHAPTER 9

ANTIGENICITY OF FeLV EXPOSED TO CHEMICAL INACTIVATING AGENTS

Introduction

It was considered that it would be useful in formulating FeLV vaccines to have an *in vitro* method to test the antigenicity of FeLV antigens which had been treated with the chemical inactivating agents used to prepare vaccines in this study. It was believed that treatment of virus with agents like paraformaldehyde might result in some loss of antigenicity and that it would not be possible for reasons of time and cost to test this in cats in order to find an optimum concentration of the agent to use in vaccine preparation. When this study began, the method which seemed most appropriate in testing antigenicity was one which used virus neutralisation since the neutralisation reaction involves the antigens which are thought to be important in immunity to FeLV. Attempts were made to develop a neutralisation inhibition test in which chemically treated FeLV antigen might be used to inhibit the antigen-antibody reaction measured by neutralisation. Similar tests to quantitate FeLV cellular antigens were described previously (Russell and Jarrett, 1978a). For reasons discussed below, the present experiments were not successful. However, during the course of this study, several mouse monoclonal antibodies became available which neutralised FeLV. Therefore they recognised FeLV epitopes which were involved in neutralisation. These antibodies were tested and found to bind to FeLV in an enzyme-linked immunosorbent assay (ELISA) and could, therefore, be used to assess the antigenicity

of FeLV determinants relevant to vaccination after exposure to chemical inactivating agents. This Chapter outlines attempts to setup a neutralisation inhibition test and then describes experiments using mouse antibodies and an ELISA to test the antigenicity of FeLVs from F422 and FL74 cells which were treated with varying concentrations of paraformaldehyde, formaldehyde, AEI and beta-propriolactone (BPL).

Materials and Methods

Neutralisation Inhibition

Three FeLV antigens were used in an attempt to inhibit neutralisation of MSV (FeLV-A): F422 cells, F422 culture fluid, and combined cells and fluid. These were prepared by seeding F422 cells at a density of 1.2 X 10^6/ml in 30 ml L-M medium containing 15% FCS in 75 cm^2 plastic flasks (Nunc) and harvesting these cells 48 hours later. The culture fluid was spun at 1000 rpm for 15 minutes and the cells were resuspended in L-15 medium, spun again and resuspended in L-15 medium with 10% FCS at a cells concentration of 5 X 10^6/ml.

The antigens were diluted with L-15 medium containing 10% FCS to give dilutions of 4, 8, 16, 32, 64 and 128 in 96-well U-bottomed plastic plates. To all dilutions an equal volume (25 ul) of cat FeLV immune serum diluted 1:4 in L-15 medium with 10% FCS was added. The controls consisted of 25 ul of diluent with 25 ul of serum as the serum control and 50 ul of diluent as the virus control. The plate was incubated at 37° for 2 hours to allow any antigen-antibody reaction to take place and then 50 ul of MSV (FeLV-A) diluted in L-15 medium with 10% FCS to give 20-30

foci in the test was added to each well. The plates were shaken, incubated at 37° for 1 hour and then 25 ul of each reaction was inoculated into one well of a 24-well plastic plate which had been seeded 24 hours in advance with FeLV-B-infected FEA cells. The remainder of the test was carried out as described for the neutralisation test in Chapter 1. The same experiment was repeated using the same procedure except that instead of incubation of the antigen-antibody reaction at 37° for 2 hours, this reaction was incubated at 4° for 16 hours.

ELISA inhibition

1. Preparation of virus

The concentrated and purified FeLV used in this test were FeLV-A/F422 and FeLV-ABC/FL74 which were prepared as follows. One litre of FL74 cell culture fluid was prepared by seeding FL74 cells at 1.2 X 10^6/ml in L-M medium and harvesting 48 hours later. The fluid was centrifuged at 7,000 rpm for 30 minutes and the supernatent was frozen at -70°. It was thawed and concentrated in an Amicon CH4 hollow fibre concentrator at 4° to 100 ml which was then spun at 20,000 rpm for 2 hours in the SW27 Rotor. The supernatent fluid was discarded while the virus pellet was resuspended in 30 ml of PBS. This suspension was centrifuged in 3 potassium tartrate gradients (1.08-1.23 $g.cm^{-3}$) in the SW41 Rotor at 40,000 rpm for 1 hour. The virus bands were recovered, combined and diluted to 10 ml in PBS and spun again in the SW50.1 rotor at 30,000 rpm for 2 hours. The pelleted virus was resuspended in 2 ml of 0.15 M NaCl and stored at 4° before use. The final concentration of this virus was approximately

500-fold.

The FeLV/F422 virus was concentrated and purified in the same way.

2. ELISA test

The coating buffer was 0.05 M Na_2CO_3: $NaHCO_3$, pH 9.6.

The composition of solutions A, B, C and D was as shown in Table 9.1.

A Hybridoma Screening Kit was purchased from Bethesda Research Laboratories Ltd. which contained sheep anti-mouse IgG $(Fab')_2$ conjugated with B-galactosidase (Hybridoma Screening Reagent), and p-nitrophenol-B-D-galactoside as a substrate.

3. Antibodies

Two antibodies were used.

a. Mouse monoclonal antibody to FeLV-A

A monoclonal antibody which neutralised MSV (FeLV-A/Glasgow-1) (O. Jarrett, personal communication) was obtained from C.K. Grant, Harvard School of Public Health, Boston, U.S.A. This antibody is designated I9B4

b. Mouse monoclonal antibody to FeLV-B and FeLV-C

A monoclonal antibody which neutralised MSV (FeLV-B/Sarma) and MSV (FeLV-C/Sarma) (O. Jarrett, personal communication) was obtained from H. Lutz, Veterinary School, Zurich, Switzerland. This antibody is designated B9B12. The antibody recognises the major antigenic determinant of the virus produced by FL74 cells described by Russell and Jarrett (1978a). Further characteristics of this antibody have been described by Vedbrat

TABLE 9.1

Solutions used in ELISA test

Solution A	0.01 M PBS, pH 7,2
	1.5 mM $MgCl_2$
	2.0 mM 2-mercaptoethanol
	0.05% Tween 20
Solution B	0.01 M PBS, pH 7.2
	1.5 mM $MgCl_2$
	2.0 mM 2-mercaptoethanol
Solution C	50 mM Na_2PO_4 buffer, pH 7.2
	1.5 mM $MgCl_2$
Solution D	0.5 M $NaCO_3$

et al (1983).

4. Optimum conditions for the ELISA test

The first procedure was to establish the optimum dilutions of both antigen and antibody. Doubling dilutions of antigen from 1:10 to 1:640 were made in coating buffer. A volume of 50 ul of each dilution was placed in each of the seven horizontal wells of a 96-well Linbro enzyme immunoassay (EIA) plate. A volume of 50 ul of coating buffer was put into each of the last six wells of the top horizontal row as an antigen control. The plate was incubated overnight at 4°.

Twenty four hours later the plate was washed 4 times with solution A and 50 ul of four-fold dilutions of antibody was dispensed into the vertical wells starting from the second row at the left side. These dilutions were from 1:20 to 1:1280 made by using solution A as diluent. A volume of 50 ul of solution A was dispensed in each well of the first vertical row as an antibody control. The plate was then incubated at room temperature for one hour to allow binding of antibody to antigen.

The plate was washed 4 times with solution A and 50 ul of a 1:200 dilution of Hybridoma Screening Reagent was dispensed into all the wells. The plate was incubated at room temperature with constant shaking for 2 hours. The plate was then washed 4 times with solution A and 50 ul p-nitrophenyl, prepared in solution C at a final concentration of 1 mg/ml and 100 ul of 2-mercaptoethanol, was added to all the wells. The plate was again incubated at room temperature for one hour with constant shaking. Finally 50 ul of solution D was added to all the wells to stop

the reaction and the optical density (OD) of each well was read in a Micro-ELISA Autoreader MR580 (Dynatech) at a wavelength of 410 nm. The optimum dilutions of antigen and antibody were taken as those which produced an optical density of approximately 0.4-1.0.

5. Inactivating agents

The chemicals used were Pf, formaldehyde (BDH), AEI and beta-propiolactone (BPL) (Sigma).

6. The exposure of antigen to chemical inactivating agents

The second procedure was to establish the effects of chemical inactivating agents on the capacity of immobilised antigen to bind antibody. The steps of this procedure were as follows.

A volume of 50 ul of coating buffer containing the optimum antigen dilution established as above was dispensed in all 96-wells of an EIA plate, except in the top left hand well in which 50 ul of coating buffer was dispensed as a blank. The plate was incubated overnight at 4^{o}. The wells were then washed 4 times with solution A and the chemical inactivating agents were dispensed. Eleven doubling dilutions in PBS starting with 2% were made and dispensed in the horizontal wells. The left hand wells of each row were kept as antibody controls. In one plate horizontal rows 1-4 contained dilutions of one chemical and rows 5-8 contained dilutions of a second. The plates were incubated at room temperature.

After 24 hours the plates were washed 10 times with solution A to remove any traces of the chemicals and then 50 ul of the

optimum dilution of the appropriate monoclonal antibody in solution A was added to all the wells in horizontal rows 1, 2, 5 and 6 while 50 ul of solution A was added to the wells in rows 3, 4, 7 and 8 as an antigen control. The plates were incubated at room temperature for 1 hour and were then washed 4 times with solution A.

The remainder of the test was then carried out as described above.

Results

Neutralisation inhibition test

Results of a characteristic neutralisation inhibition test are shown in Table 9.2. In this initial experiment live F422 cells and/or virus were used as it was expected that untreated antigen would be most likely to produce inhibition of neutralisation by binding to antibody. It is clear, however, from the results in Table 9.2 that very little inhibition occurred and then only in the reaction using F422 cells. Because there appeared to be no effect with unconcentrated F422 culture fluid, which was the source of vaccine in these studies, the test was not considered to be useful.

ELISA inhibition

Effect of inactivating agents on FeLV-A/F422

The results are shown in Tables 9.3 - 9.6. Each reaction was done in duplicate and the mean of the optical density at 410 nm was calculated. The mean of each of the control reactions

TABLE 9.2

Neutralisation inhibition test

Conditions of incubation	Antigen	Foci at these dilutions:[a] 4	8	16	32	64	128
37° for 2 hours	F422 cells	20	15	11	7	6	5
	F422 fluid	11	13	13	13	11	9
	F422 cells and fluid	14	15	14	9	8	10
4° for 16 hours	F422 cells	12	12	14	14	8	11
	F422 fluid	12	14	16	16	8	13
	F422 cells and fluid	14	15	11	15	13	13

a. In Experiment 1 the no. of foci in the virus control wells were 36, 37 and 38 (mean 37) and in the serum control wells, 9, 10 and 11 (mean 10).

In Experiment 2 the no. of foci in the virus control wells were 33, 35 and 37 (mean 35) and in the serum control wells, 12, 12 and 12 (mean 12).

TABLE 9.3

Effect of Paraformaldehyde on Antigenicity of FeLV-A/F422

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1	0.373	0.049	0.086	0.153	0.187	0.214	0.273	0.272	0.309	0.367	0.404	0.421
Result-2	0.343	0.024	0.051	0.102	0.139	0.168	0.292	0.225	0.293	0.302	0.407	0.373
Average	0.358	0.036	0.067	0.238	0.163	0.191	0.258	0.248	0.301	0.335	0.406	0.397
Control-1	-0.041	-0.053	-0.055	-0.054	-0.059	-0.058	-0.046	-0.046	-0.049	-0.051	-0.043	-0.038
Control-2	-0.039	-0.058	-0.059	-0.054	-0.041	-0.054	-0.055	-0.055	-0.052	-0.049	-0.041	-0.043
Average	-0.040	-0.055	-0.057	-0.054	-0.050	-0.056	-0.050	-0.050	-0.050	-0.050	-0.042	-0.040
Difference	0.398	0.091	0.124	0.182	0.213	0.247	0.308	0.298	0.351	0.385	0.448	0.437
% of control	100.0	22.9	31.2	45.7	53.5	62.0	77.4	74.9	88.2	96.7	112.6	109.8

TABLE 9.4

Effect of Formaldehyde on Antigenicity of FeLV-A/F422

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1	-	0.186	0.096	0.099	0.151	0.203	0.225	0.255	0.335	0.462	0.541	0.521
Result-2	0.337	0.172	0.078	0.112	0.179	0.207	0.282	0.325	0.360	0.457	0.397	0.437
Average	0.337	0.179	0.087	0.106	0.165	0.205	0.253	0.290	0.347	0.460	0.469	0.479
Control-1	-0.011	-0.012	-0.053	-0.059	-0.031	-0.046	-0.054	-0.048	-0.027	-0.039	-0.042	-0.006
Control-2	-0.022	-0.032	-0.055	-0.055	-0.053	-0.056	-0.023	-0.048	-0.051	-0.042	-0.044	-0.033
Average	-0.017	-0.022	-0.054	-0.057	-0.042	-0.051	-0.038	-0.048	-0.039	-0.041	-0.043	-0.019
Difference	0.354	0.201	0.141	0.163	0.207	0.256	0.291	0.338	0.386	0.501	0.512	0.493
% of Control	100.0	56.8	39.8	46.0	58.5	72.3	82.2	95.5	109.0	141.5	144.6	139.3

TABLE 9.5

Effect of AEI on Antigenicity of FeLV-A/F422

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1		0.527	0.516	0.460	0.436	0.375	0.385	0.389	0.372	0.349	0.373	0.308
Result-2	0.437	0.327	0.357	0.443	0.378	0.332	0.343	0.375	0.346	0.262	0.271	0.252
Average	0.437	0.427	0.436	0.451	0.407	0.353	0.364	0.382	0.359	0.305	0.322	0.280
Control-1	-0.023	-0.004	-0.028	-0.033	-0.037	-0.037	-0.033	-0.036	-0.030	-0.033	-0.029	-0.024
Control-2	-0.016	-0.010	-0.015	-0.037	-0.013	-0.014	-0.037	-0.034	-0.033	-0.027	-0.024	-0.023
Average	-0.020	-0.003	-0.021	-0.035	-0.025	-0.025	-0.035	-0.035	-0.031	-0.030	-0.026	-0.023
Difference	0.457	0.430	0.457	0.486	0.432	0.378	0.399	0.417	0.390	0.335	0.348	0.303
% of Control	100.0	94.0	100.0	106.3	94.5	82.7	87.3	91.2	85.3	73.3	76.1	66.3

TABLE 9.6

Effect of BPL on Antigenicity of FeLV-A/F422

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1	0.368	0.258	0.286	0.331	0.440	0.297	0.172	0.158	0.174	0.222	0.198	0.197
Result-2	0.353	0.261	0.196	0.303	0.290	0.306	0.160	0.146	0.201	0.233	0.278	0.217
Average	0.360	0.260	0.241	0.317	0.365	0.301	0.166	0.152	0.188	0.228	0.238	0.207
Control-1	-0.023	-0.045	-0.045	-0.045	-0.045	-0.040	-0.039	-0.036	-0.035	-0.036	-0.036	-0.027
Control-2	-0.023	-0.040	-0.039	-0.043	-0.041	-0.041	-0.032	-0.034	-0.027	-0.003	-0.025	-0.026
Average	-0.023	-0.042	-0.042	-0.044	-0.043	-0.040	-0.035	-0.035	-0.031	-0.020	-0.030	-0.026
Difference	0.383	0.302	0.283	0.361	0.408	0.341	0.201	0.187	0.219	0.248	0.268	0.233
% of Control	100	78.9	73.9	94.3	106.5	89.0	52.5	48.8	57.2	64.8	70.0	60.8

(without antibody) was subtracted from the mean of the corresponding test reactions and the difference was then expressed as a percentage of the value for the untreated control (no inactivating agent). As shown in the Tables, the variation between duplicates was relatively small and there was little difference between the values among all of the antigen-free controls indicating that the inactivating agent did not produce non-specific binding of antibody to the wells.

As shown in Table 9.3, high concentrations of PF inhibited the binding of antibody to the antigen-coated wells. At a concentration of 2% the binding was only 23% of the untreated control. As the concentration of PF decreased, the extent of binding increased steadily until at concentrations of 0.003% and 0.001% the binding was slightly greater than in the controls (113% and 110% respectively).

A similar result was obtained with formaldehyde as shown in Table 9.4. In this case, the inhibition of binding at 2% formaldehyde was less than with PF (57% as against 23%). A level of binding equal to that in the untreated control was found at formaldehyde concentrations between 0.03 and 0.015% and the peak binding of 145% of the control was again at 0.003%.

The results using AEI and BPL are shown in Tables 9.5 and 9.6. The pattern observed with these agents was quite different from that using PF or formaldehyde. The range of differences in binding of antibody between concentrations of these agents was not so marked as with PF or formaldehyde, ranging from approximately 60% to 106% of the level of the untreated control.

High concentrations did not inhibit binding as with PF or formaldehyde.

Effect of inactivating agents on FeLV-ABC/FL74

Similar experiments to those described above for F422 virus were done using purified FL74 virus and a monoclonal antibody which neutralised that virus. The results are shown in Tables 9.7 - 9.10.

Using PF it is seen from Table 9.7 that, as in the previous experiment using F422 virus, the binding of antibody to the antigen was severely inhibited at high concentrations. At 2% concentration, binding was only 10% of the control. As the PF was diluted, the binding increased until the control level was reached at 0.06%. The peak of binding was at 0.03% at which concentration binding was 133% of the control, and with further dilution the level of binding fell off.

The effect of formaldehyde is shown in Table 9.8. As in the previous experiment with F422 virus, the result was very similar to that obtained with PF. Also the damaging effect on the virus appeared to be less than with PF. A peak of binding at 165% of the control was found at 0.015% and, as with PF, the level of binding fell off to 106% at a formaldehyde concentration of 0.001%.

The results with AEI and BPL were again quite different from those obtained with PF or formaldehyde. There was little variation in the extent of binding at all concentrations. With AEI, the level was between 98% and 135% of the control and with BPL was between 72% and 92% of the control. There was no

TABLE 9.7

Effect of Paraformaldehyde on Antigenicity of FeLV-ABC/FL74

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1	1.050	0.257	0.311	0.494	0.642	0.848	1.072	1.354	1.131	0.960	0.466	0.684
Result-2	0.873	0.290	0.328	0.461	0.648	0.863	1.031	1.234	1.098	0.919	0.656	0.758
Average	0.961	0.273	0.320	0.477	0.645	0.855	1.051	1.294	1.115	0.940	0.561	0.726
Control-1	0.075	0.166	0.067	0.084	0.142	0.088	0.117	0.059	0.092	0.053	0.065	0.135
Control-2	0.046	0.193	0.225	0.259	0.218	0.167	0.203	0.121	0.104	0.091	0.102	0.034
Average	0.060	0.180	0.146	0.171	0.180	0.127	0.160	0.090	0.098	0.072	0.083	0.084
Difference	0.901	0.093	0.174	0.306	0.465	0.728	0.891	1.204	1.017	0.868	0.478	0.642
% of Control	100.0	10.3	19.3	34.0	51.6	80.8	98.9	133.6	112.9	96.3	53.0	71.2

TABLE 9.8

Effect of Formaldehyde on Antigenicity of FeLV-ABC/FL74

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1	-	0.731	0.316	0.450	0.705	0.827	1.111	1.327	1.500	1.489	1.309	0.895
Result-2	0.935	0.653	0.317	0.523	0.718	0.849	1.072	1.354	1.416	1.327	1.309	1.024
Average	0.935	0.692	0.317	0.486	0.711	0.838	1.091	1.340	1.458	1.408	1.309	0.960
Control-1	0.093	0.081	0.051	0.037	0.039	0.031	0.138	0.048	0.053	0.043	0.000	0.084
Control-2	0.061	0.095	0.061	0.057	0.113	0;017	0.046	0.034	0.023	0.058	0.017	0.024
Average	0.077	0.088	0.058	0.047	0.076	0.024	0.092	0.041	0.038	0.050	0.008	0.054
Difference	0.858	0.604	0,259	0.439	0.635	0.814	0.999	1.299	1.420	1.358	1.300	0.906
% of Control	100	70.4	30.2	51.1	74.0	94.8	116.4	151.4	165.5	158.3	151.5	105.6

TABLE 9.9

Effect of AEI on Antigenicity of FeLV-ABC/FL74

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1	-	0.670	0.921	0.854	0.750	0.729	0.811	0.787	0.783	0.830	0.919	0.724
Result-2	0.713	0.668	0.830	0.746	0.694	0.621	0.657	0.683	0.770	0.705	0.662	0.746
Average	0.713	0.669	0.875	0.800	0.722	0.675	0.734	0.735	0.776	0.767	0.790	0.735
Control-1	0.082	0.097	0.069	0.031	0.030	0.069	0.045	0.102	0.028	0.025	0.020	0.057
Control-2	0.135	0.057	0.048	0.076	0.045	0.034	0.060	0.040	0.027	0.052	0.042	0.044
Average	0.108	0.077	0.058	0.053	0.037	0.051	0.053	0.071	0.028	0.038	0.031	0.050
Difference	0.605	0.592	0.817	0.747	0.685	0.624	0.681	0.664	0.748	0.729	0.759	0.685
% of Control	100.0	97.9	135.0	123.5	113.2	103.1	112.6	109.8	123.6	120.5	125.5	113.2

TABLE 9.10

Effect of BPL on Antigenicity of FeLV-ABC/FL74

Sample	A_{410} at these concentrations											
	None	2	1	0.5	0.25	0.125	0.060	0.030	0.015	0.007	0.003	0.001
Result-1	0.709	0.651	0.610	0.705	0.660	0.627	0.604	0.579	0.557	0.524	0.619	0.547
Result-2	0.704	0.623	0.562	0.692	0.589	0.578	0.513	0.521	0.501	0.508	0.557	0.563
Average	0.707	0.637	0.586	0.698	0.624	0.602	0.558	0.550	0.529	0.516	0.588	0.555
Control-1	0.037	0.010	0.003	0.045	0.009	0.031	0.017	0.089	0.034	0.056	0.026	0.008
Control-2	0.034	0.029	0.101	0.090	0.035	0.060	0.054	0.052	0.049	0.065	0.051	0.021
Average	0.036	0.020	0.049	0.067	0.022	0.045	0.035	0.070	0.042	0.060	0.038	0.015
Difference	0.671	0.617	0.537	0.631	0.602	0.557	0.523	0.480	0.487	0.456	0.550	0.540
% of Control	100.0	91.6	80.0	94.0	89.7	83.0	77.9	71.5	72.6	68.0	82.0	80.5

distinct peak as with PF and formaldehyde.

DISCUSSION

This chapter describes experiments to assess the effects of four chemical inactivating agents on the FeLV antigen involved in neutralisation. It was hoped that an assay could be developed which might determine the concentrations of an inactivating agent which would be least damaging to the antigen. These experiments were initiated with a neutralisation inhibition test in which F422 cells and its released FeLV-A were used but the experiment was not successful, because it became obvious that large amounts of antigen would be required in order to inhibit the neutralisation reaction. This test was not considered technically feasible without concentration of virus and was not therefore not continued.

Another opportunity to carry out this study occurred later when monoclonal antibodies which neutralised FeLV became available during the course of this work. In the second and third experiments, these were used with FeLV-A/F422 and FeLV-ABC/FL74. These antibodies were obviously directed against the antigens which were involved in neutralisation reactions to the virus and which were presumably also involved in immunity. Assays were developed using the ELISA technique which assessed antigenicity by measuring the binding of the antibodies to the immobilised, chemically-treated antigens. This test was successful and interesting results were obtained which indicated that the effect of PF and formaldehyde were quite different from

the effects of AEI and BPL and that at some concentrations, PF and formaldehyde enhanced the binding of antibody to antigen.

In experiments with F422 or FL74 virus, antigen treated with PF or formaldehyde, there was reduced binding at high concentration of the inactivating agent. This reduction was attributed to the damaging effects of the inactivants. A similar effect was observed previously when one-half of the antigenicity of the glycoprotein of bovine leukaemia virus was lost following treatment with 0.05% formaldehyde while treatment with AEI had no effect (Miller and van der Maaten, 1978). The effect of PF and formaldehyde is probably due to cross-linking of adjacent glycoproteins and destruction of the antibody binding sites. In this study it is assumed that inhibition is caused by a direct effect of the agent on the antigen bound to the plastic. An alternative explanation is that the PF or formaldehyde removed the antigen from the plastic, so that there was a reduced amount of antigen left for binding to the antibody. While it is not possible to eliminate or control for this possibility, it is considered unlikely. Indeed, it is more likely that these fixatives would bind the antigen more closely to the plastic.

With dilution of PF or formaldehyde, there was a reduction in inhibition until a level of binding was observed which was greater than that of the untreated antigen. The concentration of PF which gave binding equal to the control was 0.007-0.003% for F422 virus and 0.06-0.03% for FL74 virus. For formaldehyde these concentrations were 0.03-0.015% for F422 virus and 0.125-0.06% for FL74 virus. Hence, formaldehyde was less inhibiting than paraformaldehyde at the same concentrations.

With further dilution, enhancement of antibody binding was seen with both PF and formaldehyde. The concentrations which produced greatest binding were slightly different for PF and formaldehyde, and for F422 virus and FL74 virus. In the case of FL74 virus there was a very marked peak of binding at 0.03% PF and 0.015% of formaldehyde. The reasons for enhancement are unclear. There may be cross-linking within the glycoprotein which preserves the antigenic determinant from degradation as is seen with some strains of foot-and-mouth disease virus (Rowlands et al, 1972).

There was very little damaging effect of AEI or BPL on the antigenicityof either F422 or FL74 virus. However, neither was there a clear enhancing effect of these agents. This difference may be due to the different mode of action of AEI and BPL compared to the aldehydes. These are more reactive with virus nucleic acid than with viral proteins (Wild and Brown, 1968) and might not be expected to damage antibody binding sites.

The relevance of these results to vaccine production is that they may give an indication of the antigenicity of virus following chemical inactivation. The results may therefore act as a guide in the choice of the concentration of inactivating agent that should be used, assuming that this concentration inactivates the virus. It should be remembered, however, that the antibodies which were used were mouse monoclonal antibodies and the reaction of these antibodies with FeLV might be different from that of cat antibodies, although both of the mouse antibodies neutralised FeLV. A second point is that antigenicity

is not necessarily the same as immunogenicity and without testing the response of animals to virus treated with different concentrations of each inactivating agent it is not possible to answer this question. It would be useful to study this question in future. It is interesting, however, that the concentration of PF which was used in the successful vaccine experiments (0.02%) was similar to that which caused least damage to the FeLV antigen in the ELISA inhibition test, and in some cases gave an increase in antigenicity.

CHAPTER 10

PERSISTENCE OF FeLV NEUTRALISING ANTIBODIES IN CATS

Introduction

Once a vaccine is produced which protects against virus challenge following a primary course of vaccination it is desirable to determine how long immunity lasts after vaccination in order to know if and when a booster dose of vaccine might be required. It was the intention in this study to carry out such an investigation. As a comparison to artificially produced immunity it was also considered valuable to attempt to find how long immunity lasted following recovery from natural exposure to FeLV. In the event it was not possible for reasons of time to complete the first part of the proposed study but the second part was begun.

It was considered that the presence of virus neutralising antibodies in the serum was a good indicator of the immune status of cats to FeLV since it has been shown that maternal antibodies protect against FeLV challenge (Hoover et al, 1977a; Jarrett et al, 1977) and that cats which have neutralising antibodies following recovery from natural or experimental infection and are then housed together with viraemic cats do not become viraemic (Jarrett et al, 1982a. However, there is very little existing information about the persistence of antibodies to FeLV. Both Hoover et al (1977a)and Jarrett et al (1977) studied maternal antibodies in kittens of FeLV-immune queens and showed that the half-life of virus neutralising antibodies was approximately 14 days. There is, however, no published data on persistence of

antibodies after natural infection in the absence of continuing exposure to virus-excreting cats.

The plan of the present experiment was to expose a group of 16-week old cats to natural challenge with FeLV and to observe their response. It was expected that most of the cats would recover from the infection and develop neutralising antibodies (Hoover et al, 1976; Pedersen et al, 1979). When the pattern became established, half of the recovered cats would be housed with any cats which developed persistent viraemia and half would be housed separately. The cats would then be sampled at intervals to measure their levels of virus neutralising antibodies and the influence of the presence or absence of constant exposure to FeLV on the persistence of antibodies would be determined. During the course of the experiment the phenomenon of FeLV latency was discovered (Post and Warren, 1980; Rojko et al, 1982) and latently infected cats were subsequently found in the cats in the present experiment by Madewell and Jarrett (1983).

Materials and Methods

Four, 8-week old cats were infected by intraperitoneal inoculation of 1 ml FeLV containing 1.8×10^5 FIU. These cats became persistently viraemic and when 6 months old were used as donors. The cats were mixed with 19, 16 week-old kittens which were free of FeLV and virus neutralising antibodies. All of the 23 cats were housed in a room with a floor area of 10 m^2. The 19 recipient cats were sampled at 4, 16, 20, 22, 27, 35, 60, 70 and

88 weeks after exposure to the viraemic cats and were tested for viraemia and virus neutralising antibodies. Femoral bone marrow biopsies were taken at 20, 35, 60 and 88 weeks by B.R. Madewell or O. Jarrett and cultures were set up as described in Chapter 8. The culture fluids were tested for infectious FeLV. At 20 and 35 weeks after mixing, these cats were tested for viraemia and for the presence of latent FeLV in the bone marrow. In the light of these results the cats were distributed in three houses as follows. The cat numbers are shown in Table 10.1.

House 1 contained all 3 cats which were viraemic (cats 17, 18 and 19) together with cats 3 and 4 which were classed as having recovered from the infection, and cats 6, 13 and 16 which had a latent infection. These cats were kept in the original room.

There were no viraemic cats in Houses 2 or 3.

House 2 contained cats 1 and 2 which were recovered and cats 10 and 11 which were latently infected.

House 3 contained cats 5, 9, 14 and 15 which had latent infections. The area of Houses 2 and 3 was 4 m^2.

Results

The virus neutralising antibody titres of the cats over a period of 88 weeks from the time of first exposure to FeLV-infected donor cats are shown in Table 10.1. The cats have been placed in five classes depending on whether or not FeLV was isolated from their bone marrow and on the number of occasions on which virus was isolated from the bone marrow. Bone marrow

TABLE 10.1

Persistence of virus neutralising antibodies in cats exposed to FeLV

Class	Cat No.	Status[1]	Housing	VNA[2] at weeks 4	16	20	22	27	35	60	70	88	Weeks FeLV isolated from bone marrow
1	1	R	Isolated	0	0	2	8	16	2	8	4	2	-
	2	R	"	0	0	0	0	4	32	64	32	64	-
	3	R	Viraemic	0	4	0	2	4	8	4	8	8	-
	4	R	"	0	64	32	0	64	16	16	32	32	-
2	5	L	Isolated	0	0	32	128	16	32	32	64	32	20
	6	L	"	0	16		8	64	64	32	16	-	20
	7	L	Viraemic	0	0	0	2	16	64	32	32	64	35
	8	L	"	0	0	0	0	16	64	64	32	64	88
3	9	L	Isolated	0	4	16	8	16	64	16	32	64	20, 35
	10	L	"	0	0		8	16	8	8	8	16	20, 88
	11	L	"	0	0		32	64	128	32	32	64	35, 60
	12	L	Viraemic	0	4	32	8	64	32	16	8	64	20, 35
	13	L	"	0	0	32	8	64	128	128	64	64	20, 35
4	14	L	Isolated	0	0		8	16	8	8	8	2	20, 35, 60, 88
	15	L	"	0	4		32	16	64	16	64	64	20, 35, 60, 88
	16	L	Viraemic	0	64	32	128	64	8	32	64	64	20, 35, 60, 88
5	17	V	Viraemic	0	0	0	0	0	0	0	0	0	
	18		"	0	0	0	0	0	0	0	0	0	
	19		"	0	0	0	0	0	0	0	0	0	

1: Status R = recovered (non-viraemic; no virus isolated from bone marrow)
L = latent (non-viraemic; virus isolated from bone marrow)
V = viraemic (viraemic; virus isolated from bone marrow)

2: VNA = virus neutralising antibodies to FeLV-A/Glasgow-1

examination was done at weeks 20, 35, 60 and 88.

Class 1 consists of cats from which no virus was isolated in the cultures of bone marrow. These cats were considered to have recovered from the infection and their status in Table 10.1 is given as "R". Classes 2, 3 and 4 include cats from which virus was isolated in bone marrow cultures on 1, 2 or 4 occasions, respectively. All of these cats were considered to have a latent infection and were designated "L" in Table 10.1. The reason why virus was found on only one or two occasions in some cats may mean that at least some of these animals might have recovered from the latent infection and should be included in the "R" group. However, another reason might be that there is considerable sampling error in obtaining latently infected cells in the bone marrow biopsies, and that in fact all of these cats have a latent infection. Class 5 contains the three cats which developed a persistent viraemia. Although not shown in the Table, FeLV was isolated from bone marrow cultures of these cats on each occasion.

The housing of the cats is indicated in Table 10.1 by either "isolated" or "viraemic". "Isolated" means that the cats were housed with other recovered or latently-infected cats and were not in contact with viraemic cats so were not continuously exposed to exogenous FeLV. "Viraemic" means that the cats were kept in the same house as the 3 persistently viraemic cats.

The conclusions from the results in Table 10.1 were that non-viraemic cats appeared to fall into two categories of antibody producers. Some cats, like cats 1, 3, 10 and 14,

responded with low titres of virus neutralising antibodies (titres of 2–16) while the remainder produced higher titres (up to 128). These two categories did not correspond to any other classification used in this experiment. For example, the high responders were not exclusively latently infected cats or cats which were continuously exposed to viraemic cats. In fact, these cats were distributed between all of the classes.

Another important finding was that, once established, the level of the virus neutralising antibody titre did not vary significantly throughout the period of the experiment. This result means that exposure to viraemic cats is not necessary to maintain the level of virus neutralising antibodies in the serum of cats which have been exposed to natural FeLV infection.

Discussion

The results of this experiment appear to show that the titre of FeLV neutralising antibodies is maintained in cats for a period of over one year whether or not the cats are continuously exposed to cats excreting FeLV. The results indicate that following contact infection a cat becomes a low or a high responder. In this experiment the reason for this is not clear. It does not appear to depend on the time taken for a cat to be infected and produce antibodies since cat 2 did not have antibodies until 27 weeks and was then a relatively high responder, while cat 3 had antibodies at 16 weeks and became a low responder. If the period taken for the animal to produce antibodies is dependent on the dose of virus to which it was

exposed, this result suggests that the level of antibodies might not necessarily be correlated with the infecting dose of virus.

The results also show that cats which appear to have recovered from the infection (in that no virus was isolated from their bone marrow cultures) or latently infected cats may have either low or high antibody levels. This result suggests that virus continuously being released in latently-infected cats may not be required to maintain antibody levels. A major problem in interpreting these results is that it is not possible to completely eliminate the possibility that all cats which become non-viraemic following exposure to FeLV are latent carriers even though the virus cannot be isolated from their bone marrow. It is possible that virus may be latent in other cells in the body. This would mean that cats, like cats 1-4, which are considered to have recovered from the infection and eliminated the infection, might have a latent infection. This might explain why in these cats the antibody levels were maintained. If, however, it is assumed that the "recovered" cats were not latently infected, the relevance of these findings for vaccination is that once an antibody titre is established, it might be maintained for a long period. Unfortunately, in none of the experiments described in Chapters 4, 7 and 8, were cats found with virus neutralising antibodies after vaccination but before challenge. Therefore, although the vaccinated cats appeared to be immunologically primed by the vaccine and many made antibodies following challenge, it is not possible to predict from the results presented here whether or not, without challenge, their immune status would persist for as long as the antibodies found in this

experiment. To obtain this information, vaccinated cats would have to be maintained in isolation for an appropriate period and then challenged with live virus. The major problem in such an experiment is that if the cats were kept until they were adults and then challenged, only a small proportion of the unvaccinated cats would become viraemic, so it would be very difficult to show any difference between vaccinates and controls unless very large numbers of animals were employed.

The results in Chapter 8 showed that following challenge, most of the vaccinated cats became latently infected. The experiment was not carried on long enough to know if this was a permanent state. However, these cats developed titres of virus neutralising antibodies. From the results of this chapter, it seems likely that the antibody titres following challenge would persist over a long period of time.

CHAPTER 11

GENERAL DISCUSSION

In the experiments described in this thesis, a viral vaccine was prepared against FeLV infection which protected over 80% of vaccinated kittens against oronasal challenge with a dose of virus which caused persistent viraemia in all or most of the unvaccinated controls. This vaccine was more effective than any previous inactivated FeLV vaccine prepared from virus and was as effective as previous non-infectious cellular vaccines. In addition, the quantity of the viral antigen in the present vaccine was much less than in previous viral vaccines and therefore the vaccince would be relatively inexpensive to manufacture. The vaccine is now at a stage where it could be tested for its capacity to protect cats from FeLV under field conditions.

There were three stages in the development of the vaccine. First, several chemical inactivating agents were tested and paraformaldehyde was chosen for further study (Chapter 4). Secondly, two adjuvants, saponin and incomplete Freund's were compared and the latter was selected (Chapter 7). Thirdly, the effect of antigen dose and antigen type was studied (Chapter 8). The final vaccine was produced from the culture fluid of F422 cells, was inactivated with 0.02% paraformaldehyde, formulated with $Al(OH)_3$ and incomplete Freund's adjuvant and was given by two intramuscular injections, three weeks apart.

During the development of the vaccine, consideration had to be given to the following matters: first, the source of antigen

and choice of inactivating agent; secondly, the vaccination and challenge schedule; and thirdly, the measurement of the response of vaccinated cats to the challenge. In this chapter, these points are discussed in relation to the present results and the previous results of others.

1. Vaccine preparation

a. Source of virus for FeLV vaccine

F422 cells growing in suspension and producing large quantities of FeLV-A (Rickard et al, 1969) were used. Most previous vaccines have used FL74 cells which produced FeLV-ABC (Jarrett et al, 1974, 1975; Pedersen et al, 1979). The reasons why many earlier experiments were done with FL74 cells was because these produced FeLV of all three subgroups of FeLV, so it was considered that immunity might develop against all three. It is now known that most of the gp70 antigen in FL74 virus is FeLV-C (Russell, 1977) and very little is FeLV-A. Since it is likely that protection against FeLV-A is most important (Jarrett and Russell, 1978), in this work a cell line producing FeLV-A was used as a source of vaccine virus. The choice of virus-producing cells was between F422 and FeLV-A-infected FEA cells. F422 cells were favoured because they produced about 10 times more virus, in terms of viral protein, than FEA cells. Since FeLV-A isolates appear to belong to one serotype (Russell and Jarrett, 1978a), immunity to one FeLV-A might be expected to protect against infection by any other.

The conditions for the production of F422 cells were studied

(Chapter 2) and it was found that by maintaining the cells at high concentration (greater than 10^6 per ml) and harvesting the virus 24 or 48 hours after setting up the culture, good yields of infectious virus were obtained. In this way large quantities of virus could be produced very rapidly for vaccine production. However, attempts to use a less expensive medium (RPMI-1640) in place of L-M were not entirely successful. It is possible, however, that in future F422 cells could be selected to grow in RPMI or another medium and in lower concentrations of FCS. The content of FCS in the vaccine might be a problem for two reasons. First, these serum proteins might cause hypersensitivity reactions in cats given several doses of vaccine. Secondly, the proteins might inhibit the adsorption of inactivated virus to $Al(OH)_3$ in the vaccine. Further studies to reduce the content of FCS in the F422 culture medium would be useful.

In this work it was decided to use unconcentrated culture fluid (Chapter 8) as far as possible since this would be more economical in vaccine production than concentrated virus. In previous studies of viral vaccines, others have used very concentrated virus. For example, Yohn et al (1976) used a dose of 3×10^{10} virus particles of FeSV and a similar quantity of Rickard FeLV inactivated with formalin or UV-light. Olsen et al (1977) used 10^{11} particles of UV-irradiated FeLV from FL74 cells, while Salerno et al (1979) used 100-times concentrated formalin-inactivated FeLV-ABC/FL74 and Pedersen et al (1979) used 200-times concentrated formalin-inactivated FeLV-ABC/FL74.

The experiment in Chapter 8 showed that there was little difference between a vaccine which contained the equivalent of

unconcentrated virus and one which was ten-times concentrated. Although in these experiments the vaccine virus was not concentrated, in fact the virus was concentrated and then diluted in the course of washing to remove the toxic inactivating agents in order to assay for residual infectivity. In future it would be of interest to find if washing is necessary to obtain an efficient vaccine. This would also determine whether there were any substances in the vaccine which were immunosuppressive (e.g. p15E). Avoidance of washing the virus would, of course, be more economical in the production of a commercial vaccine. Antigens used in the present studies were FeLV-A/F422, FeLV-A/Glasgow-1 and F422 cells. The viruses were used concentrated tenfold or unconcentrated, while F422 cells were used at 3.5×10^6 cells per dose. The general indication was that the best antigen was that obtained by using unconcentrated FeLV-A/F422.

b. Inactivating agent

In these experiments paraformaldehyde was chosen for several reasons. First, there was experience of PF in previous cellular vaccines produced in this laboratory which induced anti-FOCMA antibodies (Jarrett et al, 1975) and protected cats against FeLV challenge (W.F.H. Jarrett - personal communication). Therefore, PF appeared to preserve the antigenicity of viral antigens. Secondly, the kinetics of inactivation of both MSV(FeLV-A) and F422 virus were less complicated than with the other inactivating agents tested (Chapters 3 and 7). Other advantages are that PF is relatively less toxic for man than AEI and BPL and is inexpensive. Disadvantages of PF and formaldehyde are that with

some viruses there is a persistent fraction of virus which is resistant to their action. Gard (1960), in a classical study, showed that this occurred during inactivation of poliovirus with formaldehyde and suggested that the reason was that the initial reaction of the agent with viral capsid protein made the virus particle less permeable to the agent so that the rate of inactivation decreased. A recent example of the tragic consequences of the failure of formaldehyde to inactivate virus completely was the recent outbreaks of foot-and-mouth disease in France, the Channel Islands and the Isle of Wight (Donaldson et al, 1982). It was obviously very important to ensure that all of the virus used in the present vaccine was inactivated. Under the conditions used (0.02% PF for 24 hours at 25°) no infectious virus remained in the volume of vaccine tested which was about 10% of the total volume. Another potential disadvantage of PF is that it may decrease the antigenicity of virus by cross-linking adjacent molecules and destroying antigenic sites (Miller and Van der Maaten, 1978). This appeared to occur with FeLV in this study when treated with PF or formaldehyde at high concentrations. However, as discussed in Chapter 9, there was enhancement of antigenicity of both FL74 and F422 virus at low concentrations of these agents. At the concentrations used in the vaccine (0.02%), the antigenicity was equal to, or greater than, untreated antigen.

In previous studies Pedersen et al (1979) and Salerno et al (1979) used formaldehyde to inactivate FL74 virus. Pedersen and his co-workers inactivated FeLV-ABC/FL74 with 0.1% formaldehyde

overnight at 4° prior to use. This vaccine was tested for viral residual infectivity and was used emulsified with Freund's incomplete adjuvant or $Al(OH)_3$ to vaccinate 16-week old kitten. Salerno and his co-workers purified the virus and treated it with 0.025% formalin for 21 days at 4° and used the product with and without Freund's complete adjuvant to vaccinate guinea pigs. Other inactivating agents which have been used to prepare FeLV viral vaccines are ultraviolet (UV) irradiation and heat (Yohn et al, 1976 and Olsen et al, 1976). Yohn and his co-workers used UV-inactivated FeLV-ABC/FL74 with adjuvant-65 to vaccinate cats. This inactivation was done by an exposure of the virus surface to an UV dose rate of 150 ergs/sq. mm/sec for an accumulated total dose of 35,000 ergs/sq. mm. Olsen et al (1976) prepared their FeLV vaccine by thermal inactivation of FeLV at 56° for three minutes or 45° for varying times ranging from 0 to 50 minutes, but in the latter case the inactivation was insufficient to kill all virus. Virus was completely inactivated only after 100 minutes. In the present vaccination experiments, varying concentrations of several chemicals were used and the chemical and concentration chosen was 0.02% paraformaldehyde at 25° for 24 hours. The results of testing of the inactivated virus for viral residual infectivity were that complete inactivation was achieved.

c. Adjuvants

Two adjuvants were used in the present vaccine: $Al(OH)_3$ and incomplete Freund's adjuvant (ICFA). $Al(OH)_3$ had been used before in FeLV vaccines by Jarrett et al (1975), Pedersen et al

(1979) and Salerno et al (1978). In the study of Pedersen et al (1979) there was a comparison of $Al(OH)_3$ and ICFA used with an inactivated FL74 virus vaccine. The number of cats in each group in the experiment was small, but there did not appear to be any difference in the efficacy of either vaccine.

In all of these studies and in the present experiments, there was no indication that $Al(OH)_3$ or ICFA gave rise to side effects. In Chapter 3 evidence is presented that 75% of virus adsorbed to $Al(OH)_3$ in the concentration used (one-tenth volume of 2% $Al(OH)_3$). In some other vaccines $Al(OH)_3$ is used at higher concentrations (up to one-half) and it would be interesting in future to examine the adsorption of inactivated FeLV to higher concentrations. It might be possible by using adsorption of virus to $Al(OH)_3$ to eliminate the washing of virus by ultrafiltration in order to remove toxic products of inactivation.

Saponin was tested as an adjuvant in the early part of this work (Chapter 5) but was not considered very satisfactory. In the first experiment (Chapter 4) the saponin produced side effects of pain and swelling at the site of injection, and fever. When saponin was subsequently used at a concentration which was not harmful, as determined by the experiment described in Chapter 5, it did not give as good a response as ICFA.

ICFA did not appear to have a harmful effect on cats. Oil adjuvants are currently used in a killed combined feline calicivirus, feline herpesvirus, feline panleukopaenia virus vaccine (Vaxicat-plus; TVL Ltd) and no side effects have been reported in the field. In other studies on FeLV vaccination,

ICFA has been used (Pedersen et al, 1979). Complete Freund's adjuvant (CFA) has also been used and although it produces granulomata in many species, there was no mention in the work of Yohn et al (1976), Salerno et al (1978) or Grant et al (1980) that any harmful effects were noted in cats.

2. Vaccination schedule and challenge

A major problem which was encountered by many previous workers on FeLV vaccination is the age-related resistance to FeLV infection in cats (Hoover et al, 1976). Kittens older than about 14 weeks of age are relatively resistant to the type and dose of challenge which is usually given. Because of this problem, in some studies it has been difficult, if not impossible, to determine whether a vaccine has been successful or not since the response of the vaccinates and controls to challenge was similar (Salerno et al, 1979).

To avoid this problem, in the present study, cats were vaccinated and challenged before they were 12-13 weeks of age. The cats were vaccinated when 8 weeks old and again 2-3 weeks later. They were then challenged 1-2 weeks following the last vaccination. In this way all or most of the control cats developed a persistent viraemia and if a vaccine was effective, the resistance of the vaccinated cats should be obvious. In the first experiment of this study (Chapter 4) not all of the unvaccinated control cats became viraemic following challenge. This was almost certainly due to challenge with too low a dose of virus since in the second and third vaccination experiments, the

administration of a larger dose ($4.5 \times 10^5 - 1 \times 10^6$ FIU) produced persistent viraemia in 6/6 cats in each case.

The appropriate FeLV challenge dose was determined in an experiment described in Chapter 6. The challenge in these experiments was given by the oronasal route in order to simulate natural contact challenge. It was shown first by Hoover et al (1972) that intranasal instillation of the Rickard strain of FeLV in newborn kittens gave rise to a persistent viraemia and then to the development of lymphosarcoma. The time course of the establishment of viraemia in 8-week old and 16-week old cats following oronasal administration was subsequently determined by Jarrett et al (1982a) and their technique was used here. In that study, it was found that 4/5 8-week old kittens and 5/10 16-week old kittens became permanently viraemic following exposure to 10^5 FIU of FeLV-A/Glasgow-1. In addition, transient viraemia was found in almost all of the kittens which eventually recovered from the infection. It was found that kittens with transient viraemia could be detected 3 weeks after infection. Following challenge, therefore, the kittens in the present experiments were tested at 3 weeks for evidence of transient viraemia and later for persistent viraemia. In different experiments the timing of the testing for persistent viraemia was slightly different. It was found, however, that there was little change in the virus status of the cat from 12 weeks after challenge.

In most other studies on FeLV vaccination, the challenge has been by subcutaneous or intramuscular inoculation of FeLV or, more commonly, FeSV. Although the time course and outcome of parenteral inoculation of FeLV is similar to that following

oronasal administration, there may be differences in the speed with which the virus spreads to the bone marrow. It may be that virus given by injection will infect bone marrow cells more rapidly than if given oronasally, and might, therefore, be less susceptible to anti-viral immunity which is produced by vaccination. So far the only carefully controlled study using natural contact transmission was that of Pedersen et al (1979). In their experiments, groups of 4-6 vaccinated or unvaccinated control kittens which were approximately 20 weeks old were housed with 2-3 viraemic cats. Under these conditions, 10/25 (40%) of the unvaccinated cats became persistently viraemic. Thus, in these experiments, the proportion of cats which became persistently viraemic was much less than in the present experiments. In another experiment involving contact transmission, Olsen et al (1980a)used their soluble tumour-associated vaccine to vaccinate 45 FeLV-free cats in a household of pet cats in which FeLV was enzootic. Of these 45 cats, 12 (27%) developed persistent viraemia. Eight of these cats developed viraemia during the vaccination procedure, and if these are excluded from the results, only 4/37 (11%) appeared to become viraemic following natural challenge. This suggests a high level of protection. However, a major objection to this study is the lack of age- and sex-matched, unvaccinated controls. The only indication of the prevalence of persistent viraemia in unvaccinated cats was that that 29% of the cats in the house were FeLV-positive before the vaccination programme began.

In several other experiments the challenge FeLV was given by

injection. Jarrett et al (1975) vaccinated kittens with live FL74 cells and challenged by subcutaneous inoculation of 10^7 infectious units of FeLV-AB/Glasgow-5. The cats were 6 months old when challenged and 3/10 of the vaccinated and 9/10 of the unvaccinated cats became persistently viraemic. The reason for the success of this challenge in producing viraemia in such a high proportion of 16 month old animals is presumably the high dose of virus which was used.

In another experiment Hoover et al (1977b) vaccinated pregnant queens with FeLV-ABC/FL74 virus inactivated by UV-irradiation. Only one of ten queens developed virus neutralising antibodies. The kittens of the vaccinated queens were inoculated intraperitoneally with 10^5 FeLV-AB/Rickard when they were 2 days old and 18/19 became persistently viraemic. Similar results were obtained by Salerno et al (1979).

All of the other investigations of FeLV vaccination have used challenge by FeSV. There are three main reasons why FeSV has been used rather than FeLV. First, when experiments on vaccination began, it was believed that anti-FOCMA antibodies were responsible for, or were a measure of, immunity to FeLV (Essex et al, 1975 i). Anti-FOCMA antibodies could be raised in cats after vaccination with live FL74 cells or inactivated FL74 cells (Jarrett et al, 1975; Pedersen et al, 1979) or UV-irradiated FeSV or FeLV (Yohn et al, 1976). Essex et al (1971) showed that the level of anti-FOCMA antibodies was important in immunity to FeSV. Hence many studies have used challenge with FeSV following FeLV vaccination and have used the development, or not, of sarcomas as an indication of the efficacy of vaccination.

A second reason for the use of FeSV is that tumours occur in weeks rather than in months or years as is the case with FeLV. Therefore, experiments can be done much more rapidly with FeSV than with FeLV challenge. In the experiments described in this thesis, FeLV was used as the challenge since it was believed that the biology of FeSV and FeLV was quite different. For example, in tumour development by FeSV, the tumour is polyclonal and the sarcoma virus transforms many cells and spreads to further cells to recruit them into the tumour. On the other hand, FeLV is thought to transform a single cell which then grows into a tumour, often after a very long incubation period.

Another reason why FeLV is considered a more appropriate challenge virus is because it was subsequently shown that FeLV neutralising antibodies are more related to resistance to FeLV infection than anti-FOCMA antibodies (Hardy et al, 1976; Russell and Jarrett, 1978b).

In future it would be useful to test the present vaccine under natural conditions of challenge with FeLV since it may be that natural contact transmission might not be so severe as the oronasal challenge used in the present experiments.

3. Response of vaccinated cats to FeLV challenge

It was considered unnecessary in the present experiments to wait for lymphosarcoma to develop in cats in order to test the efficacy of a vaccine. The only experiment in which this has been done is in that of Hoover et al (1977b). Instead, the development of viraemia was taken as a marker of FeLV infection

and freedom from viraemia by vaccinated cats following challenge was considered an indication of efficacy of the vaccine.

There is very good evidence that the vast majority of cats which are FeLV-negative do not develop diseases which are believed to be FeLV-related. Hardy et al (1976.) showed that by eliminating FeLV carrier cats from households of pet cats in which the infection had been enzootic, the virus was also eliminated. In addition, there was no occurrence in the surviving cats of FeLV-associated diseases. In a follow-up study of these cats, McClelland et al (1980) showed that the fatality rate over 3.5 years was 83% for FeLV-infected cats but only 15% for FeLV-negative cats after FeLV had been eliminated from the households. From this evidence it was considered in the present experiments that the efficacy of a FeLV vaccine could be assessed by the capacity of the vaccine to prevent viraemia.

Two types of viraemia were detected: transient and persistent. Protection against persistent viraemia was the most important indication of the efficacy of a vaccine. In the later experiments described here, cats were sampled 3, 6 and 12 weeks after challenge. It was found in the experiments described in Chapter 7 that the viraemic status of a cat did not alter after 12 weeks. The method used to detect virus in the blood was virus isolation from the plasma since Jarrett et al (1982a) showed that this method was the most sensitive for demonstrating virus.

The cats were also tested for a transient viraemia at 3 weeks after infection. In a previous study in which this has been studied, Pedersen et al (1979) found that a formaldehyde-inactivated FL74 virus vaccine did not protect cats from

transient viraemia following contact infection, in that 7/26 (27%) of vaccinates developed transient viraemia which was similar to the proportion of 6/25 (24%) in the controls. However only 3/26 (12%) of the vaccinates became persistently infected compared to 10/25 (40%) of the controls. The results in the present experiments were similar to those of Pederson and colleagues. It was found that more cats became transiently viraemic than became permanently viraemic. For example, the results in the last experiment of FeLV vaccination (Chapter 8) were that in vaccinated groups C1, C2 and C3 the number of transiently viraemic cats were 1, 2 and 3 of 6 (17, 34 and 52%) respectively compared to the number of the permanently viraemic cats in the same groups which was 0, 1 and 2 (0, 17 and 34%) respectively. This was in contrast to groups C4 and C5 (culture fluid control and unvaccinated cats) in which the result was that 6 of 6 (100%) became permanently viraemic in both cases.

It would be preferable if a vaccine could prevent transient viraemia as well as permanent viraemia for two main reasons. First, it was shown by Jarrett et al (1982a) that kittens excreted virus from the mouth during the short time of a transient viraemia. They may be able to transmit virus to other cats during this short period. Secondly, following transient viraemia a latent infection may be established, as discussed below. It would be useful if both of these situations could be prevented.

The other measure of the efficacy of the vaccine was the development of virus neutralising antibodies in vaccinated cats.

None of the vaccinated cats in these experiments produced antibodies after vaccination and before challenge. This finding agrees with the results of other studies in which kittens were vaccinated. Only after vaccinating adults with six repeated doses of UV-irradiated FeSV, did Yohn et al (1976) consistently find virus neutralising antibodies, and only in low titres. However, it appeared that the vaccinated kittens were primed by viral antigens since following challenge most cats responded by making virus neutralising antibodies. Those cats were FeLV-negative, which is a similar situation to that in the field following natural challenge (Hardy et al, 1976; Russell and Jarrett, 1978b). While the presence of virus neutralising antibodies indicates that a cat is likely to be FeLV-free, it is not certain that it is these antibodies which are responsible for suppressing the growth of the virus in the cat. For example, cell mediated immunity may be very important in killing virus-infected cells. However, antibodies can also kill feline lymphosarcoma cells in the presence of cat complement (Grant et al, 1980) and recently it was shown that mouse monoclonal antibodies which were cytotoxic were also virus neutralising (Grant et al, 1983). It is likely, therefore, that neutralising antibodies are important in suppression of virus and virus-infected cells.

Antibody to FOCMA was not measured in these experiments because its presence does not indicate that a cat is resistant to FeLV infection (Hardy et al, 1976). The experience of others using inactivated virus vaccines is that anti-FOCMA antibodies do not appear in vaccinated cats before challenge (Pedersen et al,

1979). Anti-FOCMA antibody does appear, however, in cats which are vaccinated with live FeLV or FeLV-infected cells (Jarrett et al, 1974; Pedersen et al, 1979; Grant et al, 1980) or with inactivated FeLV-infected cells (Jarrett et al, 1975; Olsen et al, 1976; Yohn et al, 1976; Pedersen et al, 1979).

A scoring system was used in the studies described here in order to assist in grading the responses following vaccination. The aim of this system was to give an indication of which of several vaccine types was most efficient. As described in Chapter 4, both the viraemic status and antibody status of the cats in a group were taken into account. A summary of the results of the three vaccine experiments is shown in Table 11.1. From this, it is seen that the scoring system was a useful aid in selecting vaccines for further development. No other studies on FeLV vaccination have used such a system, but a scoring system, based on clinical signs following challenge, was used in the development of a vaccine against feline herpesvirus (Slater and York, 1975).

A third response of cats to FeLV challenge was also investigated. While this study was in progress the phenomenon of FeLV latency was discovered (Post and Warren, 1980; Rojko et al, 1982; Madewell and Jarrett, 1983). It was considered important to determine whether cats which appeared to be protected from FeLV challenge by vaccination were latent carriers of the virus. Cats which had resisted challenge in group C1 vaccinated with PF-inactivated F422 virus as described in Chapter 8 were examined and some were found to have latent virus. It is interesting that

TABLE 11.1

Summary of Efficacy Scores in Vaccination Experiments

Group		Score			Rank
No.	Vaccine	Virus	Antibody	Total	
A1	PF(1)	0	325	325	1
A2	PF(10)	75	300	375	2
A3	AEI(1)	225	600	825	6=
A4	AEI(10)	75	350	425	3
A5	DTNP/PF(1)	25	525	550	5
A6	DTNP/PF(10)	50	475	525	4
A7	F422 cells	425	600	1025	8
A8	Unvaccinated	225	600	825	6=
B1	PF(ICFA)	232	550	782	1
B2	PF(Saponin)	300	567	867	2
B3	Unvaccinated	600	600	1200	3
C1	F422(1)	64	283	347	1
C2	F422 (10)	142	270	412	2
C3	FeLV-A(10)	382	549	931	3
C4	Control Fluid	550	600	1150	4
C5	Unvaccinated	600	600	1200	5

these cats did not show even a transient viraemia but had latent virus. This indicates that the vaccine did not prevent initial virus growth or spread from the oropharynx to the bone marrow. It is not known how long latent virus is maintained in the bone marrow. Recent studies have indicated that the proportion of cats with latent infection following natural FeLV challenge decreases from about 60% at 20 weeks after exposure, to 25% after 80 weeks (O. Jarrett, personal communication). It will be important in future to test vaccinated cats with latent infections over a longer period of time than the 12 weeks in the present experiment. Another important question is whether or not latent carriers of FeLV can transmit the virus. At present there is no evidence that they do (Madewell and Jarrett, 1983).

4. Future Studies

For the future it would seem to be important to develop an improved vaccine which would induce virus neutralising antibodies in young kittens before challenge with live FeLV. The present experiments indicated that this might not be achieved simply by increasing the dose of antigen in the vaccine. Also the vaccine of Pedersen et al (1979) was even more concentrated than those used in the present experiment (equivalent to 20-fold greater than the most concentrated vaccine used here) and did not achieve better results. It may be that the use of more efficient adjuvants might increase the immunogenicity of the vaccine. However, the development of better adjuvants than those used here has been very slow. The use of saponin in the present study was not successful.

It would seem that one of the most effective ways of immunising against FeLV would be by using a modified live virus vaccine. Jarrett et al (1975) used live FL74 cells and protected 3/3 cats against a challenge of FeLV which produced persistent viraemia in 9/10 unvaccinated controls. In a similar experiment, Grant et al (1980) protected all of 11 cats against challenge with FeSV. Pedersen et al (1979) used live FL74 virus and protected 28/28 cats from a natural challenge with FeLV which produced persistent viraemia in 15/37 controls. None of the cats vaccinated with live FL74 cells or virus showed any signs of becoming viraemic following vaccination. This is because the FL74 virus has a very low specific infectivity which is only about 10^{-3} that of FeLV grown in monolayer cultures. While the use of this naturally "attenuated" virus is superficially attractive, it is unlikely that such a vaccine could be used in the field since FL74 virus caused erythroid hypoplasia when inoculated into newborn kittens, presumably because of its FeLV-C component (Hoover et al, 1974). The vaccination experiments described above have all been done in cats which were several months old and resistant to FL74 virus infection (Hoover et al, 1976). Even in 8 week old kittens which are normally resistant to FeLV-C, this virus can be fatal if the animals are immunosuppressed for some reason (O. Jarrett, personal communication).

The type of modified live vaccine that would appear to offer the best chance of success is a temperature sensitive (ts) mutant of FeLV which could be given into the upper respiratory tract. A successful intranasal vaccine has been developed using a ts

mutant of feline herpesvirus (Slater and York, 1975). This virus grows in the nose and immunises the cat but does not become systemic. Retrovirus ts mutants can be obtained although one problem is that they may revert (Wyke, 1976). However, it would be interesting to attempt to produce such a vaccine for FeLV since it might prevent the virus spreading to the bone marrow and establishing a latent infection.

Recently, viral subunit and genetically engineered vaccines have been proposed. The experience with FeLV gp70 vaccination has not been encouraging (Salerno et al, 1978). Although antibody was induced following the inoculation of 20 ug of gp70 into guinea pigs, there was no response in cats given 40 ug. The vaccine developed by Olsen et al (1980) is apparently virus-free and contains antigens which induce antibodies to FOCMA and gp70. However, the fluid from FL74 cells requires extensive concentration and it is doubtful if such a vaccine would be economic to produce for general use in the field.

By contrast, the inactivated virus vaccine which is described in this thesis would be relatively inexpensive to manufacture. It should now be possible to test this vaccine for its capacity to protect cats from natural contact challenge as a final step to developing it for use in the field.

REFERENCES

Anderson, L.J., Jarrett, W.F.H. (1971). Membranous glomerulonephritis associated with leukaemia in cats. Res. Vet. Sci., 12, 179-180.

Anderson, L.J., Jarrett, W.F.H., Jarrett, O. and Laird, H.M. (1971). Feline leukemia virus infection of kittens. Mortality associated with atrophy of the thymus and lymphoid depletion. J. Nat. Cancer Inst., 47, 807-817.

Balcarova, J., Helenius, A. and Simons, K. (1981). Antibody response to spike protein vaccines prepared from Semliki Forest virus. J. Gen. Virol., 53, 85-92.

Bittle, J.L., Houghten, R.A., Alexander, H., Shinnick, T.M., Sutcliffe, J.G., Rowlands, D.J., Brown, F. and Lerner, R.A. (1982). Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature, 298, 30-33.

Bolognesi, D.P. (1976). Potential leukemia virus subunit vaccines (Discussion). Cancer Res., 36, 655-656.

Bolognesi, D.P., Montelaro, R.C., Frank, H. and Schafer, W. (1978). Assembly of type-C oncornaviruses: A model. Science, 199, 183-186.

Brown, F., Cartwright, B. and Stewart, D.L. (1963). The effect of various inactivating agents on the viral and ribonucleic acid infectivities of foot-and-mouth disease virus and its attachment to susceptible cells. J. Gen. Microbiol., 31, 179-186.

Charney, J., Holben, J.A., Cody, C.M. and Moore, D.H. (1976). Further immunization studies with mammary tumor virus. Cancer Res., 36, 777-780.

Creemers, P., Ouwehand, J. and Bentvelzen, P. (1978). Effect of a mouse mammary tumor virus-derived protein vaccine on primary tumor development in mice. J. Nat. Cancer Inst., 60, 1461-1466.

Crighton, G.W. (1968). Clinical aspects of lymphosarcoma in the cat. Vet. Rec., 83, 122-126.

Dalsgaard, K., Jensen, M.H. and Sorensen, K.J. (1977). Saponin adjuvants, IV. Evaluation of the adjuvant Quil. A in the vaccination of cattle against foot-and-mouth disease. Acta. Vet. Scanda., 18, 349-360.

Doel, T.R. and Baccarini, P.J. (1981). Thermal stability of foot-and-mouth disease virus. Arch. Virol., 70, 21-32.

Donaldson, A.I., Gloster, J., Harvey, L.D.J. and Deans, D.H. (1982). Use of prediction models to forecast and analyse airborne spread during the foot-and-mouth outbreaks in Brittany, Jersey and the Isle of Wight in 1981. Vet. Rec., 110, 53-57.

Dorn, C.R., Taylor, D.O.N., Schneider, R., Hibbard, H.H. and Klauber, M.R. (1968). Survey of animal neoplasms in Alameda and Contra Costa Counties, California. II. Cancer morbidity in dogs and cats from Alameda County. J. Nat. Cancer Inst., 40, 307-318.

Essex, M., Kelin, G., Snyder, S.P. and Harrold, J.G. (1971). Antibody to feline oncornavirus-associated cell membrane antigen in neonatal cats. Int. J. Cancer, 8, 384-390.

Essex, M., Sliski, A., Cotter, S.M.I., Jakowski, R.M. and Hardy, W.D. (1975d). Immunosurveillance of naturally occurring feline leukemia. Science, 190, 790-792.

Essex, M., Sliski, A., Hardy, W.D. Jr. and Cotter, S. (1976). Immune response to leukemia virus and tumor-associated antigens in cats. Cancer Res., 36, 640-645.

Fischinger, P.J., Blevins, C.S. and Nomura, S. (1974). Simple quantitative assay for both xenotropic murine leukemia and ecotropic feline leukemia viruses. J. Virol., 14, 177-179.

Fischinger, P.J. and O'Connor, T.E. (1969). Viral infection across species barriers: reversible alteration of murine sarcoma virus for growth in cat cells. Science, 165, 714-716.

Fischinger, P.J. and O'Connor, T.E. (1970). Productive infection and morphologic alteration of human cells by a modified sarcoma virus. J. Nat. Cancer Inst., 44, 429-438.

Francis, D.P., Essex, M. and Hardy, W.D.Jr. (1977). Excretion of feline leukaemia virus by naturally infected pet cats. Nature, 269, 252-254.

Gard, S. (1960). Theoretical considerations in the inactivation of viruses by chemical means. Annals. N.Y. Acad. Sci., 83, 513-560.

Grant, C.K., De Noronha, F., Tusch, C., Michalek, M.T. and McLane, M.F. (1980). Protection of cats against progressive fibrosarcomas and persistent leukemia virus infection by vaccination with feline leukemia cells. J. Nat. Cancer Inst., 65, 1285-1292.

Grant, C.K., Ernisse, B.J., Jarrett, O. and Jones, F.R. (1983). Feline leukemia virus envelope gp70 of subgroups B and C defined by monoclonal antibodies with cytotoxic and neutralizing functions. J. Immunol., 131, 3042-3048.

Graves, J.H. and Arlinghaus, R.B. (1967). Acetylethyleneimine in the preparation of inactivated foot-and-mouth disease vaccines. Proc. 71st Ann. Meeting, U.S. Livestock San. Assn., 396-403.

Green, R.W., Bolognesi, D.P., Schafer, W., Pister, L., Hunsmann, G. and Noronha, F. (1973). Polypeptides of mammalian oncornaviruses. Isolation and serological analysis of polypeptides from murine and feline C-type viruses. Virology, 56, 565-579.

Hardy, W.D.Jr. (1981a). Hemopoietic tumors of cats. J. Amer. Anim. Hosp. Assoc., 17, 921-940.

Hardy, W.D.Jr. (1981b). Feline leukemia virus non-neoplastic diseases. J. Amer. Anim. Hosp. Assoc., 1, 941-949.

Hardy, W.D.Jr., Hess, P.W., MacEwen, E.G., McClelland, A.J., Zuckerman, E.E., Essex, M., Cotter, S.M. and Jarrett, O. (1976). Biology of feline leukemia virus in the natural environment. Cancer Res., 36, 582-588.

Hardy, W.D.Jr., McClelland, A.J., Zuckerman, E.E., Snyder, H.W.Jr., MacEwen, E.G., Francis, D. and Essex, M. (1980). Development of virus non-producer lymphosarcomas in pet cats exposed to FeLV. Nature, 288, 90-92.

Hardy, W.D.Jr., Old, L.J., Hess, P.W., Essex, M. and Cotter, S.M. (1973). Horizontal transmission of feline leukaemia virus. Nature, 244, 266-269.

Hardy, W.D.Jr., Zuckerman, E.E., MacEwen, E.G. and Essex, M. (1977). Evidence that the feline oncornavirus associated cell membrane antigen (FOCMA) is a FeLV and FeSV induced tumor antigen. (Abstract) Cold Spring Harbor Symposium on Oncornaviruses, pp 177. Cold Spring Harbor.

Hoover, E.A., Schaller, J.P., Mathes, L.E. and Olsen, R.G. (1977a). Passive immunity to feline leukemia: evaluation of immunity from dams naturally infected and experimentally vaccinated. Infection and Immunity, 16, 54-59.

Hoover, E.A., Olsen, R.G., Mathes, L.E. and Schaller, J.P. (1977b). Relationship between feline leukemia virus antigen expression and viral infectivity in blood, bone marrow and saliva of cats. Cancer Res., 37, 3707-3710.

Hoover, E.A., Olsen, R.G., Hardy, W.D.Jr., Schaller, J.P. and Mathes, L.E. (1976). Feline leukemia virus infection: age related variation in response of cats to experimental infection. J. Nat. Cancer Inst., 57, 365-370.

Hoover, E.A., Kociba, G.J., Hardy, W.D.Jr. and Yohn, D.S. (1974). Erythroid hypoplasia in cats inoculated with feline leukemia virus. J. Nat. Cancer Inst., 53, 1271-1276.

Hoover, E.A., McCullough, C.B. and Griesmer, R.A. (1972). Intranasal transmission of feline leukemia. J. Nat. Cancer Inst., 48, 973-983.

Jarrett, O. (1980). Natural occurence of subgroups of feline leukemia virus. In Viruses in Naturally Occurring Cancers. Cold Spring Harbor Conferences on Cell Proliferation, Vol. 7., 603-611. Cold Spring Harbor.

Jarrett, O., Golder, M.C. and Stewart, M.F. (1982a). Detection of transient and persistent feline leukaemia virus infections. Vet. Rec., 110, 225-228.

Jarrett, O., Golder, M.C., Weijer, K. (1982b). A comparison of three methods of feline leukaemia virus diagnosis. Vet. Rec., 110, 325-328.

Jarrett, O., Hardy, W.D.Jr., Golder, M.C. and Hay, D. (1978). The frequency of occurrence of feline leukemia virus subgroups in cats. Int. J. Cancer, 21, 334-337.

Jarrett, O., Laird, H.M. and Hay, D. (1973). Determinants of the host range of feline leukaemia viruses. J. Gen. Virol., 20, 169-175.

Jarrett, O., Laird, H.M., Hay, D. and Crighton, G.W. (1968). Replication of cat leukaemia virus in cell cultures. Nature, 219, 521-522.

Jarrett, O., Pitts, J.D., Whalley, J.M., Clason, A.W. and Hay, J. (1971). Isolation of the nucleic acid of feline leukaemia virus. Virology, 43, 317-320.

Jarrett, O. and Russell, P.H. (1978). Differential growthand transmission in cats of feline leukaemia viruses of subgroups A and B. Int. J. Cancer, 21, 466-472.

Jarrett, O., Russell, P.H. and Stewart, M.F. (1977). Protection of kittens from feline leukaemia virus infection by maternally-derived antibody. Vet. Rec., 101, 304-305.

Jarrett, W.F.H., Anderson, L.J., Jarrett, O., Laird, H.M. and Stewart, M.F. (1971). Myeloid leukaemia in a cat produced experimentally by feline leukaemia virus. Res. Vet. Sci., 12, 385-387.

Jarrett, W.F.H., Crawford, E.M., Martin, W.B. and Davie, F.A. (1964a). Virus-like particle associated with leukaemia (lymphosarcoma). Nature, 202, 567-569.

Jarrett, W.F.H., Jarrett, O., Mackey, L., Laird, H.M., Hardy, W. and Essex, M. (1973). Horizontal transmission of leukaemia virus and leukaemia in the cat. J. Nat. Cancer Inst., 51, 833-841.

Jarrett, W.F.H., Jarrett, O., Mackey, L., Laird, H.M., Hood, C. and Hay, D. (1975). Vaccination against feline leukaemia virus using a cell membrane antigen system. Int. J. Cancer, 16, 134-141.

Jarrett, W.F.H., Mackey, L.J. (1974). Neoplastic diseases of the haemopoietic and lymphoid tissues. Bulletin of the W.H.O., 50, 21-34.

Jarrett, W.F.H., Mackey, L.J., Jarrett, O., Laird, H.M. and Hood, C. (1974). Antibody response and virus survival in cats vaccinated against feline leukaemia. Nature, 248, 230-232.

Jarrett, W.F.H., Martin, W.B., Crighton, G.W., Dalton, R.G. and Stewart, M.F. (1964b). Leukaemia in the cat: trasmission experiments with leukaemia (lymphosarcoma). Nature, 202, 566-567.

Laird, H.M., Jarrett, O., Crighton, G.W. and Jarrett, W.F.H. (1968). An electron microscopy study of virus particles in spontaneous leukemia in the cat. J. Nat. Cancer Inst., 41, 867-878.

Laird, H.M., Jarrett, O. and Crighton, G.W. (1968). Replication of leukemogenic-type virus in cats inoculated with feline lymphosarcoma extracts. J. Nat. Cancer Inst., 54, 209-217.

Laird, H.M., Jarrett, W.F.H., Jarrett, O. and Crighton, G.W. (1967). Virus-like particles in three field cases of feline lymphosarcoma. Vet. Rec., 80, 606.

McClelland, A.J., Hardy, W.D.Jr. and Zuckerman, E.E. (1980). Prognosis of healthy feline leukemia virus infected cats. In Hardy, W.D.Jr., Essex, M., McClelland, A.J. (eds) Feline Leukemia Virus. New York: Elsevier-North Holland, pp 121-131.

Mackey, L. (1975). Feline leukaemia virus and its clinical effects in cats. Vet. Rec., 4, 5-11.

Mackey, L., Jarrett, W.F.H., Jarrett, O. and Laird, H.M. (1972). An experimental study of virus leukemia in cats. J. Nat. Cancer Inst., 48, 1663-1670.

Mackey, L., Jarrett, W.F.H., Jarrett, O. and Laird, H.M. (1975). Anemia associated with feline leukemia virus infection in cats. J. Nat. Cancer. Inst., 54, 209-217.

Madewell, B.R. and Jarrett, O. (1983). Recovery of feline leukaemia virus from non-viraemic cats. Vet. Rec., 112, 339-342.

Miller, J.M. and Van Der Maaten (1978). Evaluation of an inactivated bovine leukemia virus preparation as an immunogen in cattle. Ann. Rech. Vet., 9, 871-877.

Neil, J.C. (1978). Feline leukaemia virus-specific proteins. Ph.D. Thesis, University of Glasgow.

Okabe, H., Twiddy, E., Gilden, R.V., Hatanaka, M., Hoover, E.A. and Olsen, R.G. (1976). FeLV-related sequences in DNA from a FeLV-free cat colony. Virology, 69, 798-801.

Olsen, R.G. and Lewis, M.G. (1981). Immunoprophylaxis: experimental vaccine development and testing. In Feline Leukaemia. Ed. R.G. Olsen. CRC Press, Florida, pp 135-148.

Olsen, R.G., Lewis, M.G., Mathes, L.E. and Hause, W. (1980a). Feline leukemia vaccine: efficacy testing in a large multicat household. Feline Practice, 10, 13-16.

Olsen, R.G., Hoover, E.A., Schaller, J.P., Mathes, L.E. and Wolff, L.H. (1977). Abrogation of resistance to feline oncornavirus disease by immunization with killed feline leukemia virus. Cancer Res., 37, 2082-2085.

Olsen, R.G., Hoover, E.A., Mathes, L.E., Heding, L. and Schaller, J.P. (1976). Immunization against feline oncornavirus disease using a killed tumor cell vaccine. Cancer Res., 36, 3642-3646.

Olsen, R.G., Mathes, L.E., Lewis, M.G. and Hoover, E.A. (1980b). Feline leukaemia virus: influence by an immunosuppressive retrovirus protein. In Advances in Comparative Leukemia Research, 1979. Eds. Yohn, D.S., Lapin, B.A. and Blakeslee, Jr. Elsevier/North Holland, pp 281-284.

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

Pedersen, N.C., Theilen, G.H. and Werner, L.L. (1979). Safety and efficacy studies of living and killed feline leukemia virus vaccines. Amer. J. Vet. Res., 40, 1120-1126.

Perryman, L.E., Hoover, E.A. and Yohn, D.S. (1972). Immunosuppression in experimental feline leukemia. J. Nat. Cancer Inst., 49, 1357-1365.

Pinter, A., Lieman-Hurwitz, J. and Fleissner, E. (1978). The nature of the association between the murine leukemia virus envelope proteins. Virology, 91, 345-351.

Pinter, A. and Fleissner, E. (1977). The presence of disulfide-linked gp70-p15(E) complexes in AKR murine leukemia virus. Virology, 83, 417-422.

Post, J.E. and Warren, L. (1980). Reactivation of latent feline leukemia virus. In Feline Leukaemia Virus, eds. Hardy, W.D.Jr., Essex, M., McClelland, A.J. Elsevier/North Holland, New York, pp 151-155.

Rickard, C.G., Post, J.E., Noronha, F. and Barr, I.M. (1969). A transmissible virus-induced lymphocytic leukemia of the cat. J. Nat. Cancer Inst., 42, 987-1014.

Riggs, J.L. (1971). An immunofluorescent test for feline leukemia and sarcoma virus antigens and antibodies. J. Amer. Vet. Med. Assoc., 158, 1085-1067.

Rogerson, P., Jarrett, W.F.H. and Mackey, L. (1975). Epidemiological studies on feline leukemia virus infection, I. A serological survey in urban cats. Int. J. Cancer, 15, 781-785.

Rojko, J.L., Hoover, E.A., Mathes, L.E., Olsen, R.G. and Schaller, J.P. (1979). Pathogenesis of experimental feline leukemia virus infection. J. Nat. Cancer Inst., 63, 759-768.

Rojko, J.L., Hoover, E.A., Quackenbush, S.L. and Olsen, R.G. (1982). Reactivation of latent feline leukaemia virus infection. Nature, 298, 385-388.

Romanova, L.N. (1981). Standardization of methods used for testing the potency of rabies vaccines. In: Cell Culture Rabies Vaccines and their Protective Effect in Man. Eds. Kuwert, E.K., Wiktor, T.J., Koprowski, H. pp. 242-244. International Green Cross, Geneva.

Rowlands, D.J., Sangar, D.V. and Brown, F. (1972). Stabilizing the immunizing antigen of foot-and-mouth disease virus by fixation with formaldehyde. Archiv. fur die ges. Virusforschung, 39, 274-283.

Russell, P.H. (1977). Neutralising antibodies in feline leukaemia virus infections. Ph.D. Thesis, University of Glasgow.

Russell, P.H. and Jarrett, O. (1978a). The specificity of neutralizing antibodies to feline leukaemia viruses. Int. J. Cancer, 21, 768-778.

Russell, P.H. and Jarrett, O. (1978b). The occurrence of feline leukaemia virus neutralizing antibodies in cats. Int. J. Cancer, 22, 351-357.

Russell, P.H. and Jarrett, O. (1976). An improved assay for feline leukaemia virus pseudotypes of murine sarcoma virus. J. Gen. Virol., 31 139-143.

Salerno, R.A., Larson, V.M., Phelps, A.H. and Hilleman, M.R. (1979). Infection and immunization of cats with the Kawakami-Theilen strain of feline leukemia virus (40379). Proc. Soc. Exp. Biol. Med., 160, 18-23.

Salerno, R.A., Lechman, E.D., Larson, V.M. and Hilleman, M.R. (1978). Feline leukemia virus envelope glycoprotein vaccine: preparation and evaluation of immunizing potency in guinea-pigs and cats. J. Nat. Cancer Inst., 61, 1487.

Sarma, P.S. and Log, T. (1971). Viral interference in feline leukemia - sarcoma complex. Virology, 44, 352-358.

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

Slater, E. and York, C. (1975). Comparative studies on parenteral and intranasal inoculation of an attenuated feline herpes virus. Develop. Biol. Standard, 33, 410-416.

Sliski, A.H. and Essex, M. (1979). Sarcoma virus-induced transformation specific antigen: the presence of antibodies in cats that were naturally exposed to leukaemia virus. Virology, 95, 581-586.

Stephenson, J.R., Khan, A.S., Sliski, A.H. and Essex, M. (1977). Feline oncornavirus-associated cell membrane antigen (FOCMA): evidence for an immunologically cross-reactive feline sarcoma virus-coded protein. Proc. Nat. Acad. Sci. U.S.A., 74, 5608-5612.

Theilen, G.H., Kawakami, T.G., Rush, J.D. and Munn, R.J. (1969). Replication of cat leukaemia virus in cell suspension cultures. Nature, 222, 589-590.

Toth, S.R. (1980). Cell cycle-dependent changes in lymphoid cells infected with feline leukaemia virus. Ph.D. Thesis, University of Glasgow.

Vedbrat, S.S., Rasheed, S., Lutz, H., Gonda, M., Ruscetti, S., Gardner, M.B. and Prensky, W.C. (1983). Feline oncornavirus-associated cell membrane antigen: a viral and not a cellularly coded transformation-specific antigen of cat lymphomas. Virology, 124, 445-461.

Weijer, K. and Daams, J.H. (1978). The control of lymphosarcoma/leukaemia and feline leukaemia virus. J. Small Animal Practice, 19, 631-637.

Weksler, M.E., Ryning, F.W. and Hardy, W.D.Jr., (1975). Immune complex disease in cancer. Clinical Bull., 5, 109-113.

Wild, T.F. and Brown, F. (1968). A study of the physical properties of the immunising antigen of foot-and-mouth disease virus and the effect of various inactivating agents on its structure. Archiv. fur die Gesamte Virusforschung, 24, 86-103.

Wolff, L.H., Mathes, L.E., Schaller, J.P., Hoover, E.A. and Olsen, R.G. (1977). Detection and evaluation of feline oncornavirus-induced cell surface antigen(s) shed from cells *in vitro*. Cancer Res., 37, 4134-4137.

Wolff, L.H., Olsen, R.G., Hoover, E.A., Yohn, D.S. and Schaller, J.P. (1977). Preparation of cell-free feline oncornavirus associated cell-membrane antigen. J. Nat. Cancer Inst., 58, 791-793.

Wyke, J.A. (1975). Selection, screening and isolation of temperature-sensitive mutants of avian sarcoma viruses. Methods in Cell Biology, 14, 251-264. Academic Press, New York.

Yohn, D.S., Olsen, R.G., Schaller, J.P., Hoover, E.A., Mathes, L.E., Heding, L. and Davis, G.W. (1976). Experimental oncornavirus vaccines in the cat. Cancer Res., 36, 646-651.

Zuckerman, A.J. (1982). Developing synthetic vaccines. Nature, 295, 98-99.