# STUDIES ON FELINE IMMUNODEFICIENCY VIRUS

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## DECLARATION

The studies described in this thesis were carried out in the Department of Veterinary Pathology at the University of Glasgow Veterinary School between October 1987 and September 1990. The author was responsible for all results except where it is stated otherwise.

No part of this thesis has been presented to any university but it has been reproduced in parts in the following scientific papers:

Hosie MJ, Robertson C and Jarrett O (1989) Prevalence of feline leukaemia virus and antibodies to feline immunodeficiency virus in cats in the United Kingdom. Veterinary Record **125**, 293-29

Hosie MJ and Jarrett O (1990) Serological responses to feline immunodeficiency virus. AIDS **4**, 215-220

## SUMMARY

The two main objectives of the studies described in this thesis were to assess the value of the recently discovered feline lentivirus, feline immunodeficiency virus (FIV), as a model for human immunodeficiency virus (HIV) infection and to use this model towards the development of an effective vaccine.

Chapter 1 introduces the classification of the retrovirus family and gives an account of certain aspects of each of the lentiviruses. The literature relevant to the five areas of epidemiology, clinical signs, diagnosis, cell tropism and vaccine development is reviewed for each lentivirus.

In chapter 2 the materials and methods which are used throughout this thesis are discussed. Specific materials and methods are described at the beginning of the relevant chapter.

Chapter 3 describes an epidemiological survey of the prevalence of FIV in the UK. Blood samples from 1204 sick and 1007 healthy cats of known health status, breed, age and sex were tested for antibodies to FIV and for feline leukaemia virus (FeLV).

The prevalence of FIV was 19% in sick cats and 6% in healthy cats. For FeLV, the prevalence was 18% in sick cats and 5% in healthy cats. Both infections were found to be more common in domestic cats than in pedigree cats. FIV was more prevalent in older cats in contrast to FeLV which was more prevalent in young cats. Whereas there was no sex effect for FeLV, male cats were more likely to be infected with FIV than female cats. No epidemiological interaction was demonstrated between FIV and FeLV infection.

Of the cats which were in contact with FIV in multi-cat households, 21% had seroconverted. The prevalence of FeLV viraemia in cats in contact with FeLV was 14%. Those clinical signs which were associated with FIV were pyrexia,

gingivitis/stomatitis and respiratory signs, and with FeLV, pyrexia and anaemia. It was concluded that both viruses were significant causes of disease, and that the groups most likely to be infected with FIV were older, free-roaming male cats and for FeLV, younger, free-roaming cats.

In chapter 4 the serological responses of cats to FIV infection are examined. Firstly, the proteins of FIV were identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Purified $^{35}$S-methionine/cysteine labelled virus contained proteins of $M_r$ 120K, 24K, 17K and 10K, of which the most prominent were p24 and p17, and minor components of 62K, 54K, 52K, 41K and 32K. Sera from FIV-infected cats precipitated two glycoproteins of $M_r$ 120K (gp120) and 41K (gp41) from lysates of $^{14}$C-glucosamine labelled infected cells. Purified virus contained very little or no detectable glycoproteins.

Secondly, the serological response to individual viral proteins was followed in experimentally infected cats by immunoblotting. Since purified virus was a poor source of gp120, a method using FIV-infected cell lysates was developed. Cats produced antibodies to gp120, p55, p24 and p17. (The p55 was presumed to be a precursor of p24 and p17.) Following infection, antibodies developed first to p24 and subsequently to p17, p55 and gp120. Sera from cats infected with three separate isolates of FIV, two from the UK and one from the USA, had cross-reacting antibodies to all of these viral proteins.

Thirdly, the criteria for identification of seropositive cats were defined. The minimum requirement for a positive immunoblot was antibody to gp120 or to at least three core proteins (p55, p24 and p17). Comparison of two commercial enzyme-linked immunosorbent assay (ELISA) kits and immunoblotting indicated that false positive results occurred as a result of non-specific reactions in the ELISA systems.

Chapter 5 describes the initial isolation of FIV-8 followed by studies to optimise the production of FIV proteins for vaccine formulations. The in vitro cell tropism of two isolates of FIV was examined, both by infection with cell-free FIV-infected supernatants and by co-cultivation with FIV-infected cells. Q201 cells, which were most readily infected with FIV-8, were examined by flow cytometric analysis for changes in expression of surface markers after FIV infection. As the expression of fCD4 and the molecule recognised by monoclonal antibody vpg15 decreased with FIV-8 infection, experiments were designed to identify the receptor for FIV-8. Subsequently, vpg15 was shown to block FIV-8 infection of both Q201 cells and CRFK cells. This provided strong evidence that the molecule recognised by vpg15 was the receptor for FIV-8.

In chapter 6 the development of a system to evaluate the efficacy of vaccines against FIV is described. The minimum infectious dose of FIV-8 was determined by titration in cats and was subsequently used in challenge experiments. Post-challenge, cats were monitored for infection by virus isolation and seroconversion (measured by ELISA and immunoblot). Passive immunisation of 2 cats did not give protection against 40 infectious units of FIV. An immune stimulating complex (ISCOM) vaccine was constructed which contained FIV core proteins. A very good antibody response was made to the core proteins but there was no protection against 20 infectious units of FIV.

Chapter 7 gives an account of the attempts to induce antibodies against the envelope glycoprotein of FIV. Lysates of infected cells were used as a source of gp120. Purification of the gp120 using gel filtration and affinity chromatography was shown to be very inefficient. Therefore a vaccine was constructed which contained fixed, inactivated FIV-infected cells. Animals vaccinated with this preparation produced antibodies against the envelope proteins of FIV but only low levels of virus neutralising antibodies were achieved. None of the vaccinated

cats were protected against challenge with 20 infectious units of FIV.

Chapter 8 is a general discussion which reviews the results in this thesis and compares them with those of experiments by others. The advantages of FIV as an animal model for HIV are discussed and suggestions for future work are proposed.

# 1. INTRODUCTION

This thesis describes studies which were undertaken to assess feline immunodeficiency virus (FIV) as a model for human immunodeficiency virus (HIV) infection. The areas which were investigated were the epidemiology of FIV infection, the serological response to the infection and its diagnosis and the cell tropism of FIV. The final part of the thesis describes studies towards the development of a vacccine against FIV.

FIV belongs to the lentivirus subfamily in the retrovirus family. The virus was first isolated from cats suffering from a condition similar to that seen previously in humans with acquired immunodeficiency syndrome (AIDS) [1]. The virus has subsequently been found to be a common infection throughout the world and to be a significant cause of disease in the cat [2,3,4]. FIV is also emerging as an important model for human immunodeficiency virus (HIV) [1,5,6].

In this chapter the properties of the retroviruses and especially the lentiviruses will be described. As interest in the animal lentiviruses has increased considerably since the isolation of the human lentivirus, each of the lentiviruses will be described. The aspects which will be reviewed are the epidemiology of the infections, the clinical signs of the diseases, the diagnosis of the infections, the cell tropisms of the viruses (both in vivo and in vitro) and studies towards vaccination.

## 1.1. RETROVIRIDAE

The Retroviridae family includes all viruses containing an RNA genome and an RNA-dependent DNA polymerase (reverse transcriptase) enzymatic activity [7]. All retroviruses have common morphological, biochemical and physical properties that justify their inclusion into a single virus family. These properties are shown in table 1.1. A detailed description is

**Table 1.1**

**Taxonomic features of Retroviridae**

| | |
|---|---|
| Nucleic acid | linear, positive-sense single-stranded RNA<br>methylated cap structure at 5′ end<br>polyadenylate tract at 3′ end<br>contains 3 genes encoding structural proteins:<br>5′-gag-pol-env-3′ |
| Protein | approx. 60% by weight;<br>gag: internal structural proteins<br>pol: reverse transcriptase<br>env: envelope proteins |
| Lipid | approx. 35% by weight; derived from cell membrane |
| Carbohydrate | approx. 4% by weight; associated with env proteins |
| Density | 1.16-1.18 $gml^{-1}$ in sucrose |
| Morphology | spherical enveloped virions (80-120 nm diameter)<br>variable surface projections<br>icosahedral capsid<br>ribonucleoprotein complex with a core shell |

given by Weiss et al [8]. The family is divided into 3 subfamilies:

**i. Oncovirinae**

This subfamily includes the oncogenic and closely related non-oncogenic viruses. There are 4 distinct subgroups: type-A, type-B, type-C and type-D viruses. The oncoviruses of human and veterinary importance are C-type and are exogenous, being spread either by contact or congenitally. There are also many endogenous oncoviruses which do not normally occur in an infectious form. These viruses exist in cellular DNA as proviruses and are transmitted genetically. They may be entirely latent, partially expressed or some may be spontaneously activated and cause disease.

**ii. Lentivirinae**

Lentiviruses establish persistent infections and characteristically cause chronic, slowly progressive diseases. The members of the group are human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), visna virus (VV), caprine arthritis encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and bovine immunodeficiency virus (BIV).

**ii. Spumavirinae**

Spumaviruses are the "foamy" viruses which establish persistent infections in several species but are generally considered apathogenic. They may contaminate primary cell cultures and cause intense vacuolation of the cell cytoplasm and very marked syncytium formation.

## 1.2. LENTIVIRINAE

### 1.2.1. Morphology

Lentiviruses are typically 100-150nm in diameter with a bar or cone shaped core. The virus particles bud from cell membranes and the envelope has short spikes which can be seen on the cell surface but which are lost on budding.

### 1.2.2. Genetic Organisation

The genome of lentiviruses is a positive-stranded polyadenylated RNA of 9 to 10 kilobases. Although the structure and function of the viral genes has been studied most extensively for HIV-1, it is likely that all lentiviruses are similar. Figure 1.1 shows the genetic organisation of HIV-1. The genome contains three genes (gag, pol and env) which are common to all retroviruses. The genome is flanked by long terminal repeats (LTRs) which do not code for any protein but contain regulatory elements. In addition, lentiviruses have unique open reading frames (ORFs) 3′ to the pol gene which have regulatory functions. Whereas other retroviruses require dividing cells, lentiviruses require activation and/or differentiation of the host cell for productive replication. These additional ORFs probably account for the ability of lentiviruses to replicate in non-dividing, end-stage cells by providing either additional virus-encoded replication functions or by activating the non-dividing cells to synthesise proteins and other factors necessary for DNA replication and integration.

In HIV, the gag-pol region is transcribed in a single genome length mRNA from which 2 proteins are translated: the gag precursor (p55) and a gag-pol fusion protein of $M_r$ 160 x $10^3$. The gag gene product, p55, is cleaved to the 3 core proteins, the matrix protein p18 (MA), the capsid protein p24 (CA) and the nucleocapsid protein p13 (NC). The pol gene encodes the enzymes reverse transcriptase (RT), endonuclease (EN) and integrase (IN).

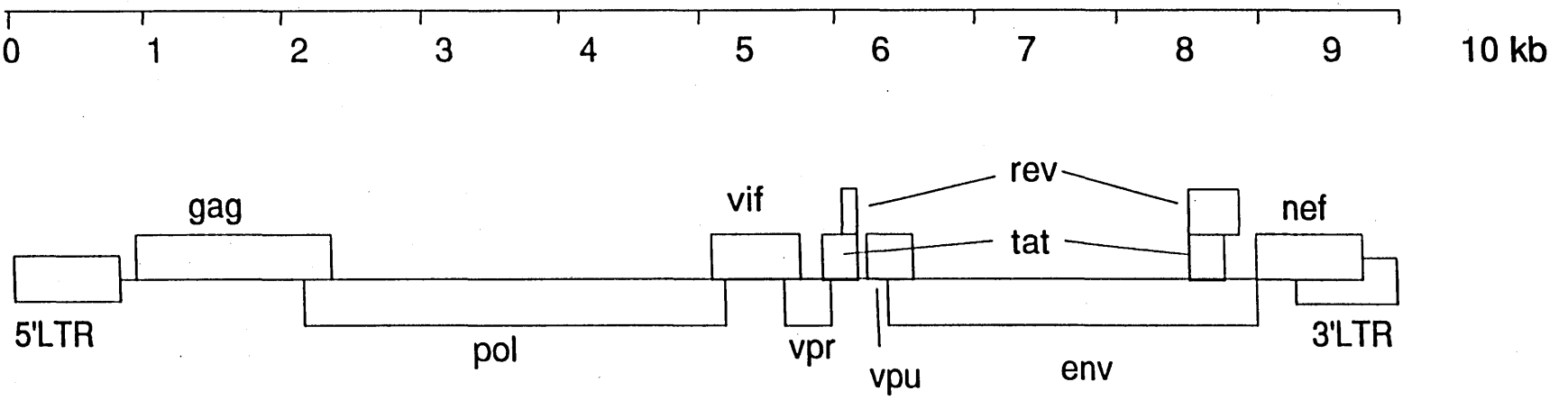



Figure 1.1

Genomic organisation of HIV-1 (courtesy of JC Neil)

The *env* gene encodes a large polyprotein of $M_r$ 100 x $10^3$ which becomes glycosylated to give the envelope precursor gp160. The precursor is cleaved to give the surface glycoprotein gp120 (SU) and the transmembrane protein gp41 (TM). The only notable antigenic cross-reactivities and nucleic acid sequence homologies among lentiviruses have been demonstrated for the *gag* and *pol* genes.

The regulatory genes are *tat*, *rev*, *vif*, *nef*, *vpr* and *vpu* (Figure 1.1). In HIV-2 and SIV there is an additional gene, *vpx*. The *tat* gene codes for a transactivator of viral gene expression which binds the transactivator receptor (TAR) element in the R region of the LTR and thereby promotes the expression of other viral proteins. The products of both *tat* and *rev* are necessary for viral protein synthesis and the *vif* protein is necessary for the production of fully infectious virus particles. The *nef* product is thought to reduce virus expression. The functions of *vpr*, *vpu* and *vpx* are unknown.

At both the 5′ and 3′ LTRs are sequences containing the cap site, the polyadenylation signal and the termination signal for viral transcription (R region). Sequences at the 5′ LTR in the U3 region contain the enhancer-promoter elements for initiation of RNA transcription. The primer binding site for the synthesis of minus strand viral DNA is immediately downstream of the U5 region and for the plus strand is immediately upstream of the U3 region.

### 1.2.3. Replication

Whereas other retroviruses synthesise proviral DNA in the cytoplasm, lentiviruses replicate in the nucleus, the site of cellular DNA synthesis. Also, in contrast to other retroviruses, lentivirus-infected cells contain only a small proportion of circularised DNA and little integrated DNA. There are large amounts of unintegrated, linear DNA in the nucleus of the infected cell which may be associated with cytopathogenicity [9].

The mechanism of control of latency in lentiviral infections is not known but it has been shown that signals which can activate cell division and differentiation may lead to proviral expression and the release of large amounts of infectious virus, causing the cell lysis which is typical of lentiviruses [10].

## 1.3. REVIEW OF LENTIVIRUS LITERATURE

### 1.3.1. Human immunodeficiency virus

The acquired immunodeficiency syndrome (AIDS) was first described in 1981 when cases of Pneumocystis carinii pneumonia (PCP) and Kaposi's sarcoma (KS) were seen in previously healthy young male homosexuals. The syndrome was soon found in other groups, including intravenous drug users, recent Haitian immigrants, haemophiliacs, recipients of blood transfusions, sexual partners of individuals either having or at risk of having the disease and offspring of mothers with or at risk of the disease. In 1983-1984, researchers in France and the United States independently identified the cause of AIDS. Termed lymphadenopathy-associated virus (LAV) [11], human T cell lymphotropic virus-III (HTLV-III) [12] and the AIDS associated retrovirus (ARV) [13], the virus was renamed human immunodeficiency virus (HIV) in 1986 [14].

There are two distinct serotypes of HIV, HIV-1 and HIV-2. Whereas HIV-1 is primarily found in Central Africa, Europe and the United States, HIV-2 has mainly infected individuals in countries in West Africa. HIV-2 has a similar ultrastructure and demonstrates the same cell tropism as HIV-1, but the overall nucleotide sequence homology between the two serotypes is only 42% [15]. Indeed HIV-2 shows greater sequence homology to the simian immunodeficiency virus ($SIV_{mac}$) which was isolated from captive rhesus macaques. $SIV_{agm}$ was isolated from wild African Green monkeys and is approximately equally related to HIV-1 and HIV-2 [16]. A virus ($SIV_{cpz}$) isolated from a chimpanzee in Gabon has been shown to be closely related to HIV-1 [17].

#### 1.3.1.1. Epidemiology

The infection is worldwide and it is estimated that 1 to 2 million people in the United States and 5 to 10 million in Africa and other countries are infected with HIV [18]. It appears that the majority of people infected with HIV will eventually develop AIDS.

HIV is transmitted by direct contact of genital or rectal mucosa with infected semen or vaginal secretions, by direct inoculation of contaminated blood or by perinatal infection. Extensive evidence suggests that the virus is rarely, if ever, transmitted via sneezing, coughing, contact with skin or oral mucosal membranes or by insect vectors [18].

There are 3 epidemiological patterns [19]. The first pattern arises from heterosexual transmission and occurs in Africa, the Caribbean and parts of South America. In these areas there is an equal prevalence in males and females and the major risk factor is the number of sexual partners. Perinatal and parenteral transmission via contaminated blood are also important routes of infection. The second pattern of transmission arises where homosexual transmission occurs and where there is intravenous drug use. In this situation the major risk factors are male homosexuality, intravenous drug use, blood transfusion and haemophilia. The third pattern is seen in areas where HIV has been recently introduced, such as Asia, and the Middle East. In these areas the incidence of infection is low and the rate of spread will depend on the prevalent social habits.

#### 1.3.1.2. Clinical Signs

The clinical manifestations of AIDS are reviewed by Volberding and McCutchan [18]. After infection with HIV, there is an assymptomatic phase lasting 5 to 10 years. A minority of infected individuals will develop AIDS within 1 to 5 years. Shortly after infection there may be an influenza-like illness lasting 3 to 14

days. There may be fever, lethargy, lymphadenopathy and other non-specific signs. The lymphadenopathy may persist. As the immune system becomes increasingly impaired, syndromes such as oral candidiasis, herpes zoster and oral hairy leukoplasia may appear and resolve. The clinical forms of AIDS vary depending on risk group and region. KS is more common in homosexual men compared to haemophiliacs and PCP is more common in recipients of blood transfusions compared to homosexuals. Toxoplasmosis is more common in Europe than in the United States and tuberculosis and cryptococcal meningitis are more common in Africa.

1.3.1.3. Diagnosis

Infection is diagnosed by the demonstration of antibodies or, less commonly, antigen in the blood. Serum antibody is detected by ELISA and positive samples are confirmed by immunoblotting. Antibodies may be detected between 2 weeks and 3 months after infection [20]. The antigen tests for p24 are useful in early infection, before antibodies have developed, and in rare individuals who do not develop antibodies.

1.3.1.4. Cell tropism

Infected haematopoietic cells are the most frequent source of infectious virus in infected individuals [21]. HIV has been detected in lymphocytes, macrophages, follicular dendritic cells and megakaryocytes. To recover HIV, the optimal culture procedure involves co-cultivation of the cells or tissue with mitogen-stimulated peripheral blood mononuclear cells from normal donors, propagated in medium containing interleukin-2 (IL-2).

The in vivo depletion of CD4+ helper T-cells in HIV-infected individuals [22] and the in vitro tropism of HIV for cells bearing the CD4+ surface antigen [23] suggested that the receptor for HIV was CD4. HIV infects T-cells via an interaction between the envelope protein gp120 and CD4. This interaction can be blocked by monoclonal antibodies against CD4 [23,24,] and by

recombinant soluble CD4 [25,26]. Human monocytes and macrophages also express CD4 and can be infected with HIV, possibly becoming the main reservoir of infection and transporting the virus to various organs, including the brain.

Since a number of CD4- cells have been shown to be infected in vitro, it is possible that alternative receptors for HIV exist. McKeating et al [27] demonstrated that HIV can infect CD4- cells via $F_c$ receptors when complexed with low levels of antibody. $F_c$ receptors on macrophages have been implicated in enhancement of HIV infection.

#### 1.3.1.5. Vaccination

Chimpanzees have been protected from infection by HIV-1 after vaccination with recombinant gp120, showing no signs of infection after more than 6 months [28]. The immunogen was prepared in an aluminium hydroxide adjuvant and inoculated at 0, 1 and 8 months. Significant levels of neutralising antibodies were achieved. The animals were challenged at week 35 with 10 infectious units of homologous virus.

### 1.3.2. Simian immunodeficiency virus

SIV was first isolated from captive rhesus macaque monkeys, Macaca mulatta ($SIV_{mac}$) [29]. Subsequently, many isolates of SIV have been obtained from a variety of species of captive and wild monkeys, including sooty mangabey monkeys, Cercocebus atys ($SIV_{sm}$), pig-tailed macaques, Macaca nemestrina ($SIV_{mne}$), cynomolgus monkeys, Macaca fascicularis and African green monkeys, Cercopithecus aethiops ($SIV_{agm}$). With the exception of $SIV_{agm}$ isolates, which have not been tested extensively, experimental infection of macaques with other SIV isolates results in immunosuppressive disease similar to the disease associated with HIV infection.

### 1.3.2.1. Epidemiology

Antibodies to SIV were found in 26-42% of sera from wild African green monkeys from east Africa [30,31] although there is no evidence of disease in SIV-infected African green monkeys. The prevalence of the infection and the routes of transmission in the wild are unknown. In colonies of captive monkeys where SIV is enzootic, prenatal and contact transmission occur.

None of the SIV strains characterised so far are closely related to HIV-1. HIV-2, on the other hand, is closely related to $SIV_{mac}$ and $SIV_{smm}$. $SIV_{agm}$ is eqidistant between HIV-1 and HIV-2 [32]. As Asian macaques are not infected with SIV in the wild, captive macaques probaly acquired SIV by cross-species transmission from an SIV-infected African primate in a primate centre. This hypothesis is supported by both molecular and epidemiological data [32]. The unusually close relationship between SIV from captive macaques with HIV-2 has led to the suggestion that primates in captivity became infected from a human being with HIV-2 infection (Dr Hayami, personal communication).

### 1.3.2.2. Clinical signs

The clinical signs seen in experimentally infected macaques are similar to those of HIV infection. There is a relatively short acute phase with viraemia, the level of which decreases as the antibody titre increases. There is an asymptomatic phase which may include sporadic episodes of disease and this is followed, after a variable period, by chronic progressive signs and death from immunosuppression and opportunistic infections. A detailed description of the clinical manifestations and pathology of SIV infection is given by Fultz and Anderson [33].

### 1.3.2.3. Diagnosis

SIV is diagnosed by the presence of antibody using ELISA, immunoblotting or neutralisation tests. Virus can be isolated by

co-cultivation of peripheral blood T-cells with human T-cells and virus can be identified by immunofluorescence, electron microscopy or reverse transcriptase assay of culture fluids.

1.3.2.4. Cell tropism

Studies to assess the cellular tropism of various SIV isolates have identified some differences as well as similarities between HIV and SIV. While both viruses are tropic for CD4+ lymphocytes and macrophages, in vitro replication of at least some SIV strains in human CD4+ cells is more restricted than HIV. It appears that in addition to binding CD4, another molecule is required for virus entry into cells [34].

1.3.2.5. Vaccination

Several groups have demonstrated that vaccines derived from whole virus can induce immune responses that protect against infection [35,36,37].

**1.3.3 Feline immunodeficiency virus**

FIV is the most recently discovered lentivirus and is associated with immunosuppression in domestic cats. Like the human and simian lentiviruses, FIV is T-lymphotropic, persists in infected animals and causes severe immunodeficiency in the natural host [1]. There is a significant degree of genetic relatedness between FIV and other lentiviruses, especially in the gag-pol genes [5] and common antigenic determinants are shared by the major core proteins of FIV, CAEV and VV [5]. The organisation of its small ORFs is most similar to that of VV, although the cell tropism and pathogenesis of FIV infection are most similar to those of HIV and SIV.

1.3.3.1. Epidemiology

FIV has been detected wherever attempts have been made to

identify infected cats. Several serological surveys have reported similar findings for the prevalence of infection in the USA, the UK and France; 10 to 19% of sick cats and 1 to 6% of healthy cats [4,38,39,40,41]. The prevalence was shown to be markedly higher in Japan [2] and very low in the Netherlands, Germany and Switzerland [38,42]. Male cats are more commonly infected than female cats and transmission occurs by biting.

1.3.3.2. Clinical signs

After experimental infection with FIV, kittens develop pyrexia which lasts a few days, neutropaenia which lasts a few weeks and lymphadenopathy which persists for several months [3]. Animals appear normal after this primary phase but following a long latent period, cats develop signs of immunodeficiency.

The most common clinical signs are lethargy, anorexia, weight loss, pyrexia and lymphadenopathy [43]. Other common signs are gingivitis, diarrhoea and rhinitis [4,43,44]. Neurological deficits may occur [45]. A significant correlation has been found between FIV infection and lymphoproliferative malignancies [46]. Opportunistic infections such as Toxoplasma gondii and Demodicosis have been reported [47,48]. Pre-existing infection with FeLV potentiates the severity of the transient primary and chronic secondary stages of FIV infection [49].

1.3.3.3. Diagnosis

Diagnosis of infected animals is by demonstration of antibodies by ELISA or immunoblotting [1,2,3,6,50]. Virus can be isolated from the peripheral blood T-cells of infected cats [1,51].

1.3.3.4. Cell tropism

Like HIV and SIV, FIV is T-lymphotropic. Macrophages can be infected in vivo and in vitro [52]. Some molecular clones infect feline fibroblasts in vitro but replicate less efficiently on

feline peripheral blood T-cells [53].

1.3.3.5. Vaccination

Preliminary vaccine trials have shown that inoculation with inactivated FIV-infected cells or purified virus will protect cats from infection [54].

### 1.3.4. Visna virus

Visna virus was the first lentivirus to be isolated and characterised. The virus may also be called maedi virus or maedi-visna virus. The virus is associated with a pneumo-encephalitic disease complex (maedi, laboured breathing: visna, paralysis and wasting, in Icelandic) which occurred in epidemic form in Iceland following the introduction of latently infected sheep from Europe during the 1930s.

1.3.4.1. Epidemiology

The infection is found in sheep throughout the world except in Australia or New Zealand. The prevalence of the disease is low, although certain breeds (such as Border Leicester sheep in the USA and Texel sheep in the Netherlands) seem to be more susceptible. The virus is transmitted primarily in milk although direct transmission may occur, especially where there is overcrowding. In infected flocks the prevalence increases with age, from 20% in year-old lambs to 80% in animals older than 7 years. In the majority of sheep, infection is asymptomatic [10].

1.3.4.2. Clinical signs

The main clinical feature is a chronic progressive interstitial pneumonia (maedi) with lymphoid hyperplasia. The pneumonic form is more common than the neurological form (visna) which was once common in Iceland, causing paralysis due to a severe meningoencephalitis, often with demyelination. Many infected

sheep also have an indurative mastitis.

1.3.4.3. Diagnosis

Diagnosis of infected animals is by demonstration of antibodies by immunodiffusion or ELISA.

1.3.4.5. Cell tropism

The monocyte/macrophage is the principal target for viral replication. The virus is latent in monocytes and becomes activated with cell differentiation. There is a persistent, cell-associated viraemia by which virus spreads to many other organs.

1.3.4.5. Vaccination

Attempts have been made to vaccinate sheep using whole virus inactivated by UV irradiation as well as envelope glycoprotein gp135 concentrated from cell culture supernatant and purified on lectin columns [55]. On challenge with live virus by the respiratory route there was no protection.

**1.3.5. Caprine arthritis encephalitis virus**

A close relationship exists between the visna virus of sheep and the CAEV. Persistently infected goats are common but disease is rare.

1.3.5.1. Epidemiology

CAEV occurs worldwide. Transmission studies have shown that infection usually occurs via colostrum or milk and rarely by direct contact [56]. Age does not appear to play a role in susceptibility.

#### 1.3.5.2. Clinical signs

The most common clinical sign is chronic arthritis, often accompanied by bursitis. Virus is expressed in the synovial membranes of the joints soon after experimental infection and can be isolated from synovial membrane biopsies of clinical cases. Neurological signs may occur, especially in young kids, with foci of inflammatory cells and demyelination throughout the white matter of the brain and spinal cord. Other signs which can be associated with infection are pneumonia, mastitis, interstitial nephritis, myocarditis and hyperplasia of the lymphoid tissues [56].

#### 1.3.5.3. Diagnosis

Diagnosis of infected animals is by demonstration of antibodies by immunodiffusion or ELISA. Antibody titres are higher to gp135 than to p28 and therefore tests which use gp135 as antigen are preferable [57].

#### 1.3.5.4. Cell tropism

As in VV infection, the main target cell for CAEV is the monocyte. In vitro, the virus will grow in cells from choroid plexus, foetal testicle, cornea, synovial membrane and in a permanent cell line from a Himalayan tahr ovary [56].

### 1.3.6. Equine infectious anaemia virus

#### 1.3.6.1. Epidemiology

EIAV occurs in south-eastern USA, south and central America, southern Africa and northern Australia. The virus is transmitted mechanically from viraemic horses by biting flies (Stomoxys spp.), mosquitos, midges and iatrogenically via unsterilised hypodermic needles.

### 1.3.6.2. Clinical signs

The clinical signs of EIAV have been reviewed by Narayan and Clements [10]. The disease may occur as an acute syndrome which is fatal within one month of infection or as a chronic relapsing disease causing cachexia and chronic anaemia. Some infected animals, however, never develop the disease but act as carriers of the virus. After infection, horses develop fever, anorexia and anaemia which lasts a few days and is followed by a period of clinical normality which may last for weeks or months. There are three or four cyclical recrudescences of the disease before the onset of chronic wasting. Some animals remain clinically normal although stress or the administration of corticosteroids may cause an episode of disease. Acute disease is characterised by haemolytic anaemia and haemorrhages and necrosis of hepatocytes and lymphocytes in the spleen and lymphatic tissues [58].

The disease is immune-mediated, the anaemia resulting from complement-dependent lysis of erythrocytes which have become coated in viral antigen and the pyrexia resulting from circulating immune complexes containing EIAV. Glomerulonephritis is also common as a result of the deposition of immune complexes in the kidney. The periodic onset of disease is associated with the appearance of new antigenic variants, followed by a period when the virus is latent, probably as a result of specific neutralising antibody.

### 1.3.6.3. Diagnosis

Diagnosis of infected animals is by demonstration of antibodies by immunodiffusion or ELISA.

### 1.3.6.4. Cell tropism

In the infected animal, EIAV primarily infects and destroys peripheral and tissue macrophages. In vitro, EIAV can be propagated in equine peripheral blood mononuclear cells and can

be adapted to grow in foetal equine kidney and foetal donkey dermal cells [59].

1.3.6.5. Vaccination

Vaccination using formalin-inactivated EIAV in muramyl dipeptide induced neutralising antibodies. After challenge, 9 of 12 ponies were shown to be carriers of EIAV but none had clinical signs and therefore vaccination prevented disease [60].

### 1.3.7. Bovine immunodeficiency virus

The bovine lentivirus was first described by Van Der Maaten et al [61]. There has been little research of the pathogenesis, host immune response or epidemiology of BIV infection of cattle.

1.3.7.1. Epidemiology

Preliminary seroepidemiological studies suggest that BIV is sporadically present in dairy cattle in the USA [62]. Virus may be transmitted iatrogenically via unsterilised hypodermic needles.

1.3.7.2. Clinical signs

Cattle infected with BIV have signs of lymphadenopathy, lymphocytosis, central nervous system lesions, progressive weakness and emaciation [10].

1.3.7.3. Diagnosis

Diagnosis of infected animals is by demonstration of antibodies by immunofluorescence or immunoblotting [63].

# 2. MATERIALS AND METHODS

## 2.1. MATERIALS

### 2.1.1. Cells

Normal feline T-cells were isolated from peripheral blood as described in section 2.2.2. and normal cat thymocytes were isolated as described in section 2.2.3. These cells could be maintained in culture containing recombinant human IL-2 for many months.

Q201 cells were antigen-specific T-cells against ovalbumin. A cat was immunised three times with ovalbumin ISCOMs. Each inoculation was given at 4 weekly intervals and contained 1mg ovalbumin and 50ug Quil A (Oswald Jarrett, personal communication). Two weeks after the third inoculation, peripheral blood T-cells were isolated using the method described in section 2.2.2. The cells were cultured with autologous antigen presenting cells in the presence of 50ug per ml ovalbumin. The culture was incubated for 4 days and re-cultured for a week without antigen but in the presence of IL-2. Further antigen presentations were made at monthly intervals for 4 months after which the cells were seen to proliferate without further antigen presentations. Q201 cells remained IL-2 dependent. P51 cells were developed using similar methods as for Q201 cells but in this case the antigen was FeLV rather than ovalbumin (Angela Pacitti, personal communication). P51 cells were shown to produce small amounts of FeLV (Angela Pacitti, Oswald Jarrett and Helen Laird, personal communications).

3201 is an FeLV nonproducer line established from a naturally occuring feline lymphosarcoma [64]. FL74 is also a permanent feline lymphoblastoid cell line derived from a lymphosarcoma but is productively infected with FeLV [65]. F422 was established from a thymic lymphosarcoma of a kitten inoculated with the second passage of the Rickard strain of FeLV [66]. F422-MD cells

are a variant producing non-infectious FeLV (Oswald Jarrett, personal communication). T3 was derived from an FeLV-positive feline thymic lymphosarcoma with myc-containing proviruses [67]. McCoy cells were derived from a neoplastic feline mesenteric lymph node [68] and are negative for FeLV.

Crandell feline kidney cells (CRFK) were derived from the renal cortical cells of a normal domestic kitten [69]. A clone of CRFK, designated ID10 (kindly provided by P Andersen), had been shown to support the growth of the Petaluma strain of FIV. The ID10 cells were transfected with ras and myc genes to give the HO6TI and MCGM1 lines, respectively (kindly provided by D Spandidos). AH927 cells were derived from a feline embryo culture which underwent spontaneous transformation [70].

### 2.1.2. Experimental Cats

The experimental cats were obtained from the specific pathogen free cat colony of the University of Glasgow Department of Veterinary Pathology. The cats were bred within the colony and were in housed in small groups. The cats used in the the present studies were randomised according to age and sex before being assigned to the experimental and control groups.

## 2.2. METHODS

### 2.2.1. Cell Culture

Cells were routinely grown in $25cm^3$, $80cm^3$ or $175cm^3$ plastic flasks in an atmosphere of 5% $CO_2$ in air and were incubated at $37^oC$. All cells were cultured in RPMI-1640 (Gibco, UK) with 10% foetal bovine serum (Imperial laboratories, UK), 2mM glutamine (Gibco, UK), 100 units penicillin per ml (Gibco, UK), 10ug streptomycin per ml (Gibco, UK) and $2 \times 10^{-5}$ moles per ml 2-mercaptoethanol (Sigma). This medium was supplemented with 100 units per ml recombinant human IL-2 (kindly provided by Jack Nunberg, Cetus Corporation) for peripheral blood T-cells,

thymocytes, Q201 and P51 cells.

Suspension cells (T-cells, thymocytes, Q201, P51, 3201, FL74, F422, T3 and McCoy) were grown at a concentration of between 5 x $10^5$ and 1.5 x $10^6$ cells per ml. They were subcultured every 3 to 4 days. On occasions where there was a high percentage of dead cells, Ficoll-Hypaque solution (Pharmacia, Sweden) was used to remove these cells from the culture. A volume of 10mls of suspension culture was gently layered on top of 10mls Ficoll-Hypaque in a 25ml centrifuge tube and was spun at 2000rpm for 10 minutes. The live cells, which collected in a band at the interface, were removed and diluted to 20mls in RPMI-1640 and spun again at 1000rpm for 5 minutes. The cell pellet was then resuspended at the appropriate density in culture medium.

Monolayer cells (ID10, HO6TI, MCGM1 and AH927) were subcultured at a ratio of 1:4 twice weekly. The culture fluid was removed and the adherent cell monolayers were dispersed by rinsing twice in a solution of 0.01% trypsin and 0.02% EDTA in RPMI-1640. The cells were diluted with culture medium, centrifuged at 1000rpm for 5 minutes and resuspended in fresh culture medium.

### 2.2.2. Isolation of peripheral blood T-cells

T-cells were isolated from 2 to 20mls heparinised blood using the Ficoll-Hypaque method. The heparinised blood sample was diluted in 3 times its volume of RPMI-1640 and transferred carefully as an overlay to a 25ml centrifuge tube containing 10mls Ficoll-Hypaque solution (smaller tubes were used when only small samples were available). The blood was centrifuged at 2000rpm for 10 minutes with the brake switched off to minimise swirling. The pellet contained erythrocytes and granulocytes and the interface contained the mononuclear cells. The interface was aspirated and transferred to a clean tube, diluted with at least a 5-fold excess of medium, mixed and centrifuged at 1000rpm for 5 minutes to pellet the peripheral blood mononuclear cells. The cells were resuspended in medium at a cell concentration of 5 x $10^5$ cells

per ml and the peripheral blood T-cells were stimulated to grow by the addition of 7.5ug per ml Concanavalin A (Con A) (Sigma, UK). After 2 or 3 days in Con A, the unattached cells were collected by centrifugation and were resuspended in a new flask in medium without the addition of Con A.

### 2.2.3. Isolation of Thymocytes

To prepare a culture of thymocytes, the thymus from a normal kitten was taken aseptically into RPMI-1640. The tissue was placed in a petri dish and was teased aseptically using forceps and a scalpel to expel the cells. When the fragments of tissue could be broken no further, the thymocytes were resuspended in culture medium and centrifuged at 1000rpm for 5 minutes. The cell pellet was resuspended in 10mls medium and and aspirated with a pipette to distribute the cells evenly. Any clumps which would not disperse were discarded. The thymocytes were washed twice and resuspended in normal T-cell medium containing Con A. After 3 days the thymocytes were cultured in medium without Con A.

### 2.2.4. Growth of FIV-Glasgow-8 (FIV-8)

The isolation of FIV-8 is described in chapter 5. The virus was grown in cat peripheral blood T-cells (PBTs). Approximately 6 x $10^6$ PBTs were resuspended in FIV-8-infected supernatant overnight. Five days later the medium was changed and the same volume of uninfected PBTs was added to the culture. Three days later the medium was again changed and uninfected cells were added at a ratio of 5 to 1. This was repeated 4 days later. The supernatant collected each time the medium was changed was filtered through a 0.45um filter and frozen at -70$^{o}$C.

### 2.2.5. Production of FIV-8 stock

FIV-8 was grown in normal cat thymocytes. Uninfected thymocytes were incubated overnight with 50 mls of FIV-8-infected tissue culture supernatant at a concentration of 2 x $10^6$ cells per ml.

The following day the cells were washed and the concentration of cells was adjusted to 5 x $10^5$ cells per ml. The culture was monitored for cytopathic effect and when this was evident (5 days post-infection), the cell suspension was centrifuged at 1000rpm for 5 mins and the supernatant was filtered through a 0.45 um filter and frozen in 1ml volumes at -70$^{o}$C. Assay of virus infectivity in P51 cells showed the virus titre to be $10^4$.

### 2.2.6. Detection of Viral Antigen

FIV p24 antigen in tissue culture supernatants was detected using a monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) kit (FIV antigen detection kit, Idexx) which had a sensitivity limit of 0.2ng per ml [71].

# 3. EPIDEMIOLOGICAL SURVEY

## 3.1. INTRODUCTION

The primary aim of this survey was to assess the prevalence of FIV in the UK and to compare this with the prevalence of FeLV. The survey was designed to be representative of the whole feline population. The data for each infection were examined for evidence of any associations between prevalence and disease status, sex, age and breed in order to identify those groups most likely to be infected. Data from multi-cat households were analysed to assess the relative transmissibility of each virus. The clinical signs which could be associated with each infection were examined.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Blood samples

Blood samples were taken into heparin although occasionally clotted blood was used. Heparinised blood or serum was centrifuged at 2000 rpm for 10 minutes and the fluid was separated. Plasma and serum samples were stored at -80 $^{o}$C. Repeated freezing and thawing of samples was avoided; the majority of samples were tested for both viruses before storage and the remainder were frozen and thawed once prior to testing.

**i.Sick cats**

The samples from cats showing clinical signs were submitted to the Feline Virus Unit (FVU) diagnostic laboratory at the University of Glasgow by veterinary practitioners throughout the UK. These samples were from cats of all ages and breeds, in which clinical signs had been observed which may have been associated with FeLV or FIV infection.

**ii.Healthy cats**

The samples from cats without clinical signs were from two sources. The FVU laboratory routinely received samples from cats as part of a pre-breeding screening programme for FeLV , as well as from healthy cats which may have been exposed to other cats infected with FeLV. This population consisted predominantly of young pedigree cats. In order to obtain samples from healthy non-pedigree cats, veterinary practitioners throughout the UK were requested to submit samples from any healthy cats which were presented for elective surgery, such as castration or spaying, orthopaedic operations, de-matting procedures or as a result of road traffic accidents.

### 3.2.2. Diagnostic tests

A commercial ELISA (PetChek feline T lymphotropic lentivirus antibody test kit, Idexx) was used to detect antibodies against FIV. Samples were screened for FeLV antigen using an ELISA to detect p27 antigen and positive results were confirmed by virus isolation [72].

### 3.3.3. Database

A computer database was constructed to record the details of each cat tested. The data covering health status, age, sex, and breed were complete for 2211 cats. Any clinical signs which were given on the submission form were noted for the sick cats and the reason for consultation was noted for the healthy cats. This information could be related to the results of the FIV and FeLV tests. Only single submissions were included in this analysis.

In the database 1190 cats belonged to multi-cat households, 205 were from single cat households and the remainder were from households with unknown numbers of cats. There were 72 multi-cat households, containing 301 cats, in which at least one cat was FIV-positive and 28 households, containing 137 cats, in which FeLV was present.

### 3.3.4. Statistical analysis

Four factors were considered in the analysis of the prevalence of FIV and FeLV, namely health status, age, sex and breed. Health status was recorded as either sick (if clinical signs were present) or healthy (if there were no clinical signs). Cats which were sampled while undergoing dental procedures were excluded from the analysis as gingivitis may have been present in these cases. Therefore these cats could not faithfully be categorised as healthy. Cats were divided into four groups by their age at the time of sampling, the first being less than or equal to 1 year of age, the second being less than or equal to 5 years of age and over 1 year, the third being less than or equal to 10 years of age and over 5 years and the final group of cats which were over 10 years of age. The sex was recorded as entire male, neutered male, entire female or neutered female. For the analysis, the breeds were classified into two groups. The domestic cats comprised domestic shorthaired and domestic longhaired cats and all of the pedigree cats were analysed as one group.

The analyses of the prevalence of both FIV and FeLV was by log-linear modelling [73]. The four factors of health status, age, sex and breed which were considered in the analyses gave rise to 64 combinations. For each combination, the proportion of cats which was positive in each test was recorded. Unlike a comparison of percentages, which are calculated ignoring the other factors, the model gave parameter estimates which controlled for the other factors. The parameter estimates were kindly calculated by C. Robertson. The more positive the value of the parameter estimate, the greater the likelihood of infection.Therefore it was possible to determine which of the factors influenced the proportion of positive cats, as well as the relative magnitude of their effects. The analyses also included the investigation of interactions between any two of the four factors.

The prevalence of the two viruses in in-contact cats in pedigree

and domestic households were compared by Chi-squared analysis. The occurrence of clinical signs which, from previous reports, were believed to be associated with infection by each virus, was compared in infected and uninfected cats in the same way.

## 3.3 RESULTS

### 3.3.1. Prevalence of FIV and FeLV

A total of 2211 cats was included, comprising 1204 sick cats, together with 1007 healthy cats which represented a control population. Of the sick cats, 3% were positive for both FIV and FeLV, 15% were positive for FIV alone, 14% were positive for FeLV alone and 67% were negative for both FIV and FeLV. The percentages of infected cats were statistically significantly lower in the healthy cats. Of the healthy cats 0.5% were positive for both FIV and FeLV, 5% were positive for FIV alone, 4% were positive for FeLV alone and 90% were negative for both FIV and FeLV.

A different pattern emerged for the age distribution of FIV or FeLV infected sick cats (Figure 3.1). The peak of FIV infection was seen in cats between the ages of 5 and 10 years whereas the peak of FeLV infection was in cats between 1 and 5 years of age. The percentages of FIV or FeLV infected healthy cats were much lower for each age group (Figure 3.2).

Initially the data for FIV and FeLV were analysed separately, in order to examine associations between prevalence and health status, breed, age and sex. In addition, the data for FIV and FeLV were analysed together to determine whether or not the two viruses behaved independently.

### 3.3.2. FIV Analysis

The prevalence in sick cats was 19% compared to 6% in healthy cats. All four of the factors analysed were found to have an

**Figure 3.1**

Bar chart showing the age distribution of the percentages of sick cats which tested positive for FIV and FeLV

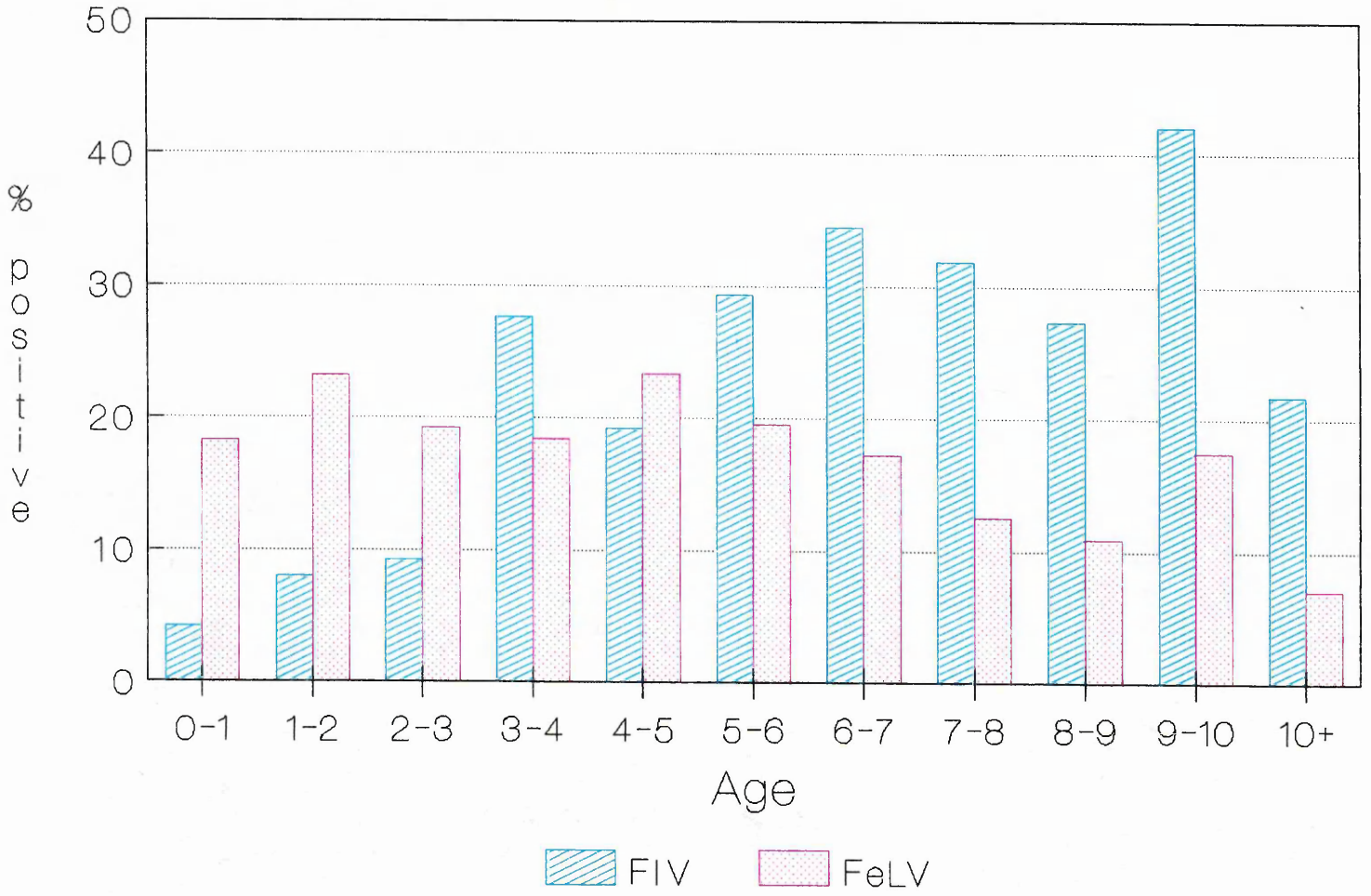
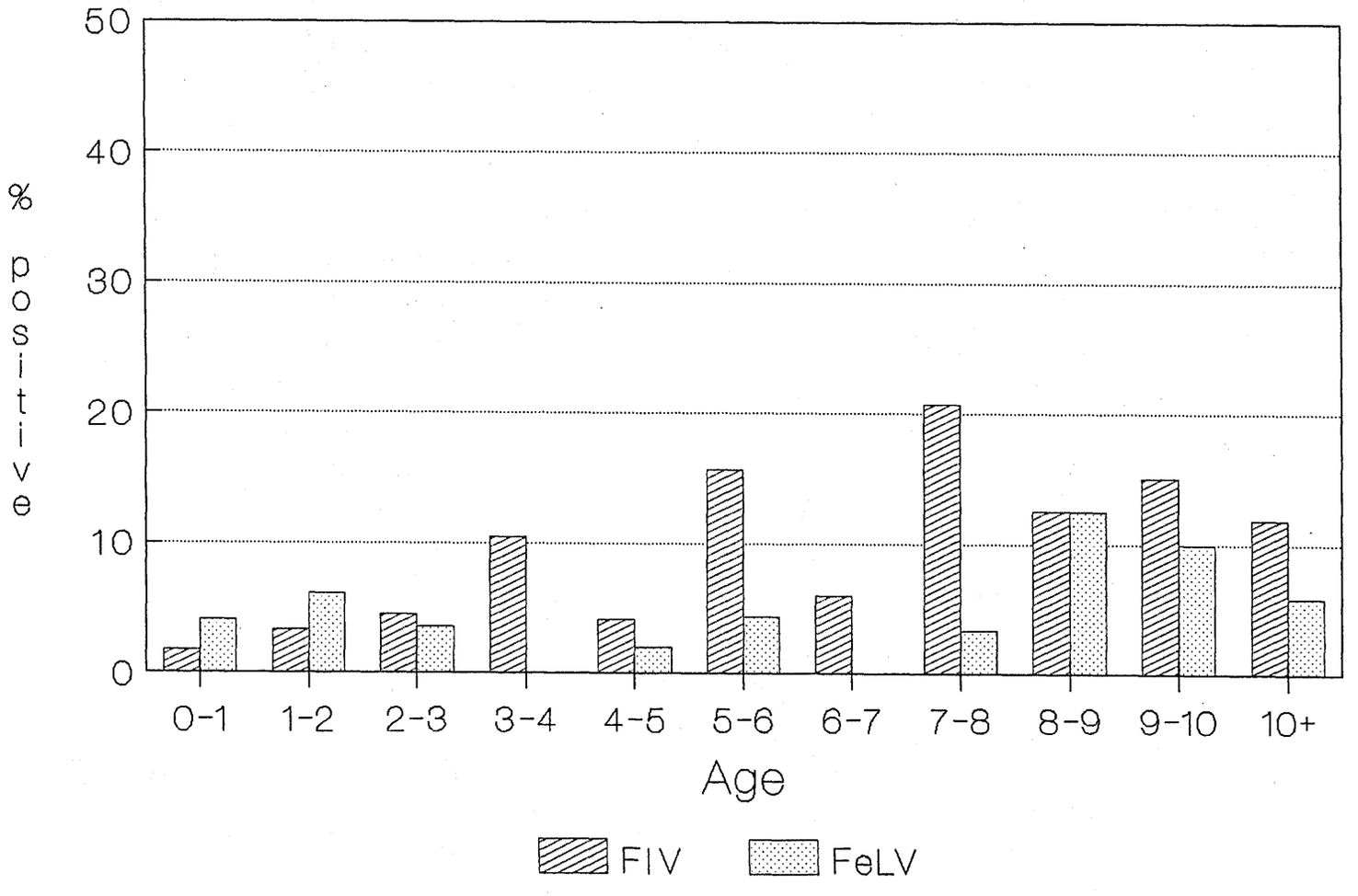

50
40
30
20
10
0
% positive
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10+
Age
FIV
FeLV

**Figure 3.2**

Bar chart showing the age distribution of the percentages of healthy cats which tested positive for FIV and FeLV


50
40
30
20
10
0
% positive
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10+
Age
FIV
FeLV

effect on the likelihood of FIV infection. Health status was the most important, followed by age, breed and sex (Table 3.1).

The likelihood of FIV infection increased with age and then decreased in the oldest age group. There was a lower probability of infection among healthy cats and also among pedigree cats. Female cats had the lowest probability of FIV infection. Neutered cats had a probability of infection which was slightly lower than that of entire males. Neutering did not alter the likelihood of a male cat being FIV-positive, but neutered females had an increased likelihood relative to entire females.

Since four factors were examined, there were six possible two-factor interactions to be investigated. Of these, the only interaction was between age and sex (Table 3.2). For entire male cats there was a steep rise in prevalence with increasing age. The other three sexes also showed increasing prevalence with increasing age, but this was followed by a decrease in the oldest age group. This age-sex interaction could be simplified by comparing the prevalences at each age for male and female cats and for neutered and entire cats. When the interaction was examined for males and females, the main difference was that for male cats the prevalence increased more steeply, peaked at a higher level and decreased to a lesser extent in the oldest age group compared to female cats. When entire cats were compared to neutered cats, it was observed that whereas the prevalence in entire cats increased between each increasing age group, the neutered cats displayed a considerable decrease in prevalence in the oldest age group.

### 3.3.3. FeLV Analysis

The prevalence of FeLV viraemia in sick cats was 18%, compared to 5% in healthy cats. The effect of sex on the proportion of cats which was FeLV-positive was not significant. There was a lower probability of infection with healthy cats and pedigree cats. The

**Table 3.1**

**The prevalence of FIV and FeLV by percentages and parameter estimates obtained by log-linear modelling**

| parameter | no tested | %FIV+ | FIV E(SE)[1] | %FeLV+ | FeLV E(SE)[2] |
|---|---|---|---|---|---|
| **health status** | | | | | |
| sick | 1204 | 18.6 | 0(-) | 17.5 | 0(-) |
| healthy | 1007 | 5.8 | -.89(.16) | 4.5 | -1.45(.18) |
| **age** | | | | | |
| 0-1 | 509 | 2.8 | 0(-) | 9.8 | 0(-) |
| 1-5 | 987 | 9.3 | 1.16(.30) | 13.0 | .20(.18) |
| 5-10 | 518 | 27.0 | 2.26(.29) | 12.6 | -.12(.21) |
| >10 | 197 | 18.3 | 1.75(.33) | 6.6 | -.84(.33) |
| **breed** | | | | | |
| domestic | 1609 | 15.5 | 0(-) | 14.2 | 0(-) |
| pedigree | 602 | 5.5 | -.89(.20) | 4.7 | -1.03(.21) |
| **sex** | | | | | |
| M | 663 | 15.4 | 0(-) | 12.8 | 0(-) |
| MN | 443 | 19.2 | -.07(.17) | 13.5 | .14(.19) |
| F | 819 | 6.1 | -.84(.19) | 9.4 | -.15(.17) |
| FN | 286 | 15.7 | -.22(.21) | 11.9 | -.16(.22) |

[1] Parameter estimates (E) derived from additive model with deviance 62.2 on 53df with the standard error (SE) in parenthesis. This model did not take the age-sex interaction into account.

[2] Parameter estimates (E) derived from additive model with deviance 41.5 on 53 df with the standard error (SE) in parenthesis.

**Table 3.2**

**Interaction of age and sex in the prevalence of FIV**

| age (years) | | sex M | MN | F | FN |
|---|---|---|---|---|---|
| 0-1 | E(SE) | 0(-) | .61(.93) | .63(.70) | 1.13(.94) |
| | % | 1.7 | 3.8 | 2.8 | 6.0 |
| 1-5 | E(SE) | 1.87(.61) | 1.77(.63) | 1.36(.63) | 2.01(.64) |
| | % | 10.7 | 11.6 | 5.6 | 13.4 |
| 5-10 | E(SE) | 3.09(.61) | 3.22(.61) | 1.86(.64) | 2.93(.64) |
| | % | 32.3 | 36.1 | 11.3 | 28.2 |
| >10 | E(SE) | 3.38(.66) | 2.20(.70) | 1.62(.73) | 1.47(.79) |
| | % | 39.1 | 16.7 | 10.9 | 8.3 |

Parameter estimates (E) with standard deviations in parenthesis and percentages for the prevalence of FIV according to age and sex, showing the interaction between these two parameters. These estimates were derived from the additive model plus age by sex interaction with deviance 40.2 on 44 degrees of freedom

main effect of age was that cats in the the oldest age group were much less likely to be FeLV-positive than younger cats. The highest probability of infection was among cats between 1 and 5 years of age.

### 3.3.4. Combined FIV and FeLV Analysis

The sick domestic cats made up 44% of the cats sampled and 87% of $FIV^+/FeLV^+$ cats were in this group. Therefore this group was analysed separately. Overall, 4% of the cats were $FIV^+/FeLV^+$ and 64% were negative for both infections. Approximately equal proportions were positive for one infection but not the other. Thus 17% were $FIV^+/FeLV^-$ and 16% were $FIV^-/FeLV^+$.

For the sick domestic cats there was no indication of any interaction between the presence or absence of the two viruses. Each appeared to behave independently. FeLV was commoner in younger cats than older cats, whereas FIV was more prevalent in older cats. Similarly, when all the data were investigated there was no interaction between FIV and FeLV.

### 3.3.5. FIV in multi-cat households

FIV infection was detected in 58 households with a total of 234 domestic cats and 14 households with a total of 67 pedigree cats (Table 3.3). Overall, 21% of cats in contact with FIV were positive. This figure ranged from 3% to 67% in different households. The prevalence of FIV in the in-contact cats was significantly higher in domestic cat households (24%) compared to pedigree cat households (9%).

Table 3.3

Prevalence of FIV antibodies in multi-cat households (MCH) in which FIV was present

| size of MCH | Domestic Cats | | Pedigree Cats | |
|---|---|---|---|---|
| | no. MCH | no.FIV+/ no. tested | no. MCH | no.FIV+/ no. tested |
| 2 | 25 | 34/50 | 8 | 9/16 |
| 3 | 12 | 16/36 | 2 | 2/6 |
| 4 | 11 | 18/44 | 1 | 1/4 |
| 5 | 3 | 5/15 | 2 | 4/10 |
| 7 | 1 | 2/7 | | |
| 8 | 1 | 3/8 | | |
| 9 | 2 | 4/18 | | |
| 11 | 1 | 4/11 | | |
| 21 | 1 | 6/21 | | |
| 24 | 1 | 9/24 | | |
| 31 | | | 1 | 3/31 |
| totals | 58 | 101/234 | 14 | 19/67 |
| minus index cases | | 43/176 (24%) | | 5/53 (9%) |

### 3.3.6. FeLV in multi-cat households

FeLV infection was detected in 20 households with a total of 84 domestic cats and 8 households with a total of 53 pedigree cats (Table 3.4). Overall, the prevalence of FeLV in the in-contacts was 14%, ranging from 0% to 100%. The difference between the prevalences in domestic and pedigree households was not significant, being 16% and 11% respectively.

### 3.3.7. Clinical signs in infected cats

The clinical signs which the veterinary surgeons noted on the submission form were ranked in order of occurrence for both FIV-positive cats and for FeLV-positive cats (Table 3.5).

The clinical signs which, from previous reports, were thought to be associated with FIV infection are listed in Table 3.6 [1,2,3,74,43]. Also listed are the clinical signs which might be expected with FeLV infection. Pyrexia, gingivitis/stomatitis and respiratory signs were noted in a significantly higher proportion of FIV-positive sick cats compared to FIV-negative sick cats (Table 3.6).

The clinical signs which were noted in a significantly higher proportion of FeLV-positive sick cats compared to FeLV-negative sick cats were pyrexia and anaemia (Table 3.6).

## 3.4. DISCUSSION

The prevalence data were examined to identify those groups of cats most likely to be infected with FIV and FeLV and to provide an indication of whether the transmissibility of FIV was different from FeLV. This survey demonstrates that both FIV and FeLV are important agents causing significant disease in cats. The prevalence of FIV was 19% in sick cats and 6% in healthy cats. Other serological surveys have reported similar findings

Table 3.4

**Prevalence of FeLV viraemia in multi-cat households (MCH) in which FeLV was present**

| size of MCH | Domestic Cats | | Pedigree Cats | |
|---|---|---|---|---|
| | no. MCH | no. FeLV+/ no. tested | no. MCH | no. FeLV+/ no. tested |
| 2 | 15 | 19/30 | 3 | 3/6 |
| 3 | 3 | 5/9 | 2 | 2/6 |
| 5 | | | 2 | 6/10 |
| 21 | 1 | 1/21 | | |
| 24 | 1 | 5/24 | | |
| 31 | | | 1 | 2/31 |
| totals | 20 | 30/84 | 8 | 13/53 |
| minus index cases | | 10/64 (16%) | | 5/45 (11%) |

Table 3.5

**Clinical signs in FIV and FeLV-positive cats, ranked in order of prevalence.**

| 1. FIV | | 2.FeLV | |
|---|---|---|---|
| clinical sign | no. of FIV+ cats with this sign | clinical sign | no. of FeLV+ cats with this sign |
| pyrexia | 115 | pyrexia | 106 |
| weight loss | 94 | anorexia | 85 |
| anorexia | 80 | weight loss | 78 |
| gingivitis/stomatitis | 67 | anaemia | 77 |
| lethargy | 59 | lethargy | 64 |
| respiratory signs | 52 | respiratory signs | 39 |
| anaemia | 47 | gingivitis/stomatitis | 35 |
| diarrhoea | 30 | lymphadenopathy | 30 |
| lymphadenopathy | 25 | diarrhoea | 22 |
| nervous signs | 15 | vomiting | 13 |
| skin infections | 8 | hepatomegaly | 8 |
| vomiting | 6 | conjunctivitis | 6 |
| conjunctivitis | 6 | nervous signs | 6 |
| jaundice | 5 | jaundice | 5 |
| neutropaenia | 5 | thymic neoplasia | 3 |
| polyphagia | 2 | neutropaenia | 4 |
| haematuria | 2 | polyphagia | 2 |
| splenomegaly | 2 | splenomegaly | 2 |
| hepatomegaly | 1 | ascites | 1 |
| cystitis | 1 | | |

Table 3.6

**Occurrence of clinical signs believed to be associated with FIV or FeLV infection**

**1.FIV**

| | no. of cats | |
|---|---|---|
| | FIV + | FIV - |
| lymphadenopathy | 25 | 131 |
| diarrhoea | 30 | 161 |
| nervous signs | 10 | 38 |
| pyrexia* | 115 | 359 |
| gingivitis/stomatitis* | 67 | 163 |
| anaemia | 47 | 185 |
| respiratory signs* | 52 | 143 |
| sick cats | 317 | 1448 |

**2.FeLV**

| | no. of cats | |
|---|---|---|
| | FeLV+ | FeLV- |
| lymphadenopathy | 30 | 120 |
| diarrhoea | 22 | 156 |
| pyrexia* | 106 | 329 |
| anaemia* | 77 | 141 |
| sick cats | 276 | 1352 |

* denotes significant difference by Chi-square analysis ($p<.05$)

for the prevalence of FIV infection in Japan [2], the USA [3] and France [38]. The levels of infection in the Netherlands, Germany and Switzerland, on the other hand, were significantly lower [38,42]. The reasons for this marked difference is not yet clear.

As well as health status having an effect on the prevalence of FIV, this survey provided evidence that age, breed and sex were also important factors. Other workers have suggested that the older, male cat is most likely to be infected with FIV [3,2]. The present findings would support these suggestions. Furthermore, this study indicated that breed has an effect and that an age-sex interaction exists. There does not appear to be any age-related susceptibility to infection with FIV as 6 month old cats are susceptible to experimental inoculation (data not shown). The higher prevalence of FIV in older cats may reflect the long latent period of this infection. An extended latent period would result in an accumulation of seropositive cats with time which would be consistent with observations on other lentiviruses, including HIV in man; and with the observation that cats experimentally inoculated with FIV did not display clinical signs of immunodeficiency within 17 months of inoculation [3]. Alternatively, continued exposure to FIV may be required before a threshold is reached and an infection can develop.

The importance of breed which was observed is less likely to be associated with any genetic effect than with managemental factors. Pedigree cats, especially those kept for breeding purposes, are often housed in small groups and prevented from roaming outdoors. Many domestic cats, on the other hand, are free-roaming with a greater opportunity to socialise with other cats than are pedigree cats. Cats which are free-roaming in this way are more likely to meet the feral cat population which is thought to have a high level of FIV infection (C Hopper, personal communication).

Differences in prevalence between sexes were noted. In male entires alone, the prevalence increased between each successive

age group. In the other three sexes there was a decrease in prevalence in the oldest age group which may indicate that infection led to mortality in this age group. Alternatively, the cats in this age group could have ceased to be exposed to the factors necessary for infection, whereas entire male cats continued to be exposed to such factors.

When all male cats were compared to all female cats, it was seen that at each age the prevalence of FIV was greater in males than females. It has previously been suggested that since male cats are more inclined to fight than female cats, transmission of FIV may occur via biting [3,41]. The successful isolation of FIV from saliva is consistent with this theory [3]. Further controlled studies are, however, required to determine methods of transmission.

When the prevalence in entire and neutered cats was compared, there was a lower prevalence in neutered cats in the oldest age group. This could reflect either mortality in the infected neutered cats at this age, or a change in their behaviour or management, such that they were no longer exposed to conditions causing new infections.

For FeLV the prevalence was 18% in sick cats and 5% in healthy cats. A recent survey conducted in the USA reported prevalences of 13% in sick cats and 4% in healthy cats [41]. The present survey clearly demonstrated the different ages at which FIV and FeLV are most common. Whereas FeLV occurred predominantly in young cats, FIV was most prevalent in older cats. These findings are consistent with the concept that cats of all ages are equally susceptible to FIV and that FIV-associated diseases have a long latent period; and that, by contrast, young cats are much more susceptible to FeLV infection and the latent period to disease caused by that virus is relatively short. While previous experimental and epidemiological observations confirm the marked age-related susceptibility of cats for FeLV [75] and indicate a period of 3.5 years from infection to death in 85% of cats [76],

there is limited information on these aspects of FIV infection.

The data from multi-cat households were examined for evidence of the relative transmissibility of FIV and FeLV. It should be noted that the multi-cat households in this study were not necessarily closed households. Cats in the smaller households were most likely free to roam outdoors, whereas the larger households, especially those containing pedigree cats, may have been closed.

Ishida et al [2] reported that 42% of 60 Japanese cats in contact with FIV were positive, whereas the level of infection was only 21% in this survey of 229 cats in the UK. The households in the Japanese survey were not closed breeding catteries but animal shelters, or households where the cats were allowed to roam outdoors. The management of the domestic cats in the households examined in this survey was comparable and yet only 24% of in-contacts had seroconverted.

One reason for the differences in the prevalence of FIV between households may be that there is variation in pathogenicity between different isolates of FIV. Some isolates may infect a higher proportion of cats in multi-cat households. In some of the large households in which FIV was detected in this survey, none of the cats , or only a single cat, had clinical signs. This contrasted with the households in the Japanese survey in which there were often chronic disease problems. Information relating to the length of time that the in-contacts had been exposed to FIV was not available.

It has been suggested that FIV is less efficiently transmitted than FeLV [77]. Our results, however, do not entirely support this view. The prevalence of FIV in in-contact cats in multi-cat households was 21% which is similar to the 16% found for FeLV. In FeLV-infected households, essentially all cats in contact with a viraemic cat become infected with the virus. From a previous study in the UK, approximately 24% were persistently infected and the remainder were recovered and were immune with serum

neutralising antibodies [78]. In FIV households, however, it is not possible to determine whether seronegative cats have been infected with the virus but have failed to become persistently infected (since there is no known marker for 'recovered' animals) or if they have not been exposed at all.

This survey also provided some information on clinical signs associated with both FIV and FeLV. There were no groups of sick cats which were actively discriminated against in the selection of samples although bias may have arisen since the samples from sick cats had been submitted for virological examination and these cats were therefore most likely to be suffering from serious or chronic disorders. Although all of the sick cats were known to be showing clinical signs, specific signs were not always recorded on the submission form. Where clinical findings were noted, it was not possible to be confident that the omission of a sign correlated with its absence. For this reason, it was not possible to examine the combinations of clinical signs in FIV- positive or FeLV- positive cats.

Previous studies have shown an association between the presence of FIV antibodies and chronic stomatitis in the UK [44]. This association was noted in the present study, as well as the associations with pyrexia and respiratory signs. The classification of respiratory signs included both upper and lower respiratory tract disorders as in many cases these could not be differentiated using the information on the submission forms.

FeLV is a well established cause of anaemia [79] and this association was found to be significant, as well as the association with pyrexia.

Later studies (reported in section 4.3.4) demonstrated that false positive results occurred in the ELISA test which was used in this survey. Therefore the false positives were classified according to health status, age, breed and sex in order to determine whether any goup was more likely to give a false

positive result. This analysis showed that the false positives were equally distributed amongst all groups. Therefore the statistical analysis of this survey remained valid.

In conclusion, this survey indicates that the older, male, free-roaming cat is most likely to be infected with FIV. There is no age-related susceptibility to FIV, in contrast to FeLV which is most prevalent in young cats. FIV and FeLV act independently and are both significant pathogens where there is contact between cats.

# 4. SEROLOGICAL RESPONSES TO FIV

## 4.1. INTRODUCTION

FIV infected cats have serum antibodies to the virus which are detected most conveniently by an ELISA. We have, however, noted that some reactions in this test are non-specific since no reactivity is demonstrable by immunoblotting. The purpose of this study was to identify the viral proteins of FIV and to examine the serological response to each protein following FIV infection in cats, using immunoblotting. The serological responses in cats naturally and experimentally infected with FIV were investigated. These immunoblot results were categorised according to reactivity of antibodies with individual viral proteins and were then compared with the results of ELISAs. In this way a set of criteria were established to identify FIV antibody-positive cats.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Gel elecrophoresis

Infected cells were incubated at a concentration of $10^6$ cells per ml in methionine-free medium for 4 hours. A mixture of $^{35}S$ L-methionine and L-cysteine (ICN Translabel) was then added to a final concentration of 100 uCi per ml in medium with 10% of the normal concentration of methionine and the culture was incubated overnight. The culture fluids were then filtered through a 0.22 um membrane and were centrifuged in a 20-50% sucrose gradient in an SW41 rotor at 35,000 rpm for 60 minutes. The peak of radiolabel at a density of 1.15 $g.cm^3$ was collected, diluted to 5 ml in tris-buffered saline and pelleted in an SW50.1 rotor at 35 000 rpm for 60 minutes. The pellets were resuspended in SDS-PAGE sample buffer containing 500mM 2-mercaptoethanol and were electrophoresed in a 8-15% gradient SDS-PAGE gel. The gels were then treated with enhancer (Enlightning, NEN Research Products), dried and autoradiographed.

### 4.2.2. Radioimmunoprecipitation

Infected cells were incubated overnight at a concentration of $10^6$ cells per ml in glucose-free medium with 5 uCi per ml $^{14}C$-glucosamine. The cells were then washed once with 0.14M NaCl, 10mM Tris pH 7.4 and resuspended in lysis buffer (100 mM Tris pH 7.4, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate and 1% aprotinin). The cell lysate was mixed with cat anti-FIV antiserum and incubated for an hour before the addition of Protein A Sepharose CL-4B (Sigma). After incubating for an hour, the sample was washed several times, resuspended in SDS-PAGE sample buffer and electrophoresed in a 10% SDS-PAGE gel. The gel was then fixed, treated with enhancer, dried and autoradiographed.

### 4.2.3. Immunoblotting

The immunoblot (Western blot) procedure was a modification of that previously described [80]. The antigen was prepared in one of two ways, either as concentrated virus or as a cell lysate.

To concentrate virus, culture fluids from infected cell cultures were clarified by centrifugation at 7 500 rpm for 20 minutes and filtered through a 0.45 um membrane. The virus was then pelleted by ultracentrifugation at 36 000 rpm for 60 minutes and resuspended in SDS-PAGE sample buffer.

To prepare a cell lysate, 2 x $10^7$ cells were washed out of culture fluid and resuspended in 40 uls of lysis buffer. The lysate was microfuged briefly to remove cell debris and SDS-PAGE sample buffer was added to the supernatant.

The antigens were resolved using the Biorad Minigel apparatus. Either 10% polyacrylamide gels or 10-20% gradient gels were used, the latter giving better resolution of the lower molecular weight proteins. The proteins were transferred to nitrocellulose and the remaining binding sites were blocked with 0.5% non-fat milk

powder (NFMP) in Tris-buffered saline.

The nitrocellulose was cut into strips which were incubated with serum diluted 1:10 in 1.0 % NFMP and 0.5% Tween-20 in Tris buffered saline for two hours. The strips were washed several times in 0.5% Tween-20 in PBS and then incubated with biotinylated protein A for an hour. After further washing, the strips were incubated with streptavidin-alkaline phosphatase for an hour. The strips were washed once again before being developed with a substrate containing 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as previously described [81].

### 4.2.4. Serum samples

Samples were obtained from specific pathogen free cats experimentally infected with the Petaluma (kindly provided by NC Pedersen), Glasgow-8 and Glasgow-14 strains of FIV. Field sera were obtained from cats by veterinarians in the UK.

All sera were tested using a commercially produced plate ELISA kit (PetChek Feline T-Lymphotropic Lentivirus Antibody Test Kit, Idexx Corp.) for the detection of anti-FIV antibodies [50]. The test was carried out according to the manufacturer's instructions and the absorbance of each well was read at 650nm. Results were expressed as a ratio (S/P) of the absorbance of each sample well to that of a control positive sample. Selected sera were also tested using the commercially produced CITE ELISA test (CITE Combo Kit, Idexx Corp., kindly provided by Pitman-Moore) for the detection of FIV antibodies and FeLV antigen.

## 4.3. RESULTS

### 4.3.1. Viral proteins

The result of SDS-PAGE of $^{35}S$ labelled virus is shown in figure 4.1a. The Mr of the major bands were 24K (p24) and 17K (p17) and of the minor bands, 120K and 10K. Additional faint bands were

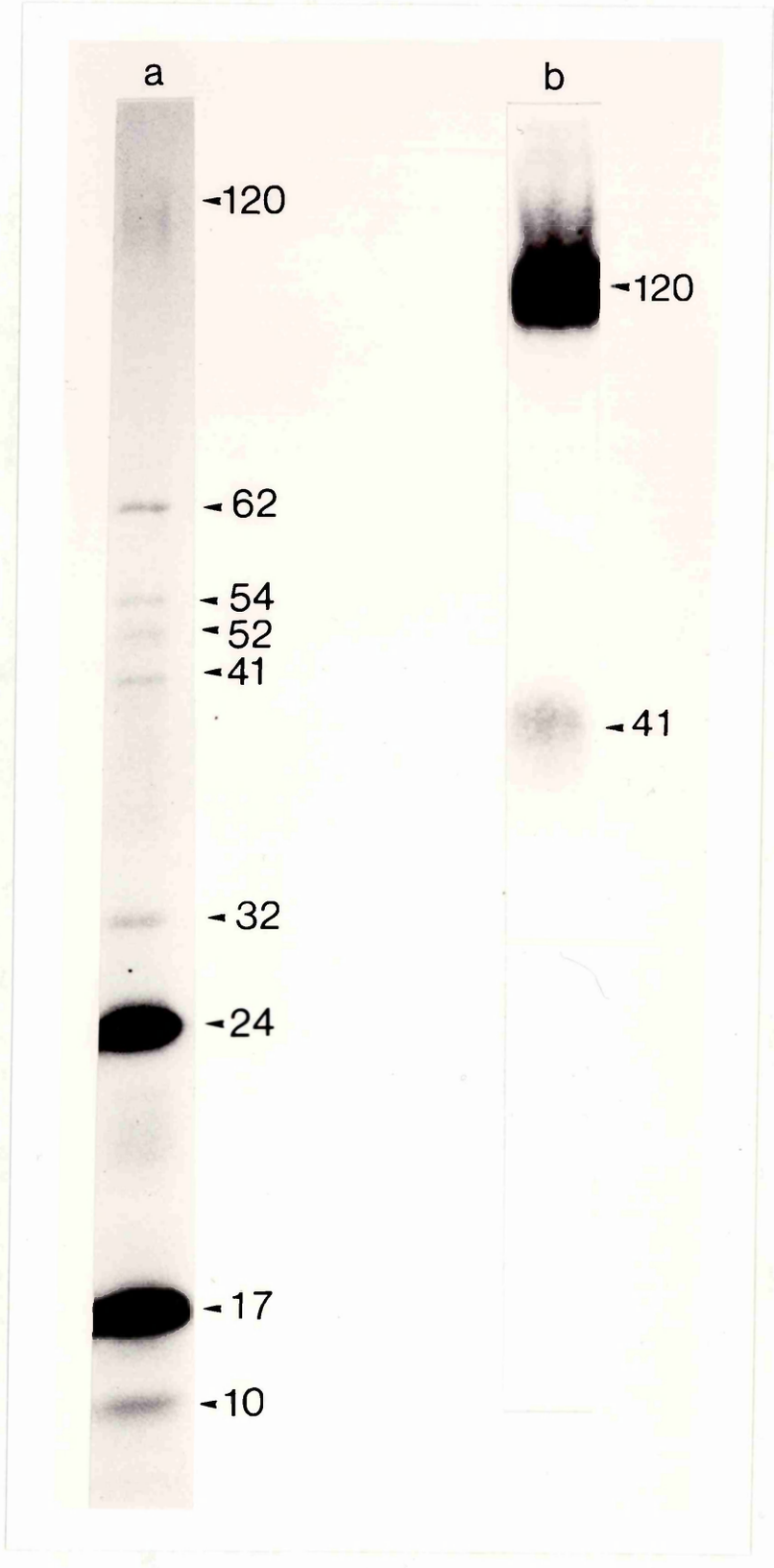



Figure 4.1

a. Autoradiograph of $^{35}$S-methionine/cysteine labelled FIV virus and b. Radioimmunoprecipitaion of $^{14}$C-glucosamine labelled FIV-infected cell lysate.

observed with Mr of 62K, 54K, 52K, 41K and 32K. Radioimmunoprecipitation of $^{14}$C-glusosamine labelled infected cell lysate with a FIV-positive antiserum indicates that the 120K and 41K proteins were glyscosylated (Figure 4.1b).

### 4.3.2. Detection of antibodies to FIV proteins by immunoblotting.

Serum samples from cats experimentally infected with FIV were reacted in immunoblots with proteins prepared from concentrated virus. Antibodies were detected to p55, p24 and p17 but there was no reactivity with a higher molecular weight putative envelope glycoprotein. It was considered that the glycoprotein may have been lost either into the culture fluid or during the purification procedure, or was not transferred to the nitrocellulose membrane. In an attempt to identify the glycoprotein on immunoblotting, the procedure was repeated using infected cell lysates as the antigen. The immunoblots prepared in this way revealed antibody to gp120 indicating a loss of the glycoprotein during preparation of the concentrated virus.

As more information could be obtained using infected cell lysates rather than concentrated virus as antigen on immunoblots, cell lysates were used in all subsequent blots. The majority of blots with field sera were easily interpreted (Figure 4.2). However, a minority exhibited a number of apparantly non-specific bands. To ensure that the reactivity in sera was virus specific, a series of field sera were blotted against uninfected cell lysate as well as infected cell lysate (Figure 4.3). Some sera showed non-specific binding to components in uninfected cell lysate, most notably at a molecular weight of approximately 60K, but this activity could be differentiated from the virus-specific bands seen at 55K. The bands at 120K, 24K and 17K were similarly shown to be virus-specific. Another band was seen with the sample in lane 3 at 10K which was assumed to be a third viral core protein.

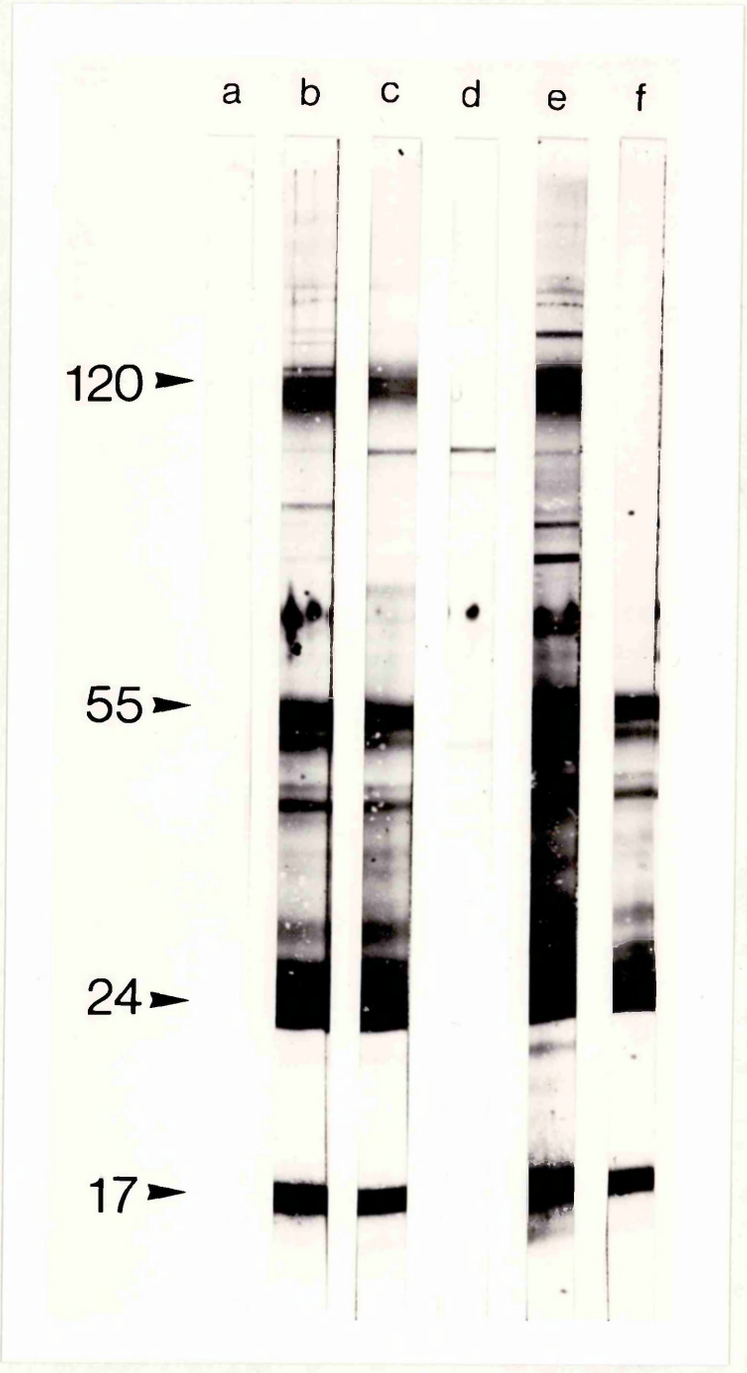



**Figure 4.2**

Immunoblots of field sera against FIV-Glasgow-8-infected cell lysates. All sera were in ELISA category A. Sera in lanes a and d were in immunoblot category 6, sera in lanes b, c and e were in immunoblot category 1 and the serum in lane f was in immunoblot category 3.

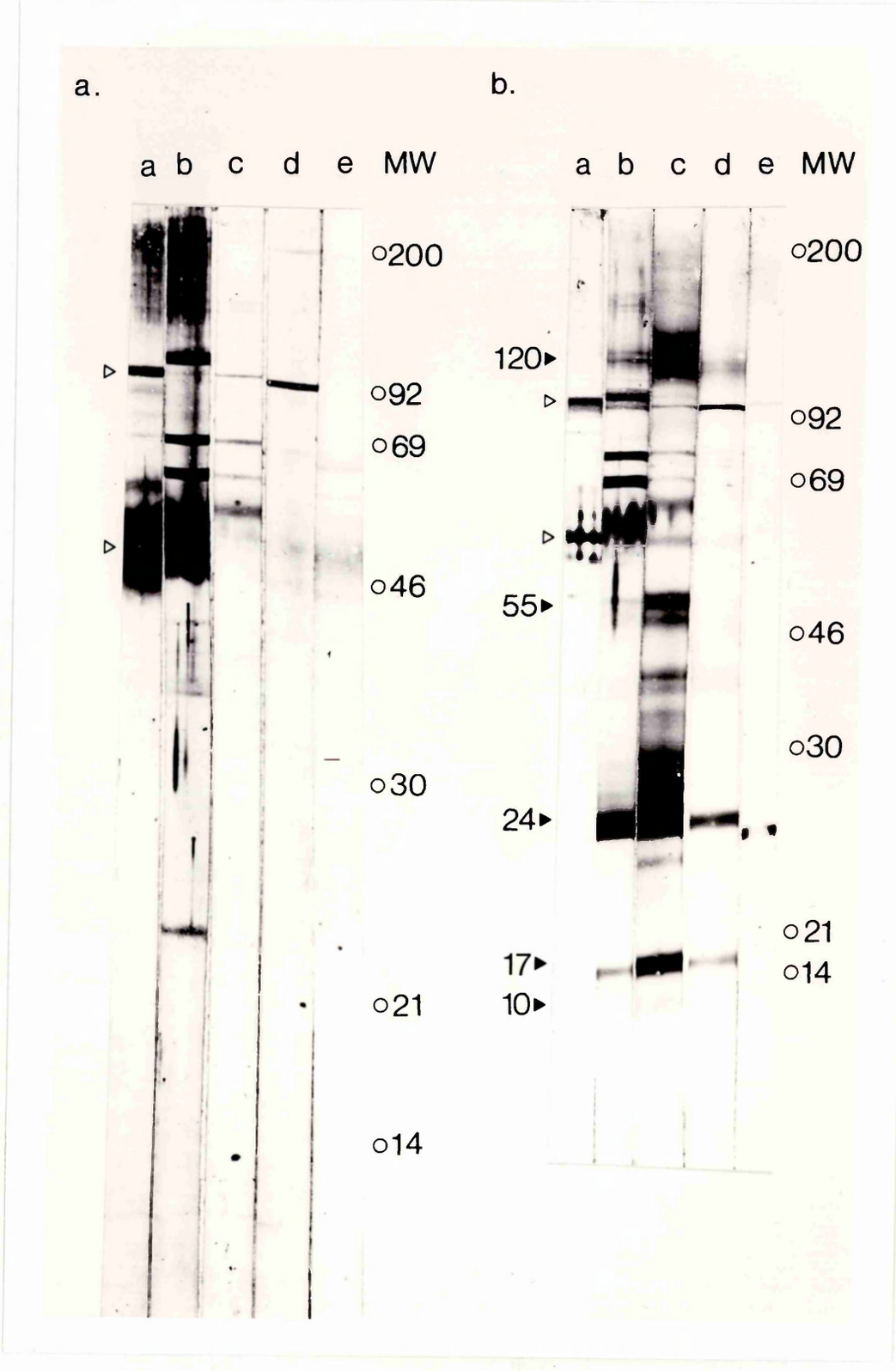



**Figure 4.3**

Immunoblots of sera against a. uninfected and b. FIV-infected cell lysates. Lane a: serum in ELISA category B, immunoblot category 6; lanes b,c and d: sera in ELISA category A, immunoblot category 1; lane e: serum in ELISA category D, immunoblot category 5. Open arrows indicate non-specific bands seen against both infected and uninfected cell lysates.

### 4.3.3. Development of immune responses to viral proteins in experimentally infected cats

The antibody response to individual viral proteins was followed in cats which were experimentally inoculated with FIV. Serum samples were taken prior to infection and then at three week intervals from cats infected with the Glasgow-8 strain of FIV. These samples were used in immunoblots against FIV-infected cell lysates. It was seen that antibodies developed first against p24 and subsequently against p17 and p55. The cats which seroconverted by 6 weeks post infection (pi) had been infected with a greater dose of virus than those which seroconverted at 9 weeks pi. Antibody to the high molecular weight glycoprotein appeared at the same time as p17 and p55 (Figure 4.4). All of these anti-FIV antibodies were still detectable 45 weeks post infection. Sequential samples taken from cats experimentally infected with both the Petaluma and Glasgow-14 strains of FIV gave similar results to the cats infected with the homologous virus (Figure 4.5).

### 4.3.4. Comparison of plate ELISA and immunoblot results

The results of the plate ELISA test were read at 650nm and the S/P ratio was calculated as previously described [50]. According to the manufacturer, samples with ratios equal to or greater than 0.5 are positive for antibody to FIV. For the purposes of this study, S/P ratios were further classified into 4 groups: group A with ratios greater than 1.5, group B with ratios between 0.5 and 1.5, group C with ratios between 0.2 and 0.5, and group D with ratios less than 0.2.

The results of the immunoblots were classified into groups according to the confidence with which they could be designated positive or negative. Group 1 contained those samples which showed bands against gp120 and at least one core protein. Group 2 contained samples with a band against gp120 but no core proteins. Group 3 samples reacted against three or four of the core

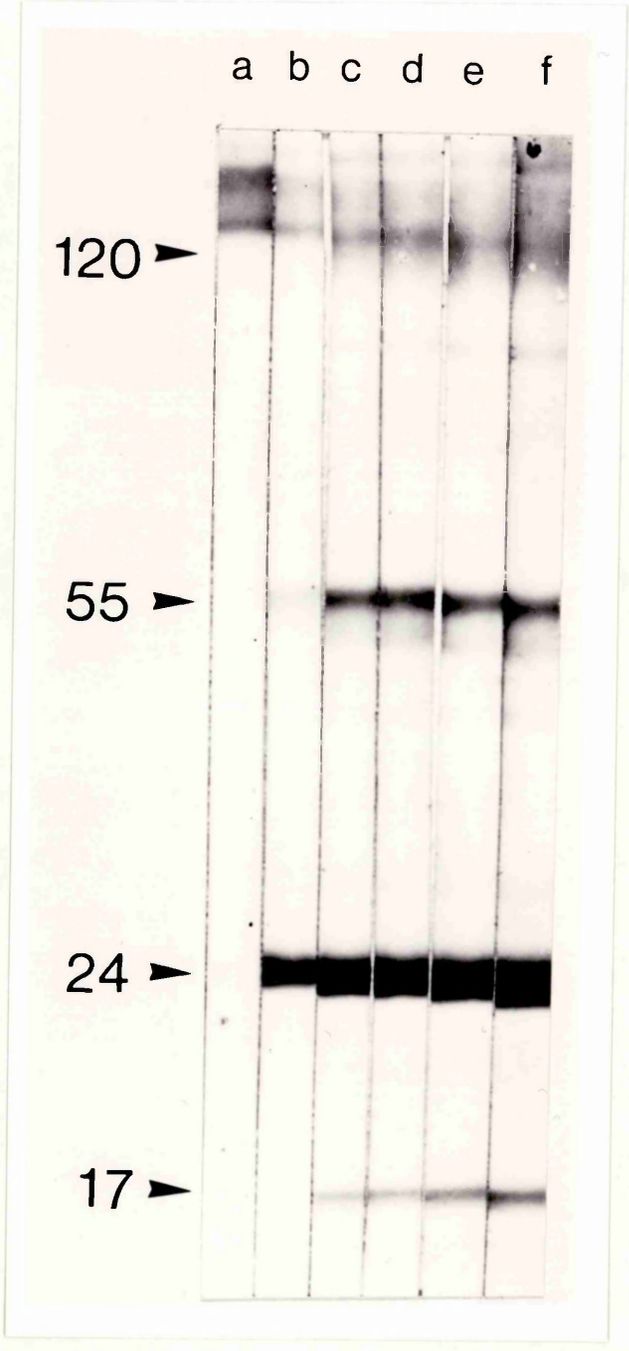



**Figure 4.4**

Sequential immunoblots of serum samples from a cat experimentally infected with FIV-Glasgow-8. Lane a: sample taken prior to infection; lanes b to f: samples taken at three weekly intervals post infection; lane g: sample taken 6 months post infection.

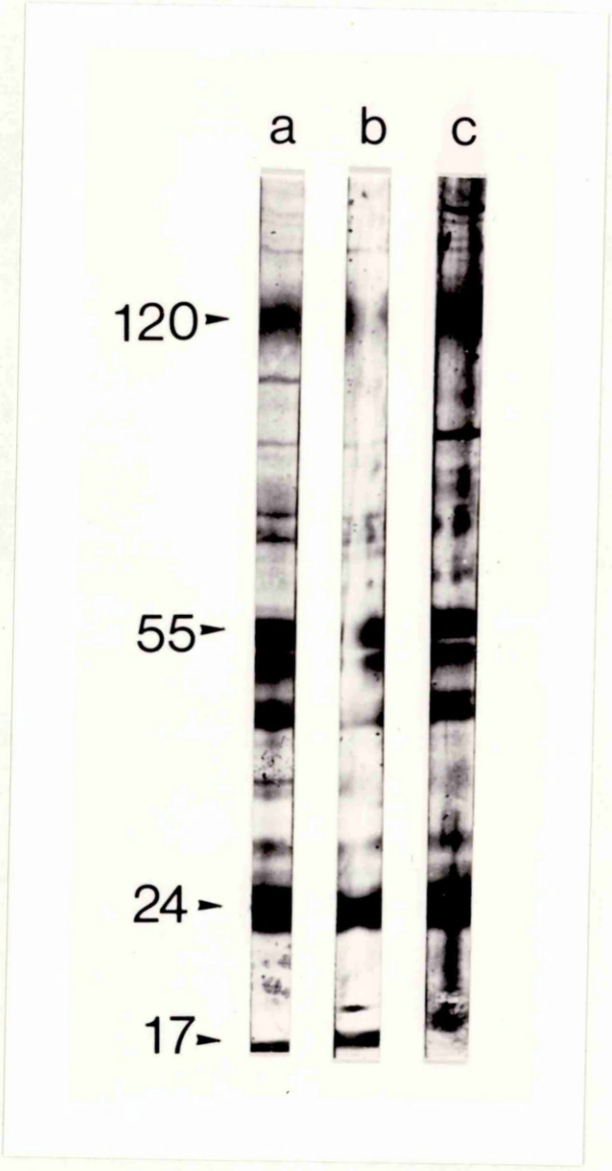



**Figure 4.5**

Immunoblots of serum samples taken from cats 15 weeks after experimental infection with a. FIV-Petaluma, b. FIV-Glasgow-8 and c. FIV-Glasgow-14 against FIV-Glasgow-8-infected cell lysates.

proteins (p55, p24, p17 and p10). Samples in groups 1 to 3 were regarded as positive. Samples in groups 4 and 5 reacted with two and one core protein respectively and samples in group 6 showed no virus-specific bands. Samples in groups 4 to 6 were regarded as negative.

### 4.3.5. Serological responses in naturally infected cats

A series of 68 samples from cats which had been diagnosed FIV antibody negative and 215 samples from cats diagnosed FIV antibody positive by ELISA were tested by immunoblotting against infected cell lysate (Table 4.1). All samples which were ELISA-negative were negative on immunoblotting. Of the samples which were ELISA-positive according to the manufacturer, 57/215 (26.5%) were in immunoblot categories 4 to 6 and were therefore regarded as negative. Of the samples in ELISA group A (with S/P ratios of >1.5), only 10/125 (8%) were negative by immunoblotting.

### 4.3.6. Comparison of three ELISA systems

A series of 30 selected samples were tested using immunoblotting, the old plate ELISA used in this study, the CITE test for FIV antibodies as well as by the new format plate ELISA. Of these 30 samples, 7 were positive and 3 were negative by all four tests.

Of the remaining 20 samples, 15 were positive by the old plate test but were negative by immunoblotting, the CITE test and the new plate test. These samples therefore appeared to be false positives by the old plate ELISA test. One sample was positive using the old plate test and the CITE test but negative using immunoblotting and the new plate test. These samples were presumably false positives by the old plate and CITE tests.

Two samples were negative by the new plate test but positive by immunoblotting, the CITE test and the old plate test. These samples therefore appeared to be false negatives by the new plate test. One sample was negative by CITE but positive by the other

Table 4.1

Comparison of plate ELISA S/P ratio and immunoblot results

| | | immunoblot category | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| ELISA category | A | 99 | 2 | 14 | 5 | 3 | 2 |
| | B | 36 | 6 | 1 | 4 | 10 | 33 |
| | C | 0 | 0 | 0 | 1 | 9 | 25 |
| | D | 0 | 0 | 0 | 0 | 5 | 28 |

three tests and was presumably a false negative by the CITE test.

One sample was positive by immunoblotting (having antibodies against both envelope and core proteins) but was negative using the other three tests.

Therefore, it appeared that false positive results were most common with the old plate test, but were occasionally seen with the other ELISA tests. Of the three ELISA tests, the CITE test correlated most closely with the results obtained by immunoblotting, followed by the new plate test. However, it was suspected that occasional false negative results arose with these two test kits.

## 4.4. DISCUSSION

This study confirmed that the protein content of FIV is similar to that of HIV [82]. Thus, the presumed major core proteins are p24, p17 and p10 with a possible precursor, p55. Two glycosylated polypeptides, gp120 and gp41, are probably the envelope glycoproteins of the virus. The additional polypeptides observed in radiolabelled virus may represent products of the pol gene.

Cats infected naturally or experimentally with FIV make antibodies to several of these proteins. This study demonstrated the order of appearance of antibodies against both core and envelope proteins of FIV whereas others have also reported the appearance solely of anti-core antibodies in sequential samples from experimentally infected cats using immunoblot analysis [3,50].

From these results it is clear that the reason for the lack of gp120 in immunoblots made with concentrated virus is that this protein is lost from the virus during production or purification. Similar findings have been made with HIV. The use of FIV infected cell lysates as antigen for blots is a convenient method of overcoming this problem and permitting the detection of anti-

gp120 antibodies.

It would appear that the timing of the appearance of antibodies following infection is dependent on the dose of virus used. Those cats which received the lowest dose of virus were first seen to seroconvert at 9 weeks pi whereas those which were infected with the highest dose began to seroconvert by 6 weeks pi. The long time course of continued antibody response demonstrated in this study has also been seen by others [50] and is consistent with the persistent nature of the infection. In this study, the cats which had been experimentally infected with FIV showed no overt signs of clinical disease, the longest period of infection being 45 weeks. This finding would suggest that FIV infection, as HIV infection, has a long incubation period. Various organs of these cats did however, display histological lesions of an early AIDS type (WFH Jarrett, personal communication).

The similar patterns of seroconversion seen in sequential samples from cats infected with different isolates of FIV suggests that there are conserved epitopes between these three isolates. Field sera, however, did not consistently react against both envelope and core proteins. It is conceivable that had the cats which had been experimentally infected with FIV been monitored until the time of death, there would have been a change in the serological reaction on immunoblots. In early and asymptomatic HIV infection in man, antibodies to p24 predominate, whereas with progression to AIDS, the antibody titre to p24 declines or disappears [83]. We are currently investigating whether field samples from seropositive cats show variable responses on immunoblots according to the stage of infection as well as whether there are possible strain differences.

The criteria for classifying samples as seropositive by immunoblotting were selected on the basis of the pattern shown by sera from experimentally infected cats as well as by analogy with HIV diagnosis. The minimum requirement of antibody to gp120 or to at least three core proteins may need to be redefined if further

studies indicate that virus can be isolated from cats which do not meet these criteria. Examining immunoblots for reactivity against gp41 may be of value in interpreting results but so far it has not been possible to consistently reproduce lysates containing gp41.

A previous study tested by immunoblotting 141 samples from field cats which were positive by plate ELISA [50]. The criteria for a positive result by immunoblotting in that report was staining of two or more FIV polypeptides. Four of the 141 samples were reactive to only a single FIV protein (p26 or p15) and the remainder showed bands to p26 and p15, with variable reactivity to proteins of 10, 32, 40, 47 and 65 kilodaltons. The four samples which were negative by immunoblotting were, however, positive by the RIPA-SDS-PAGE assay in which reaction to gp120 was required for confirmation. That report, therefore, did not reveal any false positive plate ELISA test results. In contrast, the present study demonstrated the existence of false positive results in this ELISA test. Even if the same criteria were applied as in the earlier paper (namely that the presence of reactivity against only two core proteins on an immunoblot could be regarded as positive), 48/215 (22%) of plate positive samples in the present study were negative on immunoblotting. Neither study revealed any discordance between the tests if the samples were negative in the plate ELISA.

The plate ELISA test wells are coated with FIV antigen prepared from a feline cell line [50]. In the CITE ELISA test, the antigen is membrane bound and a prefilter is used to remove insoluble material in the plasma. The comparison between the three ELISA tests suggested that the CITE test had a higher specificity compared to the old plate test. The immunoblots against uninfected cell lysates demonstrated that some cat sera contain antibodies which react with lysates from normal feline cells. Hence, it is possible that feline cell antigens on the ELISA wells bind antibodies non-specifically, resulting in false positives.

The new plate test was designed to eliminate false positive results by incubating the sample with antigen linked to horse-radish-peroxidase (HRPO) (Tom O'Connor, personal communication). Anti-FIV antibodies in the sample cross-link the plate-bound antigen and the HRPO-linked antigen, thereby giving a positive result. The results of this study confirm that the new test format has indeed eliminated the false positive results which were observed with the old test but suggest that there may be some false negative results with the new test. False negatives may occur if the assay cut-off is too stringent.

An ELISA test using recombinant FIV p24 has been developed to measure anti-p24 antibodies [84]. As the ELISA wells are coated with a pure preparation of p24 expressed in Escherichia coli (in contrast to the commercial ELISAs which use virus purified from feline cells) this test is very specific. When the anti-p24 antibody test was evaluated by comparison with immunoblotting, the results were in agreement for the majority of samples. There was, however, an intermediate range of absorbances for which the results were indeterminate. Therefore this test was valuable for screening large numbers of samples, combined with the use of immunoblotting for indeterminate samples.

The phenomenon of false positive results was observed in the early testing of human blood donor sera for anti-HIV antibodies [85]. From a sample of 3961 donors, 9 were positive on an initial ELISA test and of these, 4 remained postive when the test was repeated. Only 2 samples were confirmed as being positive by immunoblotting, having antibodies against p24, p41 and p64. Seroconversion did not occur in 2 recipients of blood that was consistently ELISA positive but immunoblot-negative. In contrast, the 2 recipients of immunoblot-positive blood seroconverted 6 and 8 weeks after transfusion.

It was important in this study to establish a set of criteria for a positive immunoblot result as not all sera contained antibodies

against both envelope and core constituents. Those sera which reacted against 3 or 4 core proteins but not against gp120 may have been from cats infected with an isolate of FIV with a gp120 antigenically distinct from that of FIV-Glasgow-8. This reaction was designated positive in this study. Sera reacting against only two or fewer core proteins, on the other hand, were classified as negative. Similarly, in HIV testing, it has been found that isolated anti-p24 and anti-p55 (without any anti-gp120/160) probably represents a false positive reaction [86,87,88,89]. A patient with a false positive immunoblot with both anti-p24 and anti-p41 has also been reported [90]. Another type of false positive has been reported which displays a strong reaction to p13 and a weak reaction with p55 [88].

Reactivity to only the p24 protein of HIV in immunoblot analysis was often observed in early HIV infection [83,85]. It has been shown, however, that when blotted against antigen enriched for gp120/160, such sera also show reactivity to gp120/160 [89].

The results of this study demonstrated further similarities between FIV and HIV, as well as providing useful information for the diagnosis of FIV infection in the cat.

# 5. IN VITRO CELL TROPISM OF FIV

## 5.1 INTRODUCTION

This chapter describes the initial isolation of FIV and the subsequent efforts to produce large amounts of virus for the studies described elsewhere in this thesis. Various feline cell lines were compared for their ability to support the growth of FIV. Q201 cells, which were shown to be highly susceptible to FIV infection, were examined by flow cytometric analysis at intervals after infection and changes in the expression of cell surface markers were detected. Subsequently a monoclonal antibody (vpg15) was identified which blocked FIV-8 infection of both Q201 cells and CRFK cells. Thus there was strong evidence that the FIV-8 receptor was recognised by vpg15.

Initially, to isolate FIV from infected cats, selected sera were screened for anti-FIV antibodies by immunofluorescence using T-cells infected with the Petaluma strain of FIV (FIV-P). Sera were selected on two criteria. Firstly the cats had to show signs of immunodeficiency and secondly their sera had to be FeLV-negative. One isolate, namely FIV-Glasgow-8, was selected and was subsequently used for all work described in this thesis.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Isolation of FIV-Glasgow-8

#### 5.2.1.1. Infection of T-cells with plasma

T-cell cultures of 2mls were set up at a cell concentration of 2 x $10^6$ cells per ml in normal T-cell medium. Samples of blood plasma were diluted in normal T-cell medium (200uls plasma per ml), filtered through a 0.45um filter and incubated with the cells overnight. The following day the cells were washed and the concentration was adjusted to 5 x $10^5$ cells per ml. The cultures were examined daily for the appearance of cytopathic effect and

when this was evident the cells were pelleted and fixed for electron microscopic examination.

FIV was isolated from the plasma of 3 cats, thus indicating that cell-free infectious virus was present in these cats. These isolates were designated FIV-Glasgow-8 (FIV-8), FIV-Glasgow-14 (FIV-14) and FIV-Glasgow-15 (FIV-15). The following information was available for each cat:

Cat 8: 6 year old entire male domestic shorthaired cat
pyrexia, respiratory signs, lethargy,
anorexia, weight loss

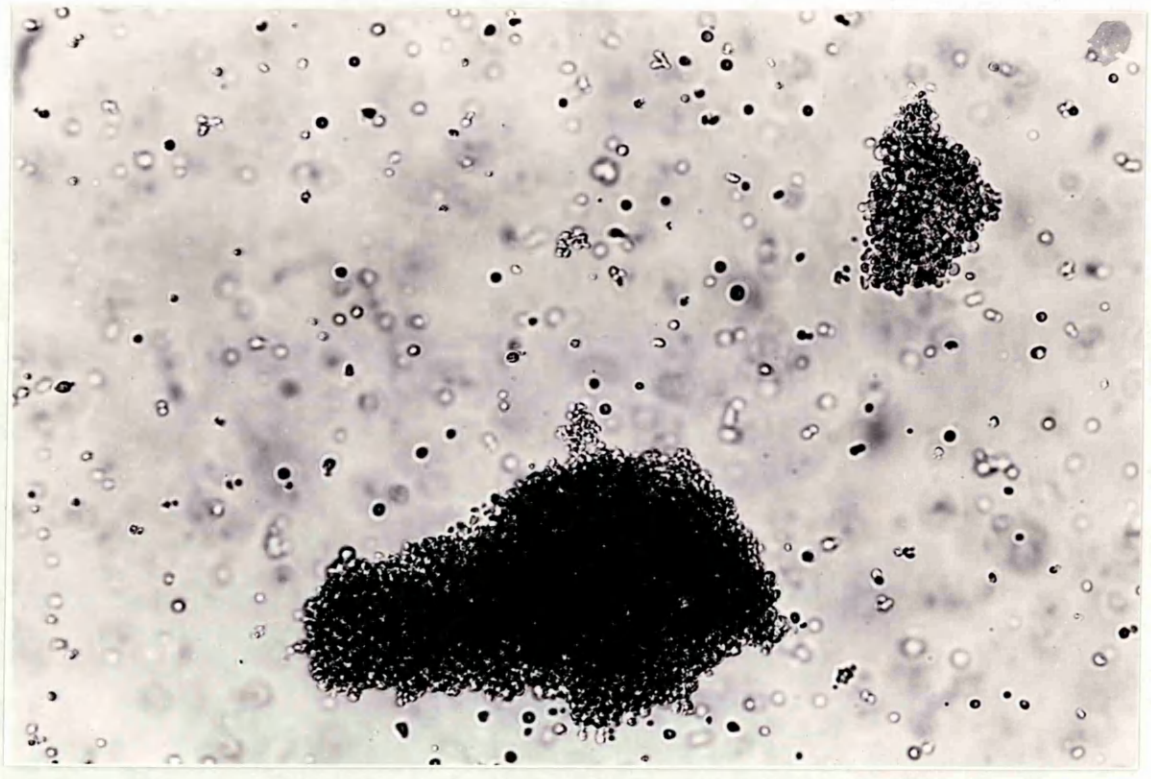
Cat 14: aged entire male domestic longhaired cat
pyrexia, weight loss, lymphadenopathy

Cat 15: 13 year old neutered male cat
weight loss, lymphadenopathy

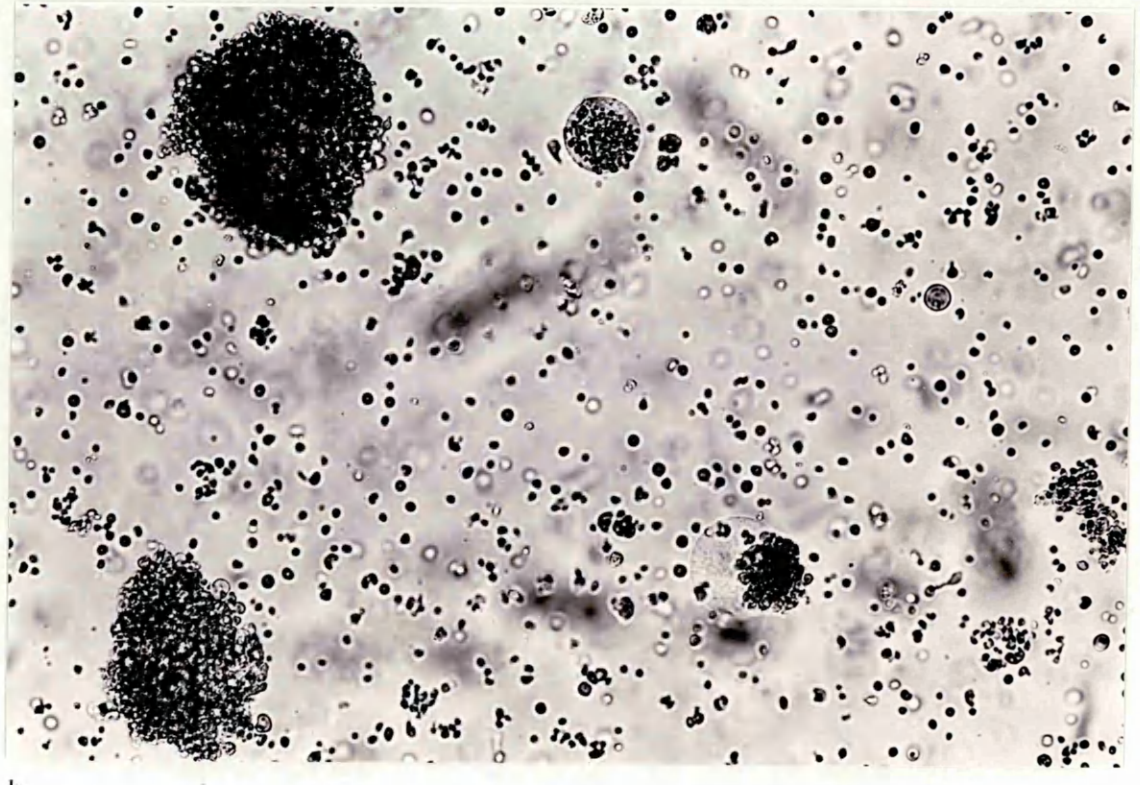
The cytopathic effect appeared in the cultures between 7 and 14 days in culture. Ballooning cells appeared first, followed by degeneration and syncytium formation (Figure 5.1). When examined using the electron microscope, particles of approximately 120nm diameter and displaying typical lentivirus morphology were found (Figure 5.2).

5.2.1.2. Isolation of FIV-8 from cultured T-cells

Two months after the initial sampling, a 5ml heparinised blood sample was obtained from cat 8 above. The sample was diluted to 20mls in RPMI-1640, layered over 5mls Ficoll-Paque solution and centrifuged at 2000rpm for 10 minutes. The white cells at the interface were carefully pipetted into a clean tube, diluted to 25mls in RPMI-1640 and centrifuged at 1000rpm for 5 minutes. The cell pellet was resuspended in 5mls normal T-cell medium to which Con A had been added (7.5ug per ml). After 2 days in culture, the unattached cells were washed out of the medium containing Con A



a.



b.

Figure 5.1

Feline peripheral blood T-cells a. uninfected and b. infected with FIV, showing a cytopathic effect with syncytium formation.

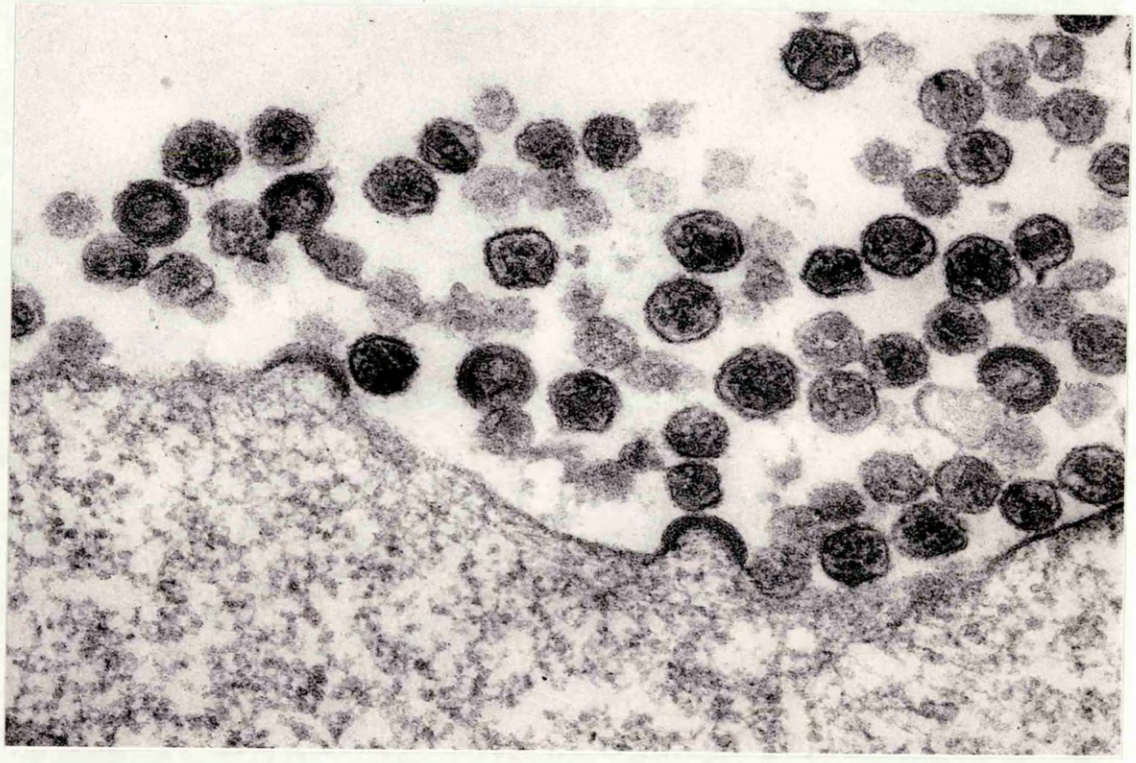


**Figure 5.2**

Electron micrograph of FIV particles in a culture of feline peripheral blood T-cells (courtesy of Dr H Laird).

and were transferred to a fresh flask containing 5mls normal T-cell medium. The culture medium was changed twice weekly and the cell concentration adjusted to 5 x $10^5$ cells per ml. Once a week susceptible cells from a normal cat were added to the culture and whenever necessary the dead cells were removed from the culture by density gradient centrifugation using Ficoll-Hypaque solution.

After 5 days in culture there was evidence of ballooning degeneration of cells and syncytium formation. The addition of uninfected T-cells resulted in a massive cytopathic effect over the following 2 or 3 days. If uninfected cells were not added to the culture, virus production decreased as the cells which were susceptible to infection died.

### 5.2.2. Titration of FIV-8 in normal T-cells and P51 cells

Two types of cell were used initially to grow FIV-8, normal cat T-cells and P51 cells. Both cells showed ballooning degeneration and syncytium formation with FIV-8 infection but this appeared to be more marked in P51 cells. To compare the relative susceptibilities of the two cell types, FIV-8 stock A was titrated in each.

Ten 5ml cultures of each cell type were set up in 25$cm^2$ flasks containing 5 x $10^6$ cells. Each culture was incubated overnight with five-fold dilutions of FIV-8 stock A, starting at 1 in 5. The medium was changed every three days. Cultures were assayed for virus production using the immunoblotting method described in section 4.2.3. Briefly, cell lysates were made (1 x $10^6$ cells per 10uls lysis buffer) at 6 and 12 days post infection. The proteins were separated by SDS-PAGE and blotted onto nitrocellulose. A polyclonal cat anti-FIV serum was used to stain the nitrocellulose membrane and infected cultures were identified by the presence of viral proteins.

### 5.2.3. Cell tropism studies

#### 5.2.3.1. Preparation of virus and virus-infected cells

Cultures of Q201 cells were infected with tissue culture supernatants containing FIV-P and FIV-8. After 24 hours the cells were washed out of the infected supernatant and the cells were resuspended in normal T-cell medium. The cultures were maintained for a week and susceptible cells were added. The culture supernatants were tested for FIV antigen by ELISA and when high titres were obtained the cells and supernatants were used for infections. Both cultures gave comparable ELISA titres.

The cells were pelleted, resuspended in 3mls normal T-cell medium and irradiated in a cobalt-60 source with a dose of 3000 rads. The cells were then washed twice in medium before co-cultivation. The infected supernatant was filtered using a 0.45um filter to remove cell debris before being used in infections.

#### 5.2.3.2. Co-cultivations with infected cells

The suspension cells used in this experiment were Q201, 3201, FL74, F422, F422-MD, T3 and McCoy cells. The monolayer cells used were 3 types of CRFK cells (ID10, HO6TI and MCGMI) and AH927 cells. Co-cultivations were set up with either $10^6$ FIV-infected Q201 cells and 4 x $10^6$ suspension cells or $10^6$ FIV-infected Q201 cells and 2 x $10^6$ freshly trypsinised monolayer cells.

#### 5.2.3.3. Infection of cells with supernatants

Cultures of 5 x $10^6$ suspension cells and 3 x $10^6$ monolayer cells were infected with 1ml FIV-infected supernatant (the supernatant equivalent of $10^6$ cells) in 4mls medium. Control cultures were set up without infected supernatnant.

5.2.3.4. Maintenance of cultures

The cultures were incubated overnight either with infected cells or with infected supernatant. The following day the suspension cells were centrifuged and were resuspended in 5 mls medium. The monolayers of cells were washed and new medium was added. The cells were subcultured every 5 days, the cell concentration of the suspension cells being adjusted to $10^6$ per ml and the monolayer cells being subcultured at a ratio of 1 in 4. Uninfected cells were treated in an identical fashion. The cultures were tested at weekly intervals for 4 weeks for the appearance of FIV antigen by ELISA.

**5.2.4. Use of monoclonal antibody to block FIV infection of Q201 cells**

Four cultures, each containing 5 x $10^6$ Q201 cells were incubated with solutions of different monoclonal antibodies in 1ml normal T-cell medium at 37$^0$C for 30 minutes. The monoclonal antibodies used were vpg30 (anti-fCD4), vpg15 and a monoclonal antibody against cat IgA. All antibodies were provided by Tom Dunsford. The antibodies were added to a final dilution of ascites of 1/200. The fourth culture was incubated in medium without the addition of antibody as a control. Each culture was centrifuged at 1000rpm for 5 minutes, the supernatant was discarded and the cells were resuspended in FIV-8-infected supernatant containing antibody as in the first incubation. The cells were incubated with the virus at 37$^0$C for 30 minutes before being washed twice and resuspended in normal T-cell medium containing a maintenance solution of antibody (1/500 dilution of ascites). The cells were subcultured 3 days post-infection and were maintained in culture for a week. Samples of supernatant were taken daily for measurement of p24 production by ELISA.

### 5.2.5. Use of monoclonal antibody to block FIV infection of CRFK cells

CRFK (ID10) cells were seeded onto 24-well plates overnight at a concentration of 2 x $10^5$ cells per well. The following day the medium was removed and the cells were incubated at 37°C for 30 minutes with two-fold dilutions of vpg30 and vpg15, starting at 1/100. The cells were then incubated with FIV-8-infected supernatant (derived from CRFK cells) containing antibody solution at 37°C for 30 minutes. The cells were then washed twice and medium containing maintenance antibody (1/5 dilution of antibody solution used to treat cells prior to infection) was added. The cells were maintained in culture for 4 days. Control wells were not incubated with antibody but were incubated with virus or were incubated with neither antibody nor virus.

The infection of CRFK cells after antibody treatment was assayed by immunohistochemical staining to detect p24 antigen. The cells were fixed in methanol for 20 minutes, washed in phosphate-buffered saline (PBS) and were incubated in 4% normal goat serum in PBS for an hour. After 3 PBS washes, an anti-p24 monoclonal antibody (kindly provided by Niels Pedersen) was added (1/3000 dilution in PBS) for an hour. After 3 PBS washes, biotinylated anti-mouse immunoglobulin (Vector Laboratories, UK) was added at a dilution of 1/2000 and was incubated for 15 minutes. The cells were washed three times in PBS and were then incubated with streptavidin-peroxidase (Sigma, UK) which was used at 1/1000 in PBS for a further 15 minutes. After 3 PBS washes the substrate solution (0.04% amino-ethyl carbazole in 0.05M acetate buffer pH 5.0 containing 0.05% hydrogen peroxide) was added for 10 minutes and the reaction was stopped by rinsing the wells in distilled water. The wells were examined under a light microscope for red-staining foci of infection expressing p24 antigen and these foci were counted.

## 5.3. RESULTS

### 5.3.1. Titration in normal cat T-cells and P51

Normal cat T-cells were consistently infected with the first four dilutions of FIV-8 stock A, ie. to 1/625. P51 cells were infected with a 1/3125 dilution on one occasion and with 1/15625 on another. Hence P51 cells were more susceptible to FIV-8 than normal T-cells.

### 5.3.2. Cell tropism studies

The susceptibility of cell lines to productive infection with FIV-8 and FIV-P are shown in Table 5.1. Each cell line was also examined for cell markers by flow cytometric analysis. The cells were stained with a panel of monclonal antibodies and the phenotypes are shown in Table 5.1 (Brian Willett, personal communication).

The cells which were susceptible to FIV-8 (Q201, F422 and T3) gave positive ELISA results from week 1, both with co-cultivation and with supernatant infection. The suspension cells which were susceptible to FIV-P (Q201, F422, F422-MD, T3 and McCoy) also gave positive results from week 1. The CRFK cells, on the other hand, became positive at various times. The ID10 cells became positive with supernatant at week 1 and with co-cultivation at week 3. The HO6TI cells became positive with supernatant at at week 3 and with co-cultivation at week 2. The MCGMI cells became positive with supernatant at week 2 and with co-cultivation at week 3.

### 5.3.3. Expression of cell markers in FIV-infected Q201 cells

Q201 cells infected with FIV-8-infected supernatant were examined daily for the expression of fCD4 and the molecule recognised by vpg15. The FIV-8-infected Q201 cells and a control culture of uninfected Q201 cells were analysed using flow cytometry. The

Table 5.1

**Cell phenotypes and susceptibility to infection with two isolates of FIV**

| cell line | cell markers | | | | | | susceptible | |
|---|---|---|---|---|---|---|---|---|
| | fpanT[1] | fCD4[2] | fCD8[3] | class I[4] | class II[5] | vpg15[6] | FIV-8 | FIV-P |
| Q201 | + | +[a] | - | + | + | +[a] | + | + |
| 3201 | + | + | + | + | + | - | - | - |
| FL74 | + | - | - | | + | + | - | - |
| F422 | + | - | - | + | + | + | + | + |
| F422-MD | + | - | - | | - | | - | + |
| T3 | + | - | - | | - | + | + | + |
| McCoy | - | - | - | + | - | + | - | + |
| ID10 | - | - | - | + | - | + | - | + |
| HO6TI | - | - | - | + | - | + | - | + |
| MCGMI | - | - | - | + | - | + | - | + |
| AH927 | - | - | - | + | - | + | - | - |

[1] monoclonal provided by Christopher Ackley [91]

[2] monoclonals provided by Christopher Ackley [92] and Tom Dunsford

[3] monoclonal provided by Max Cooper [93]

[4] monoclonal provided by John Hopkins [94]

[5] monoclonals provided by Niels Pedersen and Tom Dunsford

[6] monoclonal provided by Tom Dunsford

[a] decreased expression with infection with FIV-8

differences between the percentages of uninfected and infected cells expressing each marker are shown in figure 5.3 (data kindly provided by Brian Willett). It was demonstrated that there was a decreased expression of both fCD4 and the molecule recognised by vpg15 in FIV-infected cells in vitro. In cells which had been infected with FIV-8 for 28 days, however, the expression of fCD4 appeared to return to normal levels whereas there was still a decreased expression of the molecule recognised by vpg15.

### 5.3.4. Blocking of FIV infection

While pretreatment of Q201 cells with monoclonal antibodies against fCD4 and cat immunoglobulin A did not alter the course of infection with FIV-8, pretreatment with vpg15 blocked infection (Figure 5.4). Similarly in CRFK cells, vpg15 increasingly blocked FIV-8 infection as the antibody concentration increased (Figure 5.5), thus demonstrating that the effect was vpg15-dependent.

## 5.4. DISCUSSION

The first isolation of a local FIV isolate was made from plasma, demonstrating that virus may be free in plasma as well as cell-associated. Subsequently, it was found to be easier to isolate FIV from peripheral blood T-cells rather than serum or plasma which suggested that FIV was highly T-lymphotropic.

The clinical signs demonstrated by the cats from which these first isolates were made displayed non-specific clinical signs of an immunodeficiency-type disorder, similar to those reported by Pedersen et al [1] and Harbour et al [51]. From several isolates of FIV, FIV-8 was chosen for the studies in this thesis. This isolate was from a cat which was suffering from a very acute episode of disease and in tissue culture produced a marked cytopathic effect. It was necessary to grow large quantities of virus to examine the viral proteins, to make diagnostic reagents, for in vivo pathogenesis experiments and ultimately for constructing vaccines. Therefore, atempts were made to grow FIV-8

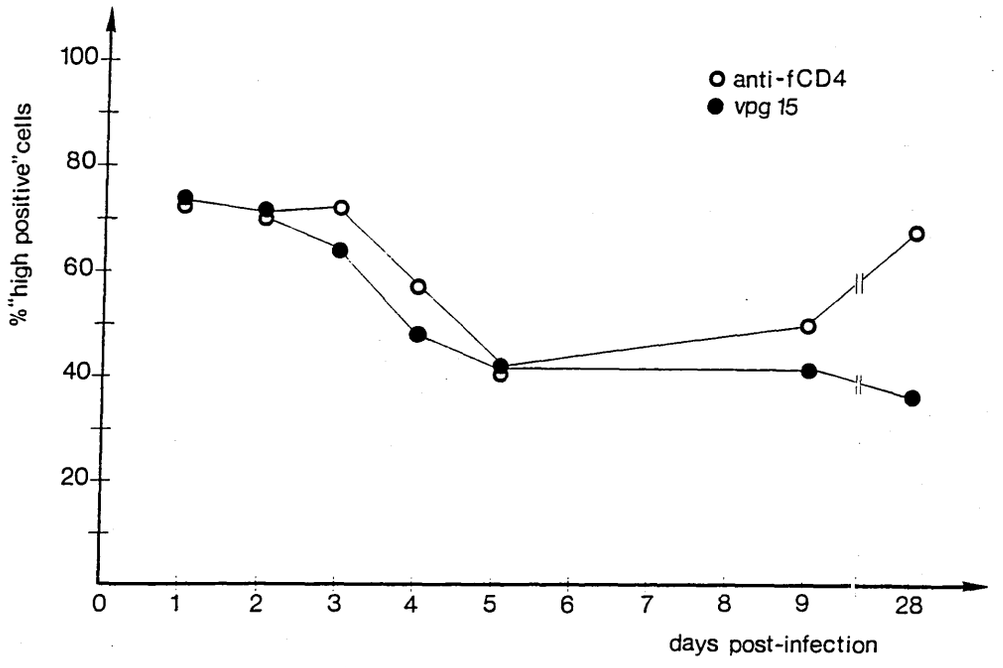



**Figure 5.3**

The differences in the percentages of uninfected and FIV-8-infected Q201 cells expressing fCD4 and the molecule recognised by vpg15 at daily intervals after infection.

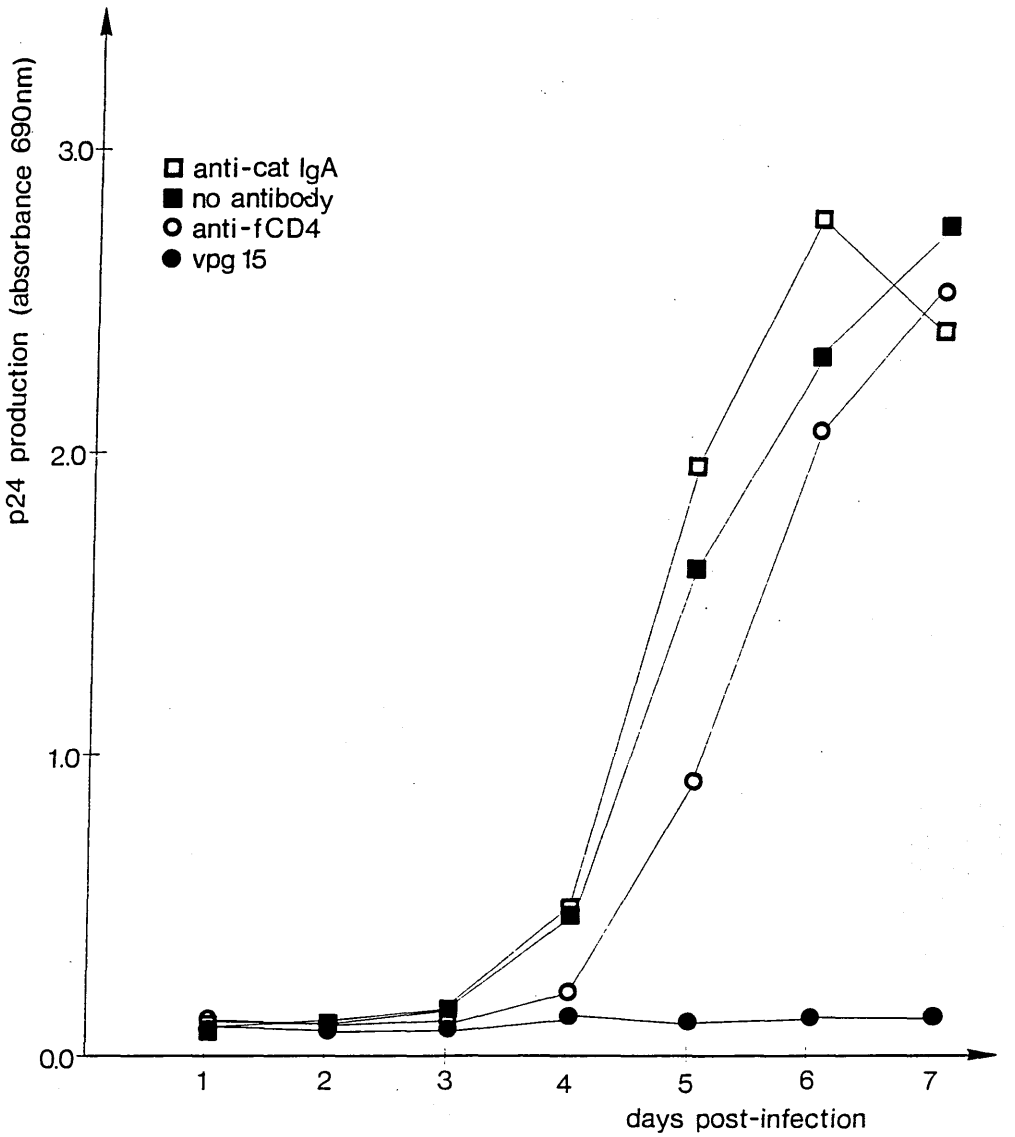



**Figure 5.4**

The levels of p24 in supernatants of Q201 cells infected with FIV-8 after incubation with anti-cat IgA, anti fCD4, vpg15 and antibody-free medium. The culture supernatants were tested daily by ELISA.

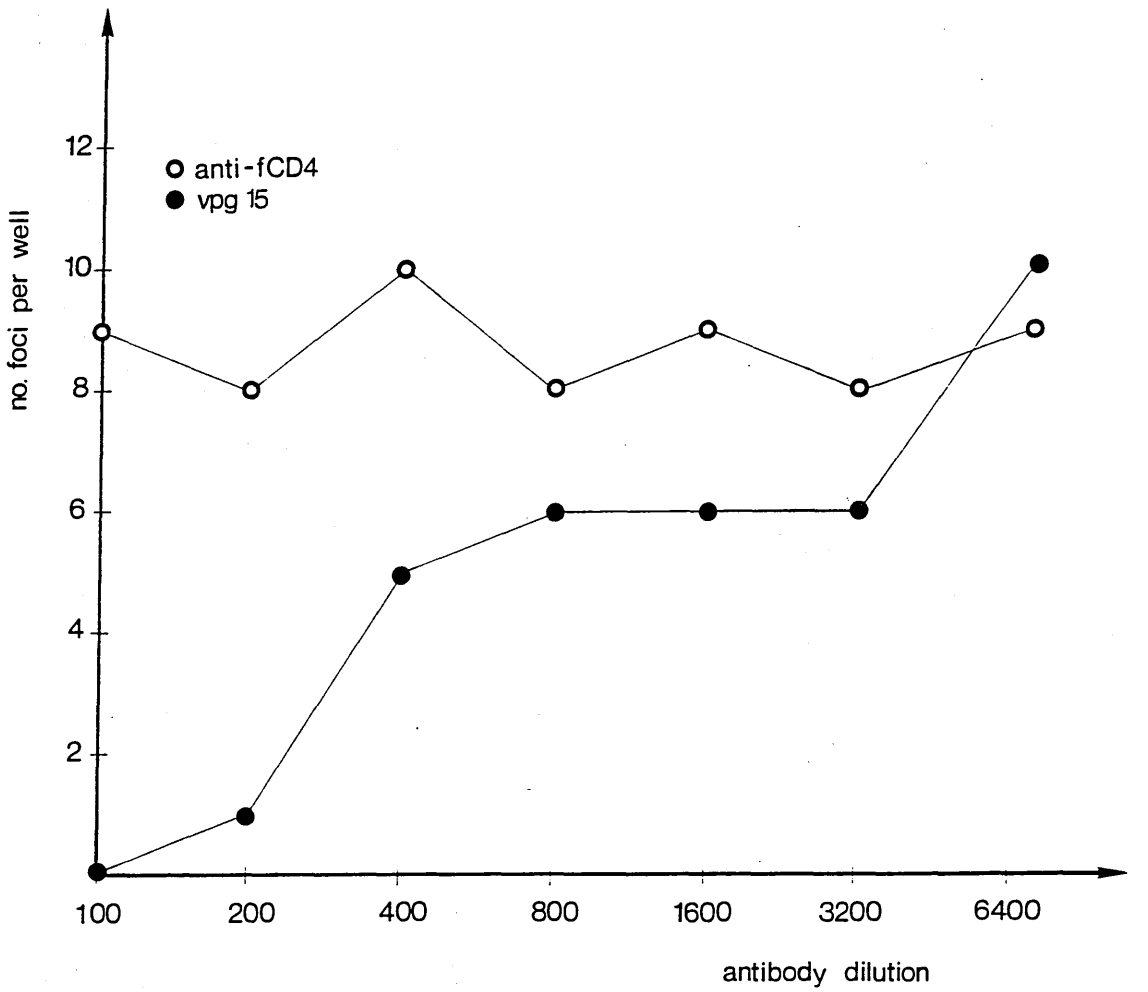



**Figure 5.5**

The number of foci staining for p24 in cultures of CRFK cells infected with FIV-8 after incubation with two-fold dilutions of vpg15 and anti-fCD4 antibody. The cells were stained 4 days post-infection.

in a variety of cell lines.

Initially, virus growth was assayed by the examination of infected cultures for the appearance of cytopathic effect, followed by electron microscopy and the identification of lentivirus particles. Subsequently, FIV antigen production was detected by immunoblotting cell lysates using the technique described in section 4.2.3. Later experiments used the recently developed commercial ELISA to measure p24 production.

As growth of FIV in peripheral blood T-cells and P51 cells required large amounts of recombinant human IL-2 as well as a supply of fresh, uninfected cells, attempts were made to grow FIV in other cell lines. The titration of FIV-8 in cells demonstrated that P51 cells were more susceptible to infection compared to normal cat T-cells. When examined by FACS analysis, it is seen that whereas normal cat T-cells are both fCD4+ and fCD8+, P51 cells are exclusively fCD4+. This suggested that fCD4 may have an important role in FIV infection.

The CD4 molecule has been shown to be the cellular receptor for HIV. It is a glycoprotein of 60 kilodaltons (kD) which is expressed on the cell membrane of mature T-lymphocytes and, to a lesser extent, on cells of the monocyte/macrophage lineage. Traditionally, CD4+ T-cells have been ascribed the function of helper/inducer cells, activating B-cells and cytotoxic T-cells. CD4+ T-cells interact with antigen-presenting cells which carry the major histocompatibility complex (MHC) class II antigen.

The relationship between CD4+ helper/inducer T-cells and infection with HIV was first suggested when the peripheral blood of AIDS patients was found to be depleted of CD4+ T-cells [22]. In vitro studies with HIV demonstrated a tropism for CD4+ cells [23] and infection of cells by HIV was inhibited by monoclonal antibodies directed against CD4 [23,24]. Furthermore, gp120, the envelope glycoprotein of HIV, binds to CD4 with high affinity [95], cells which are normally resistant to HIV infection become

susceptible when transfected with a CD4 expression vector [96] and recombinant soluble CD4 blocks HIV infection [26].

Hence it was important to investigate the role of fCD4 in FIV infection as this could provide a useful model for HIV infection. Therefore, various feline cell lines with different phenotypes were used to determine whether or not there was any correlation between the presence of fCD4 on the cell surface and susceptibility to infection with FIV. The susceptibility of cells to both FIV-P and FIV-8 were compared.

The co-cultivations in this experiment used irradiated FIV-infected cells to eliminate false positive results in the ELISA for FIV antigen arising as a result of release of virus from replicating FIV-infected cells. Also, preliminary experiments had shown that co-cultivation of T-cells with monolayer cells resulted in damage to the monolayer cells and therefore it was hoped that the use of irradiated cells would permit cell contact whilst still preserving the monolayer cells.

The results show that the two isolates used in this experiment had different cell tropisms. The differences could not be attributed to different virus concentrations as the two virus stocks gave comparable titres on ELISA.

The FIV-P stock had a wider cell range compared to FIV-8. It is possible that FIV-P had become more adapted to growth in tissue culture compared to FIV-8. Both viruses had been propagated in peripheral blood T-cells or thymocytes while in our laboratory but the early passage history of FIV-P was probably in CRFK cells.

In an earlier experiment it was possible to infect 3201 cells with FIV-8 (although the initially high level of virus production declined to negligible levels after several weeks). FIV-8-infected Q201 cells were co-cultivated with 3201 cells in the presence of recombinant human IL-2 for one week. When the IL-2

was removed from the medium FIV continued to be produced, as shown by immunoblotting. Virus produced by these 3201 cells appeared to have an envelope glycoprotein of only 110K (compared to 120K in Q201 cells) but this result was not reproducible. The FIV-8-infected 3201 cells were then co-cultivated with CRFK cells which also became infected. Neither 3201 cells nor CRFK cells could be infected with supernatants from Q201 cells although they could be infected with supernatants from FIV-infected 3201 or CRFK cells respectively.

From these early experiments and the more controlled experiment using irradiated cells for the co-cultivations, it would appear that FIV can become adapted for growth in many cell lines. In the co-cultivations where IL-2 was included there was an increased interval for cell contact as the infected cells remained viable while IL-2 was present. It could therefore be hypothesised that in the co-cultivations with irradiated infected cells there was inadequate time for infection to occur. Alternatively, each isolate of FIV-8 may contain several different strains of virus with different cell tropisms and when the virus appears to become adapted to a different cell line the predominant strain changes. The early finding that FIV-8-infected 3201 cells infected CRFK cells would be consistent with this theory.

Phillips et al [97] have shown that a molecular clone of FIV-P replicates well in CRFK cells but less efficiently in T-cells. This experiment, however, shows that our stock of FIV-P is highly infectious for T-cells and therefore supports the theory that each isolate consists of a mixture of virus strains.

Although it was possible to demonstrate a decrease in fCD4 as well as vpg15 expression in FIV-8-infected Q201 cells, it was not possible to correlate susceptibility to infection with the presence of fCD4 on the cell surface. 3201 cells which expressed high levels of fCD4 could only be infected by long-term co-cultivation in the presence of IL-2. Furthermore, some fCD4- cell lines, such as CRFK, could be infected with FIV.

Nevertheless, some CD4- cell lines (such as brain and muscle-derived cell lines) can be infected with HIV, although with low efficiency since co-cultivation with CD4+ infected cells is necessary for infection [98]. It has also been suggested that CD4 independent infection can occur when anti-HIV antibodies allow uptake of virus by Fc receptor-bearing cells [99]. Therefore these results do not rule out the possibility that fCD4 has a role in FIV infection.

However, the molecule recognised by vpg15 is present on all cell lines which have been infected with FIV so far. Therefore, this molecule was a more likely candidate for the FIV receptor than fCD4. The demonstration in both Q201 and CRFK cells that vpg15 could block infection with FIV-8 provided strong evidence that vpg15 recognised the FIV receptor. As the antibody was produced by inoculation with pelleted tissue culture supernatant derived from FIV-8-infected thymocytes, it is likely that the inoculum contained the receptor molecule attached to the virus.

Further evidence that the molecule recognised by vpg15 is the FIV receptor was provided by the demonstration of a decreased expression of the molecule in FIV-infected cells in vitro. As the expression of fCD4 was also shown to decrease, it remains possible that fCD4 also has a role in FIV infection. Although vpg30 did not block FIV-8 infection, it is possible that antibodies against different epitopes would inhibit infection. Indeed, the cells which are most susceptible to FIV infection remain Q201 cells which are exclusively fCD4+, suggesting an important role for this molecule.

Further experiments have suggested that the molecule recognised by vpg15 is the low affinity $F_C$ receptor, equivalent to human CD16 (Brian Willett, personal communication). This molecule is present on neutrophils, macrophages and activated T-cells. As Q201 cells are activated T-cells, it is possible that they are highly susceptible to FIV not as a direct result of their high

expression of fCD4 but because they also express high levels of CD16.

# 6. VACCINATION STUDIES-1

## 6.1. INTRODUCTION

As the value of FIV as a model for HIV infection in man is now well established (Pedersen et al, 1987, Olmsted et al, 1989, Hosie and Jarrett, 1990), FIV can be used to investigate both the nature of a protective immunity against infection and the viral antigens which elicit protective immunity.

In an attempt to determine the role of humoral immunity in protection from FIV infection, cats were challenged with FIV-8 after passive immunisation with anti-FIV antibodies. In an experiment to establish which viral antigens are important for protection, cats were immunised with ISCOMs containing the components of purified FIV. When there was a good serological response against the core proteins, the cats were challenged with FIV-8.

To test the efficacy of vaccination against FIV it was necessary to demonstrate protection from challenge with a known amount of virus. A large stock of FIV-8 was made and dispensed in aliquots at -70$^{o}$C. This stock was titrated in cats to establish the minimum infectious dose and was subsequently used to test the efficacy of various immunisation strategies. After challenge, cats were monitored for evidence of infection by the detection of viraemia (by isolation of FIV from cultured peripheral blood T-cells) and by the development of antibodies to FIV proteins (by ELISA and immunoblotting). Attempts were made to detect FIV in tissues using the polymerase chain reaction (PCR). This would be a valuable additional test in vaccinated cats which were protected from viraemia after challenge.

## 6.2. MATERIALS AND METHODS

### 6.2.1. Titration of FIV-8 in cats

Five groups of three 6 month old cats were used in this experiment. Each group was inoculated with tenfold dilutions of the FIV-8 stock, prepared as described in section 2.2.5. The cats were sampled at 0, 3, 6, 9, 12 and 17 weeks post infection. Serum was tested for the presence of antibody by ELISA and by immunoblotting. Virus isolation from the peripheral blood T-cells was attempted at 0, 3, 9 and 17 weeks post infection. Post-mortem examination of these cats was performed 9 months post infection. Samples of tissues were taken for histological examination and for the detection of FIV using PCR.

### 6.2.2. PCR protocol

To prepare tissues for amplification, pieces of frozen tissue of approximately 200mg were homogenised in liguid nitrogen and then lysed in 500ul 1% Triton-X-100 in 10mM Tris (pH7) with 1mM EDTA for 20 minutes. Proteinase K was added to a final concentration of 500ug per ml and the samples were incubated at 37$^{o}$C for 24 hours. The samples were then left on ice for 4 hours after the addition of 125ul 5M NaCl. After centrifugation, the high molecular weight DNA was obtained by resuspending the pellet in 100ul deionised, distilled water ($ddH_2O$) and dialysing overnight in a large volume of $ddH_2O$. The supernatant was removed and the low molecular weight DNA was extracted using phenol and chloroform followed by alcohol precipitation. The pellet thus obtained was resuspended in 100ul $ddH_2O$.

Each PCR reaction was carried out with 50ul sample in a total volume of 100ul using the Gene Amp kit (Perkin Elmer Cetus, Norwalk, CT). The final reaction mixture contained 50mM KCl, 10mM Tris-HCl pH 8.4, 1.5mM MgCl2, 0.1% w/v gelatin, nucleoside triphosphates (0.2M each) and Thermus aquaticus polymerase (1 unit per reaction). Primers were added at a concentration of

0.5uM. The primers (1 TGG GAT GAG TAT TGG AAC CCT GAA 24 and 331 TGC GAA GTT CTC GGC CCG GAT TCC 355) amplified a 355 base pair segment in the LTR of FIV. These primers were chosen as they were known to amplify a region which was highly conserved between the Petaluma and various Glasgow isolates of FIV (Fiona Thompson, personal communication).

Thirty cycles of DNA amplification were used, with a 1 minute denaturation step at 91$^{o}$C, a 1 minute annealing step at 40$^{o}$C and a 3 minute extension step at 70$^{o}$C. Positive amplification controls were FIV-8 and FIV-14 LTRs cloned into the plasmid PIC19R (Fiona Thompson, personal communication).

### 6.2.3. Southern Hybridisation

Following amplification, 10ul samples of each reaction mixture were added to 3ul loading dye (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in 0.089M Tris borate, 0.089M boric acid, 0.002M EDTA (TBE)) and the fragments were resolved in a 1% agarose gel containing ethidium bromide (0.5ug per ml TBE), alongside DNA size markers (PhiX174 RF DNA-Hae III digest, BioLabs, New England).

After electrophoresis, the gel was examined under UV light for the presence of the 355 base pair LTR fragment and was photographed. The gel was then denatured for an hour in 1.5M NaCl, 0.5M NaOH, neutralised for an hour in 1M Tris HCl pH8, 1.5M NaCl and then the DNA was transferred to a nylon membrane (Hybond-N, Amersham, UK) using the Southern blotting procedure as described by Maniatis et al [100]. The gel was then removed from the membrane and the loading wells were marked with a pencil. The membrane was wrapped in Saran film (Dow Chemical Company, UK) and placed under a UV lamp for 10 minutes.

The membrane was then placed in a sealed bag containing prehybridisation fluid (6 x SSC, 0.5% SDS, 5 x Denhardt's solution, 100ug per ml denatured salmon sperm DNA) and agitated

in a water bath at 65°C for 4 hours. After incubation, a denatured $^{32}P$- labelled LTR probe was injected into the sealed bag, the bag was resealed and the probe was hybridised with the membrane overnight at 65°C. The probe was an FIV-8 LTR fragment (kindly provided by Fiona Thompson) which was labelled with [alpha-$^{32}P$] dCTP (Amersham, UK) using a random primed DNA labelling kit (Molecular Biology Boehringer Mannheim) and purified using a NICK column (Pharmacia, Sweden).

After hybridisation, the membrane was removed from the bag and washed at 65°C in 2 x SSC, 0.5% SDS for 4 hours with several changes of buffer. The membrane was then autoradiographed.

### 6.2.4. Preparation of anti-FIV antibody

Serum was pooled from 5 cats which had been experimentally infected with FIV-8 for 9 months. All 5 cats were healthy and seropositive for FIV. The 100 ml of serum was heated twice to 56°C for 30 minutes to inactivate virus and then spun in an SW21 rotor over 20% sucrose at 21K for 3 hours. The upper lipid layer and pellet were discarded. The serum was added to 200ml of 0.06M sodium acetate and stirred before adding 7.5ml n-octanoic acid dropwise and continuing to stir for 30 minutes. The solution was spun at 2500 rpm for 20 minutes and the supernatant was filtered before dialysis against 0.154 sodium chloride. Finally, the antibody solution was concentrated to 25ml using polyethylene glycol.

To measure the antibody titre, five-fold dilutions of serum were tested for anti-FIV antibody by ELISA before and after treatment with n-octanoic acid. The titre was taken as the reciprocal of the last dilution to give a positive S/P ratio according to the manufacturer [71]. The titres obtained in the passively immunised cats were similarly measured.

A culture of T-cells was incubated with 2mls of antibody solution for 24 hours. The cells were then washed and the culture was

monitored for evidence of viral infection for 14 days by visual examination for cytopathic effect and by ELISA for production of p24.

### 6.2.5. Preparation of ISCOMS

FIV-8 was grown in cat peripheral blood T-cells and clarified by centrifugation at 7.5K for 30 minutes before storage at -70$^{o}$C. Four hundred mls of stored tissue culture fluid was thawed at 37$^{o}$C, added to the same volume of saturated ammonium sulphate and incubated at 4$^{o}$C for 10 minutes before being centrifuged at 7.5K for 10 minutes. The pellets were resuspended to 18mls in Tris-saline and layered in two SW41 centrifuge tubes containing 0.8ml 50% sucrose and 2.0mls 20% sucrose in PBS. The tubes were spun at 30K for 2 hours and the virus bands were recovered in 1.5ml. The virus was then passed through a Sephadex G-25M column (column PD10, Pharmacia, Sweden) equilibrated with Tris-saline and recovered in 2.5ml. Aliquots were analysed by SDS-PAGE followed by either immunoblotting or Coomassie blue staining.

To prepare FIV-ISCOMS, 1.5ml of virus was added to the same volume of 4% decanoyl n methyl glucamide and incubated for an hour at 37$^{o}$C to disrupt the virus. Lyophylised Quil A was added to a final concentration of 0.1% together with 150ul lipid mix (10mg phosphatidyl choline, 10mg cholesterol per ml in 20% decanoyl n methyl glucamide in Tris-saline). The preparation was dialysed extensively over two days in 50mM ammonium acetate.

The purified virus was shown to contain the gag proteins p24 and p17 as well as the precursor p55. There was also some envelope glycoprotein visible on the immunoblot (but not sufficient to be detectable by Coomasie blue staining).

The ISCOMs were analysed by electron microscopy and by density centrifugation on a 10 to 40% sucrose gradient at 35K for 12 hours. Electron microscopy showed the typical regular cage-like structures, approximately 40nm in diameter (Figure 6.1). The

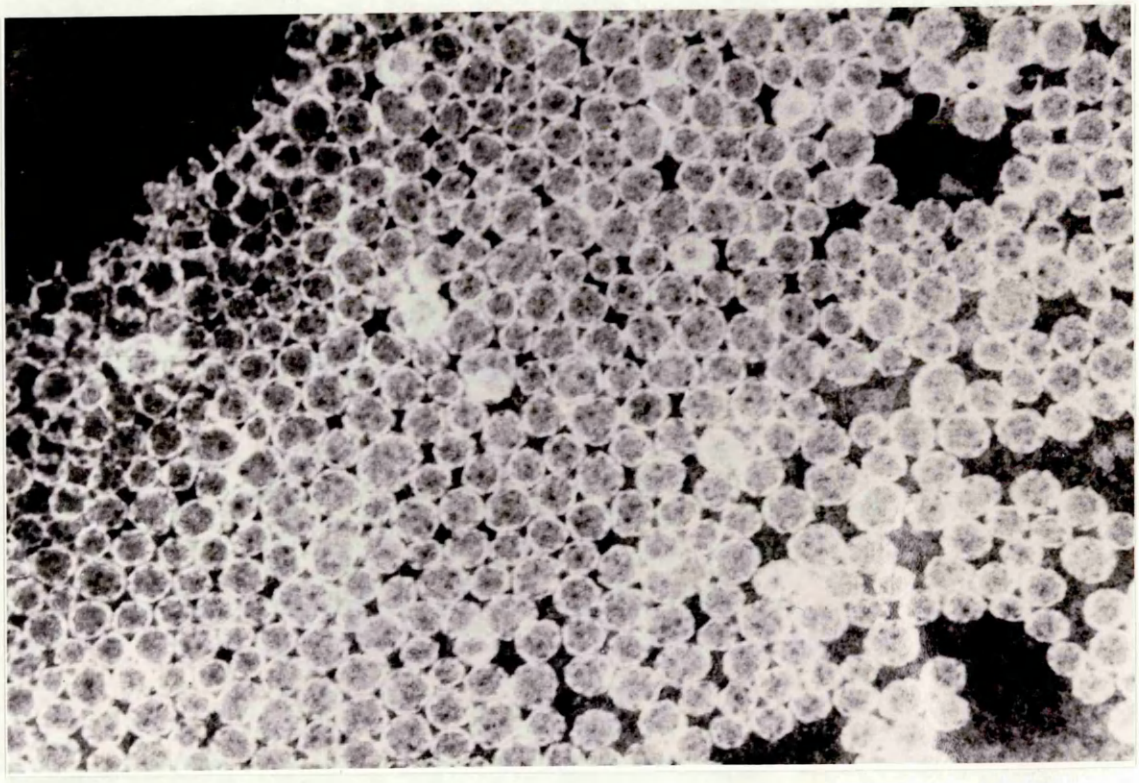


**Figure 6.1**

Electron micrograph of a sample from the FIV-ISCOM preparation (courtesy of Dr H Laird)

ISCOM preparation banded at a density of $1.087gml^{-1}$.

The composition of the virus ISCOMS is shown in the immunoblot in figure 6.2. The ISCOMs contained predominantly p24 as well as p17 and small amounts of p55 and gp120. The relative concentrations of the proteins were determined using ovalbumin as a standard. A sample of the ISCOM preparation was run in a well alongside dilutions of ovalbumin in an SDS-PAGE gel and after electrophoresis the gel was stained with Coomassie blue stain. The degree of staining of the proteins in the ISCOM preparation was compared to that of the standards and the protein content was estimated to be approximately 10ug of p24 and p17 per dose. There was negligible gp120 by Coomassie blue staining.

To determine the concentration of unincorporated Quil A in the FIV-ISCOM preparation, a method of rocket electrophoresis was used. Ten ul, 5ul and 2.5ul samples were electrophoresed in 150mM glycine, 20mM Tris buffer alongside tenfold dilutions of a reference standard of Quil A in a 0.8% agarose gel containing 2% sheep erythrocytes. After elecrophoresis the zone of haemolysis was directly proportional to the concentration of Quil A in the sample. By comparison with the standards, the level of unincorporated Quil A in the FIV-ISCOMs was estimated to be approximately 10ug per dose.

### 6.2.6. Virus isolation

To isolate FIV, peripheral blood T-cells were grown from heparinised blood as described in section 2.2.2 and the culture was monitored for the production of FIV by examination for cytopathic effect, with confirmation by ELISA for p24 antigen or immunoblot analysis of cell lysate (as described in section 4.2.3).

### 6.2.7. Measurement of anti-FIV antibodies

A commercial ELISA kit was used to measure the serological

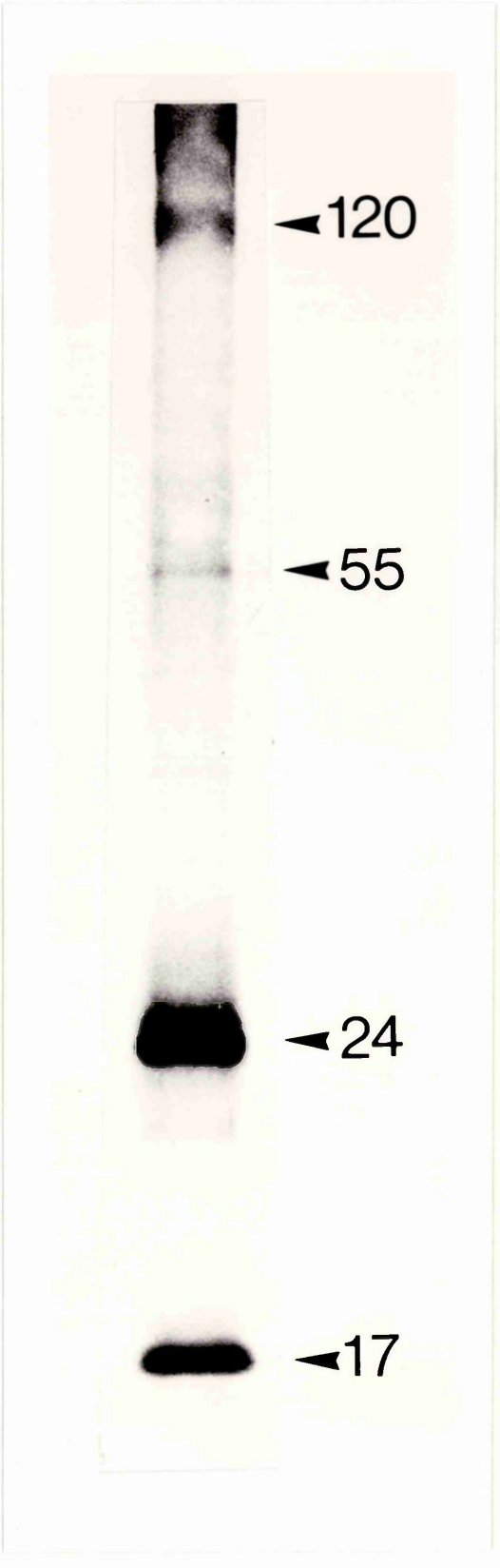



**Figure 6.2**

Immunoblot of a sample from the FIV-ISCOM preparation stained with a polyclonal cat anti-FIV serum.

response to FIV and the responses to individual proteins were determined by immunoblotting (as described in section 4.2.3).

### 6.2.8. Measurement of anti-p24 antibodies

Antibodies against p24 were measured using an ELISA in which Immulon-I wells were coated overnight with 0.2ug recombinant p24 protein which was produced by cloning the coding sequence for p24 into pGEX and expressing the protein in Escherichia coli JM83 [84]. The wells were blocked with 2% NFMP in TBS for an hour and washed twice in TBS. The sera was diluted 1 in 100 in 20% normal goat serum, 2% NFMP, 0.5% Tween-20 in TBS and incubated in the wells for one hour. The wells were washed 4 times with TBS and alkaline phosphatase conjugated goat-anti-cat immunoglobulin was incubated in the wells for one hour. After washing 6 times, disodium paranitrophenyl phosphate substrate was added and after 30 minutes the absorbance at 405 nm was measured.

## 6.3. RESULTS

### 6.3.1. Titration in Cats

The pattern of seroconversion in the 5 groups of cats is shown in Table 6.1. At 17 weeks post infection all cats in groups 1 and 2 and 1 out of 3 cats in each of groups 3 and 4 had seroconverted. None of the cats in group 5 seroconverted.

Virus could not be isolated from any of the cats at 0 or 3 weeks post infection. At 9 and 17 weeks post infection, virus could be isolated from all cats in groups 1 and 2, 1 of 3 cats in each of groups 3 and 4 and none of the cats in group 5. By applying the Karber formula and using viraemia at week 17 as the end-point, the $ID_{50}$ was $2.2 \times 10^4$.

Table 6.1

**Proportions of seropositive cats after infection with tenfold dilutions of FIV-8**

| virus dilution | weeks post infection | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 3 | 6 | 9 | 12 | 17 |
| $10^{-2}$ | 0/3 | 0/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| $10^{-3}$ | 0/3 | 0/3 | 3/3 | 3/3 | 3/3 | 3/3 |
| $10^{-4}$ | 0/3 | 0/3 | 0/3 | 1/3 | 1/3 | 1/3 |
| $10^{-5}$ | 0/3 | 0/3 | 0/3 | 1/3 | 1/3 | 1/3 |
| $10^{-6}$ | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 | 0/3 |

### 6.3.2. Histological examination of FIV-infected cats

The most striking lesion in the FIV-infected cats was lymphadenopathy. This affected the pharyngeal and mesenteric nodes especially, as well as the Peyer's patches in the alimentary tract and the bronchio-mediastinal nodes. There was a marked proliferation of the germinal centres which was indistinguishable from the lesion seen in early HIV infection (WFH Jarrett, personal communication). Lesions also occurred in the gut associated lymphoid tissue (GALT), thymus, lung, spleen, myocardium, liver, kidney and bone marrow. Table 6.2 shows the distribution of the lesions in each cat examined. The number of cats with lesions and the severity of those lesions was greater in groups 1 and 2 which received the lowest dilutions of FIV.

### 6.3.3. Comparison of tissues for the detection of FIV

Lysates were made from samples of pancreas, bone marrow, lung, lymph node, kidney and spleen taken from an SPF cat and from a cat which had been experimentally infected with FIV-P for 9 months. Agarose gel electrophoresis of the PCR products displayed bands of approximately 355 base pairs in only two wells. These were the wells containing the reaction products from the positive control and the bone marrow of the FIV-infected cat. The two bands hybridised specifically with the FIV-8 LTR probe (Figure 6.3).

### 6.3.4. Detection of FIV in bone marrow of titration cats

Lysates were made from samples of bone marrow from 14 of the 15 cats experimentally infected with tenfold dilutions of FIV-8 (described in section 6.2.1). Agarose gel electrophoresis of the PCR products showed bands with the positive control, cat 1 in group 1 and cat 3 in group 3. These bands hybridised specifically with the FIV-8 LTR probe. The autoradiograph also displayed LTR-specific bands in cats 2 and 3 in group 1, cats 2 and 3 in group 2, cat 1 in group 3, and all cats in group 4 (Figure 6.4). These

Table 6.2

a. Distribution of histological lesions in cats euthanased 9 months post infection with tenfold dilutions of FIV-8; b. virus isolation (VI) and antibody status (Ab) of samples taken post-mortem and c. detection of FIV in bone marrow biopsies using the polymerase chain reaction (PCR).

| virus dilution | $10^{-2}$ | | | $10^{-3}$ | | | $10^{-3}$ | | | $10^{-4}$ | | | $10^{-5}$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cat no. | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| **a. Organs** | | | | | | | | | | | | | | | |
| CNS | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| a/c | - | - | - | - | - | + | - | - | - | - | - | + | - | + | + |
| GALT | ++ | ++ | na | na | - | ++ | ++ | - | - | - | + | + | ++ | + | ++ |
| LNs | ++ | + | ++ | ++ | ++ | ++ | ++ | - | + | - | + | ++ | ++ | + | ++ |
| Spleen | ++ | + | ++ | + | + | + | ++ | - | - | - | + | + | na | + | - |
| BM | - | - | - | na | na | + | + | - | - | - | na | + | - | + | - |
| Thymus | ++ | ++ | + | ++ | ++ | ++ | ++ | - | - | - | - | + | + | - | - |
| Lungs | + | - | + | + | - | + | + | - | + | - | + | + | + | na | - |
| Heart | - | - | + | + | - | - | + | - | - | - | - | - | - | na | - |
| Liver | - | - | - | - | - | + | + | - | - | - | - | + | - | na | - |
| Kidneys | - | - | - | + | - | na | + | - | - | - | - | na | - | na | - |
| **b. Blood** | | | | | | | | | | | | | | | |
| VI | + | + | + | + | + | + | + | - | - | - | - | + | - | - | - |
| Ab | + | + | + | + | + | + | + | - | - | - | - | + | - | - | - |
| **c. PCR** | | | | | | | | | | | | | | | |
| BM | + | + | + | - | + | + | + | - | + | + | + | + | - | - | na |

- nothing abnormal detected
+ pathological lesions present
++ marked lesions present
na tissue not available for examination

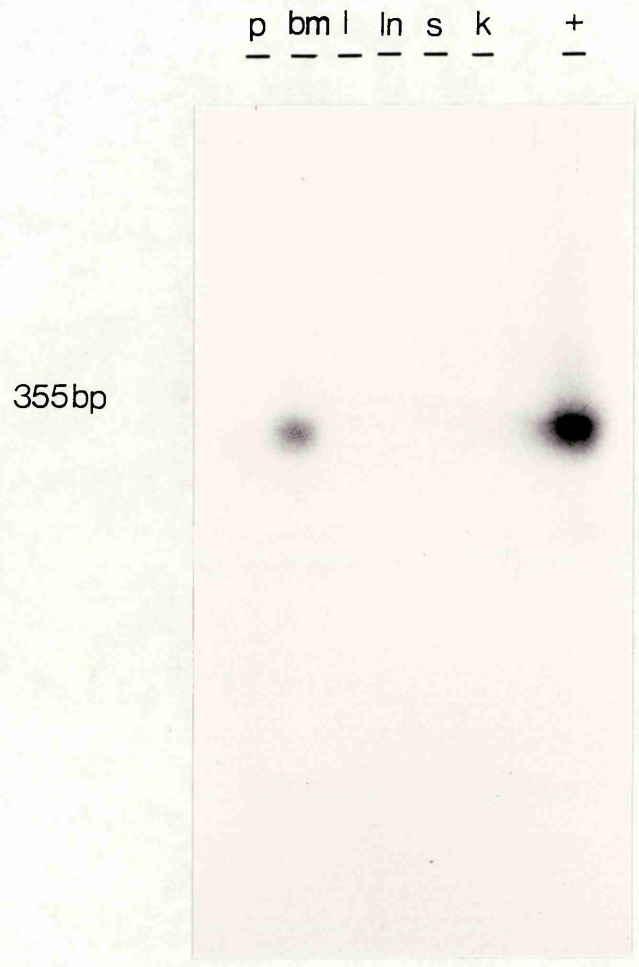



**Figure 6.3**

Southern hybridisation of PCR products from pancreas (p), bone marrow (bm), lung (l), lymph node (ln), spleen (s) and kidney (k).

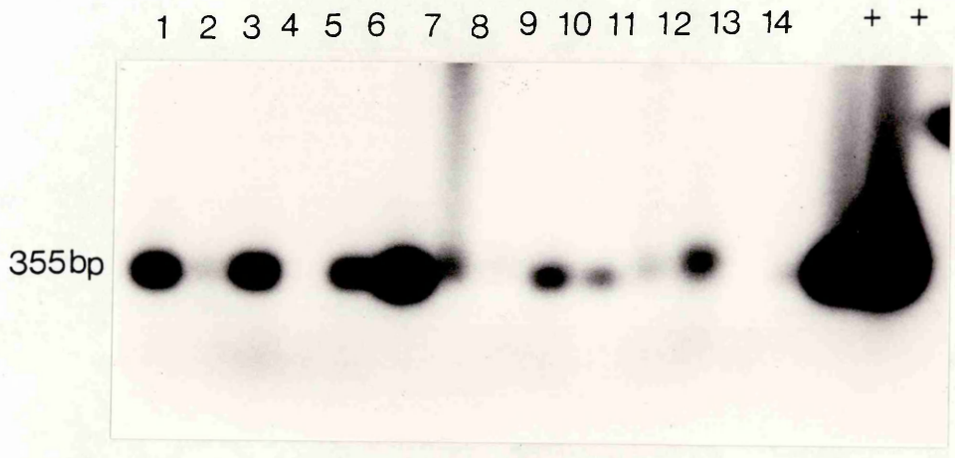



**Figure 6.4**

Southern hybridisation of products from bone marrow biopsies of cats infected with tenfold dilutions of FIV-8. Cats 1 to 3 were infected with $10^{-2}$, cats 4 to 6 with $10^{-3}$, cats 7 to 9 with $10^{-4}$, cats 10-12 with $10^{-5}$ and cats 13 and 14 with $10^{-6}$ dilutions of FIV-8.

positives were not detected on the agarose gel. Table 6.2 shows a comparison of the antibody and virus status of the cats with the detection of FIV DNA in the bone marrow.

### 6.3.5. Passive immunisation of cats

Two 8 month old specific pathogen free cats were inoculated intravenously with 10mls of anti-FIV antibody. Two hours later the two immunised cats and 2 age-matched control cats which had not received antibody were challenged with 40 infectious units of FIV-8. The cats were blood sampled prior to immunisation, prior to challenge and at weekly intervals thereafter.

The titre of the serum prior to treatment with n-octanoic acid was 500 and after treatment was 2500. Samples from the passively immunised cats 2 hours post immunisation gave titres of 100. These titres were maintained one week later but there was no antibody at 2 weeks.

Virus isolation was attempted at weekly intervals after challenge. At 4 weeks post challenge, virus could be isolated from both of the passively immunised cats but not from either of the control cats. By 5 weeks post challenge, virus could be isolated from all four cats.

### 6.3.6. Vaccination with FIV-ISCOM

#### 6.3.6.1. Virus isolation

Four 5 month old specific pathogen free cats were inoculated subcutaneously with the FIV-ISCOM at 0 weeks, 5 weeks and 18 weeks. These 4 cats, together with 4 age-matched unvaccinated controls, were challenged at 20 weeks with 20 infectious units of FIV-8. At 3 weeks post challenge 3 out of 4 vaccinated cats and one out of 4 control cats were virus-positive (Figure 6.5). By 5 weeks, all of the vaccinated cats and all but one of the control cats were virus-positive (Figure 6.6). There was no change up to

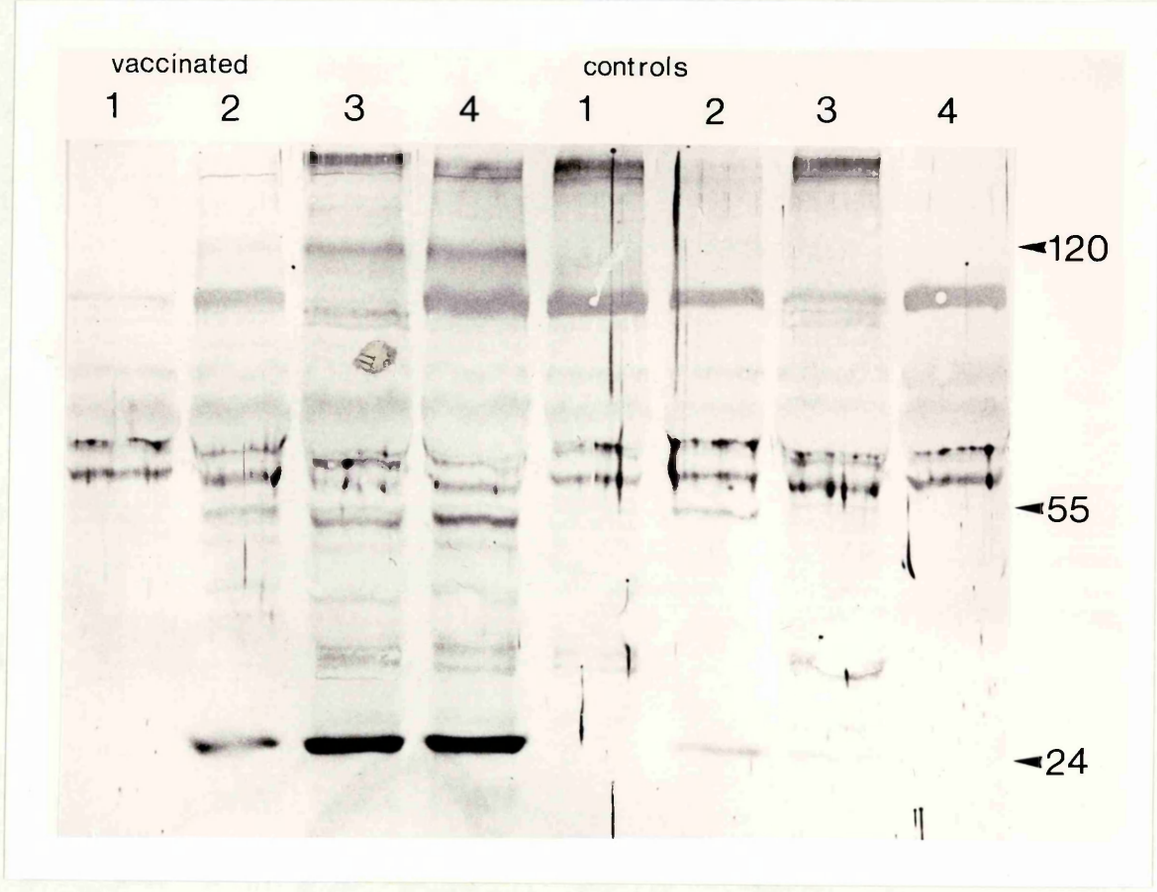



**Figure 6.5**

Immunoblot of cell lysates of peripheral blood T-cells from vaccinated and control cats taken 3 weeks post challenge and cultured for 14 days. The immunoblot was stained with a polyclonal cat anti-FIV serum.

8 weeks post challenge.

6.3.6.2. Production of anti-FIV antibodies

At 5 weeks the 4 vaccinated cats were antibody positive by ELISA and had antibodies to p55, p24 and p17 on immunoblots. At 7 weeks the levels of antibodies to the core proteins were very high as shown by the immunoblots, especially to p24 and p17, but there were no antibodies to gp120 (Figure 6.7). All 4 vaccinated cats remained ELISA-positive for the remainder of the experiment.

After challenge the unvaccinated control cats were monitored by ELISA for anti-FIV antibodies. All 4 cats were antibody-negative at 3 and 5 weeks post challenge. Three out of 4 were antibody-positive at 7 weeks post challenge. These 3 cats remained ELISA-positive for the remainder of the experiment. The three seropositive cats had antibodies against both core and envelope proteins (Figure 6.7).

6.3.6.3. Production of anti-p24 antibodies

The absorbances of the sequential samples from the vaccinated cats and the controls are shown in figure 6.8. This graph shows that very high levels of anti-p24 antibodies were achieved by vaccination with FIV-ISCOM compared to the levels achieved after challenging the controls with 20 infectious units of FIV-8.

6.4. DISCUSSION

The end-point of the titration experiment was taken as the production of persistent viraemia after infection. Every cat which was viraemic at the end of the experiment was also seropositive. Using persistent viraemia as an end-point, the $ID_{50}$ was calculated as $2.2 \times 10^4$ and hence suitable doses could be used to challenge immunised cats.

The comparison of various tissues as suitable templates for the

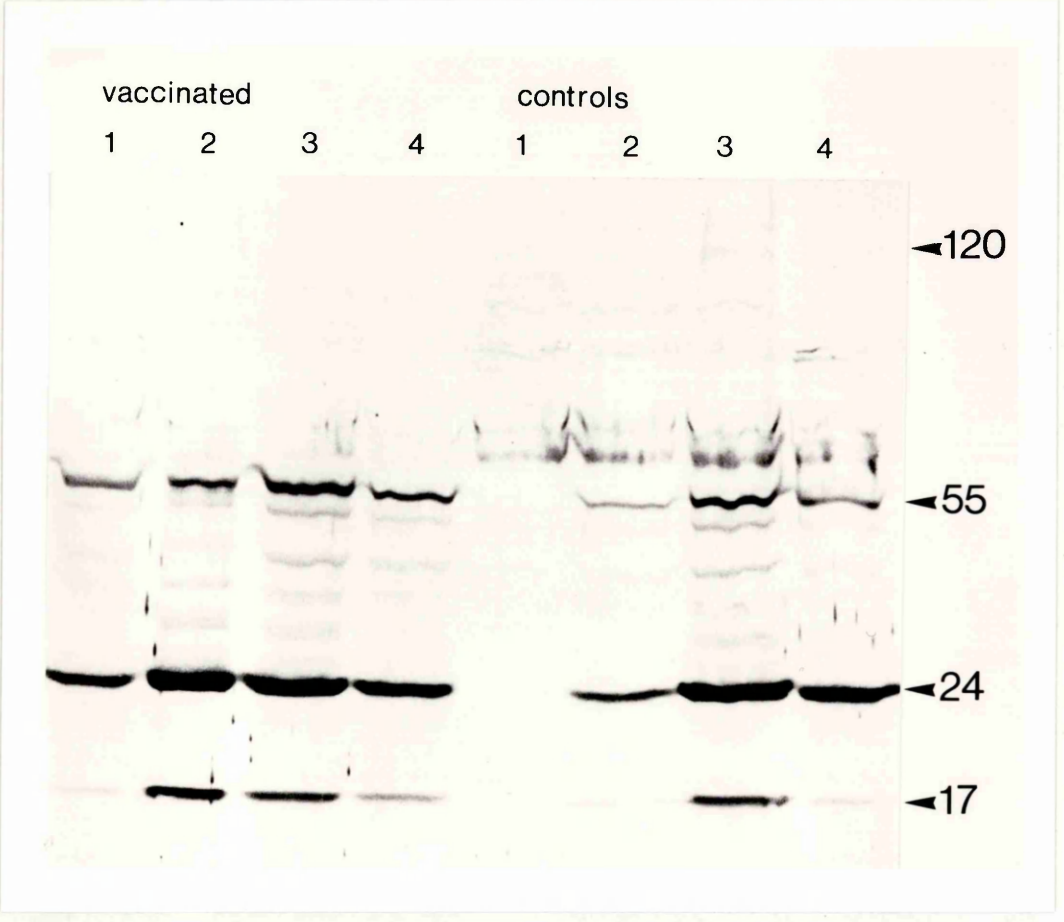



**Figure 6.6**

Immunoblot of cell lysates of peripheral blood T-cells from 4 cats vaccinated with FIV-ISCOMs and control cats taken 6 weeks post challenge and cultured for 14 days. The immunoblot was stained with a polyclonal cat anti-FIV serum.

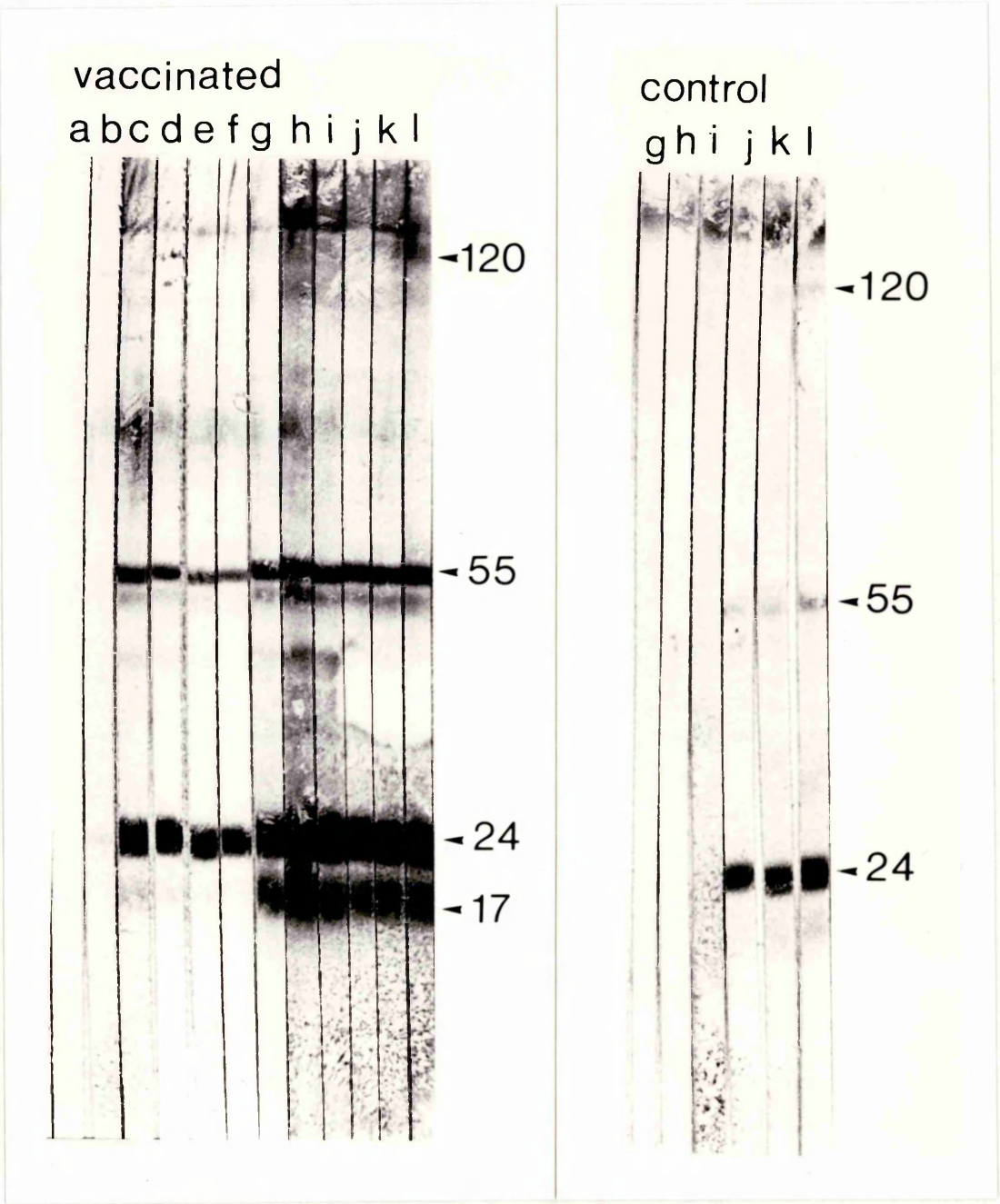



**Figure 6.7**

Sequential immunoblots of serum samples from a cat vaccinated with FIV-ISCOMs and a control cat taken at a.week 0 (inoculation 1), b. week 5 (inoculation 2), c.week 7, d.week 8, e.week 11, f.week 18 (inoculation 3), g.week 20 (challenged), h.week 23, i.week 25, j.week 27, k.week 28, l.week 30.

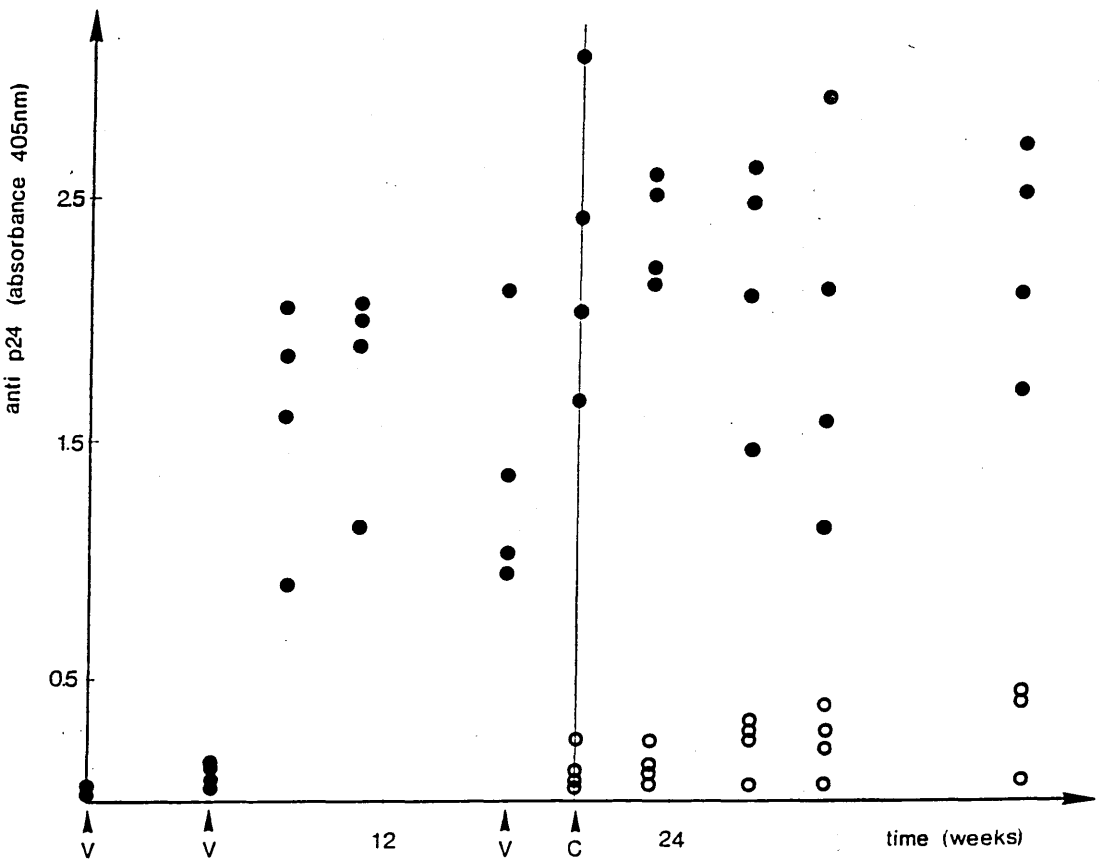



Figure 6.8

Absorbances of samples taken from 4 cats vaccinated with FIV-ISCOMs (closed circles) and 4 control cats (open circles) in an ELISA for p24 antibodies. The cats were vaccinated at weeks 0, 5 and 18 and challenged at week 20.

detection of FIV using PCR showed that the tissue of choice was bone marrow. This may be due to bone marrow containing less fibrous tissue compared to the other tissues and therefore being more easily digested by the proteinase K to release viral DNA. On the other hand, it may suggest that FIV DNA is most abundant in the cells of the bone marrow. Indeed, it has been shown by in situ hybridisation that the platelet precursor cells, the megakaryocytes, contain high levels of FIV DNA (Niels Pedersen, personal communication).

It was possible to detect FIV DNA in three cats which were negative by virus isolation. This would suggest that PCR is a more sensitive technique compared to the assays which were available to detect virus after cultivation of peripheral blood T-cells. On the other hand PCR is such a sensitive technique that false positive results can arise from contamination [101]. The possibility of contamimation was minimised by handling the reagents using positive displacement pipettes in a laminar flow hood.

One cat which was positive by virus isolation was negative using PCR. It is possible that in this case there was less proviral DNA integrated in the bone marow cells. It has been shown that the sensitivity of PCR to detect HIV genome can be significantly increased by using nested primers [102]. Bracket primers may bind to a region of the host genome similar to the intended target viral sequence. This non-specific reaction is minimised by taking a portion of the product amplified using the bracket primers and adding it to a new reaction mix containing nested primers. The use of nested primers for the amplification of FIV DNA should therefore be investigated.

In this study it was possible to passively transfer anti-FIV antibody to recipient cats, achieving titres comparable to those seen in healthy seropositive cats. These titres were maintained for one week. In spite of this, the passively immunised cats were not protected from challenge with 40 infectious units of

homologous virus. On the contrary, the virus isolation data suggest that there may have been enhancement of infection as virus could be isolated earlier from the cats which had received antibody compared to the controls.

Although it was possible to measure the titre of antibody achieved in the recipient cats by ELISA, the titre of neutralising antibodies was not known. It is posible that the neutralising antibody titre was inadequate to confer protection. Indeed the antibodies present in healthy seropositive cats may not be protective against infection. Administration of passive antibody has been successful against challenge with FeLV [103]. In addition, a study in which nine consistently HIV-antigen-positive patients were treated with hyperimmune plasma from healthy individuals with high anti-HIV titres resulted in sustained clearance of p24 antigen and clinical improvement in five patients [104]. It is possible, however, that the clearance of p24 was merely a result of the administered antibody masking the antigen from the assay. When chimpanzees were passively immunised with a preparation derived from plasma of HIV-seropositive donors, all animals became infected after challenge with 400 $TCID_{50}$ of HIV. The HIV-positive plasma had high levels of virus neutralising antibody as measured by an in vitro neutralisation assay and the virus used to challenge the chimpanzees was the same strain of HIV as had been used in the neutralisation assay [105].

In the present study, immunisation with FIV-ISCOMs produced a good antibody response to the gag proteins of FIV as shown by immunoblotting and induced extremely high anti-p24 antibody titres. But even these high levels of anti-gag antibodies did not protect against challenge.

The FIV-ISCOM vaccine contained very low amounts of gp120 and the vaccinated cats did not produce any anti-env antibodies. The major neutralising determinant of HIV is situated within the third hypervariable region, the V3 loop of the gp120 protein

[106]. Therefore it would appear (by analogy) that anti-*env* antibodies would be important in protecting against FIV infection. Also, it is known that HIV gp120 binds the host cell receptor CD4 [107] and hence if an immune response interfered with this interaction then infecton could not occur. Furthermore, data from murine retrovirus systems indicate that protective immunity is induced by the envelope protein [108]. This would suggest that an effective vaccine against FIV should contain significant levels of envelope glycoprotein.

As in the passive immunisation experiment, there was a suggestion that after vaccination with FIV-ISCOMs there was enhancement of infection in the immunised cats, the vaccinated cats becoming viraemic sooner after challenge than the controls. Antibody-dependent enhancement of viral infection can occur in vitro by at least two mechanisms. The first is via $F_C$ receptors on macrophages which mediate the uptake of virus complexed to antibody. This has been demonstrated with HIV [99,109]. The second is via complement components which bind the antigen-antibody complex and interact with complement receptors on cells, resulting in infection of the cells with virus. This type of enhancement has been demonstrated with both HIV and SIV [110,111]. To date, there have been no in vitro studies of antibody-dependent enhancement of FIV infection. The role of enhancement in lentivirus infections requires further investigation as evidence is lacking that antibody-dependent enhancement has importance in vivo. This study sugests that FIV may have an important role in the elucidation of the protective response against lentivirus infections.

# 7. VACCINATION STUDIES-2

## 7.1. INTRODUCTION

The results of the immunisation with FIV-ISCOMs, together with the information available for HIV and SIV, suggested that it was important to include a source of gp120 in a potential vaccine against FIV. As FIV-infected cells are a rich source of gp120, in contrast to purified virus, it was proposed to use fixed, inactivated FIV-infected cells as the immunogen. In this way, it was hoped that the vaccinated cats would produce anti-env antibodies and would therefore be more likely to be protected from challenge. Attempts were also made to partially purify gp120 from FIV-infected cell lysates for incorporation into ISCOMs.

## 7.2. MATERIALS AND METHODS

### 7.2.1. Preparation of fixed, inactivated cells

FIV-8 was grown in cat PBTs. The cells were infected with FIV-infected tissue culture supernatant and uninfected cells were added three times at weekly intervals. The cells were harvested when the culture was producing a large virus yield, as shown by an ELISA for antigen production.

The cells were treated with glutaraldehyde using a modification of methods previously described [109,110]. The infected culture was spun at 1000rpm for 5 mins, 6 x $10^7$ cells were resuspended in 6ml ice cold Earle's balanced salt solution and 6mls 0.15% glutaraldehyde was added. The cells were left on ice for 5 mins and 12ml 0.1M glycine was added on ice. The cells were centrifuged at 1000rpm for 5 mins, the supernatant was discarded, the cells were resuspended in 6ml of normal T-cell medium with 10% DMSO and 600ul Tris pH 8.0 and 13.2ul beta propiolactone were added. The cells were incubated at 37$^{o}$C in the dark for 2 hours, dispensed into 200ul volumes and stored at -70$^{o}$C.

A volume containing 2 x $10^6$ cells was washed and co-cultivated with 4 x $10^6$ uninfected cells and the culture was maintained for one month. The culture was examined daily for the appearance of cytopathic effect and samples of tissue culture supernatant were taken at weekly intervals to be tested for FIV-antigen by ELISA There was no evidence of cytopathic effect after co-cultivation of the fixed, inactivated cells with uninfected cells for 4 weeks. Similarly, the results of the weekly tests for antigen production were all negative.

For each dose of vaccine, 2 x $10^6$ cells were thawed and washed twice in PBS. The cells were resuspended in 1ml PBS containing 50ug Quil A and kept on ice until inoculation.

### 7.2.2. Gel Filtration

FIV-infected peripheral blood T-cells were harvested 3 days post-infection and lysed in 1% Empigen-BB dipolar ionic detergent at a cell concentration of 4 x $10^7$ per ml. A fast protein liquid chromatography apparatus (Pharmacia, Sweden) was equilibrated with 50mM Tris containing 0.1% Empigen. Two 250ul samples were loaded onto a Superose-6 column (Pharmacia, Sweden) and the proteins were eluted in 1ml fractions. Fractions in the initial high molecular weight peak were retained, the apparatus having been calibrated so that the proteins in the initial peak had apparent molecular weights of greater than 80 kilodaltons (George Reid, personal communication). The fractions were concentrated before immunoblot analysis.

### 7.2.3. Affinity Chromatography

The fractions containing gp120 were pooled and loaded onto a Lentil-Lectin-Sepharose 4B column which had been equilibrated with 50 mM Tris, 0.1% Empigen. The column was washed with equilibration buffer and any bound glycoprotein was eluted with 50mM Tris, 0.1% Empigen and 50mM alpha-methyl mannoside.

## 7.3. RESULTS

### 7.3.1. Vaccination wth cell vaccine

Five 10 week old specific-pathogen-free kittens were inoculated subcutaneously at 0, 3, 6, 9, 12, 15 and 18 weeks. Serum samples were taken at 3 weekly intervals for anti-FIV antibody testing by ELISA and by immunoblotting.

The 5 vaccinated cats and 5 age-matched control cats were challenged with 20 infectious units of FIV-8 at week 21. Prior to challenge, sera were tested for anti-env antibodies by radioimmunoprecipitaion of $C^{14}$-glucosamine labelled P51 cells, using the method described in section 4.2.2. Virus isolation was attempted from the peripheral blood lymphocytes as described in section 6.2.5 at week 18, on the day of challenge and at 3 weekly intervals thereafter.

At 0 and 3 weeks post-immunisation all 5 vaccinated cats were negative for FIV-antibody by ELISA and immunoblotting. At 6 weeks, 1 out of 5 cats was positive by ELISA and immunoblotting. By 9 weeks all 5 vaccinated cats were antibody-positive. Sera taken at week 15 contained antibodies against the env proteins gp120 and gp41 in all 5 vaccinated cats, as shown in figure 7.1. Sequential samples from one of the vaccinated cats were tested by radioimmunoprecipitaion to determine when anti-env antibodies could first be detected. There was evidence of antibody at 6 weeeks post-inoculation in this cat.

Sera taken prior to challenge were tested for neutralising antibody. All of the control cats and one of the vaccinated cats had no detectable neutralising antibody, 3 had titres of 25 and one had a titre of 200 (R. Osborne, personal communication).

All vaccinated and control cats were negative by virus isolation 3 weeks prior to and on the day of challenge. By 3 weeks post-challenge, 4 of the 5 vaccinated cats and none of the controls

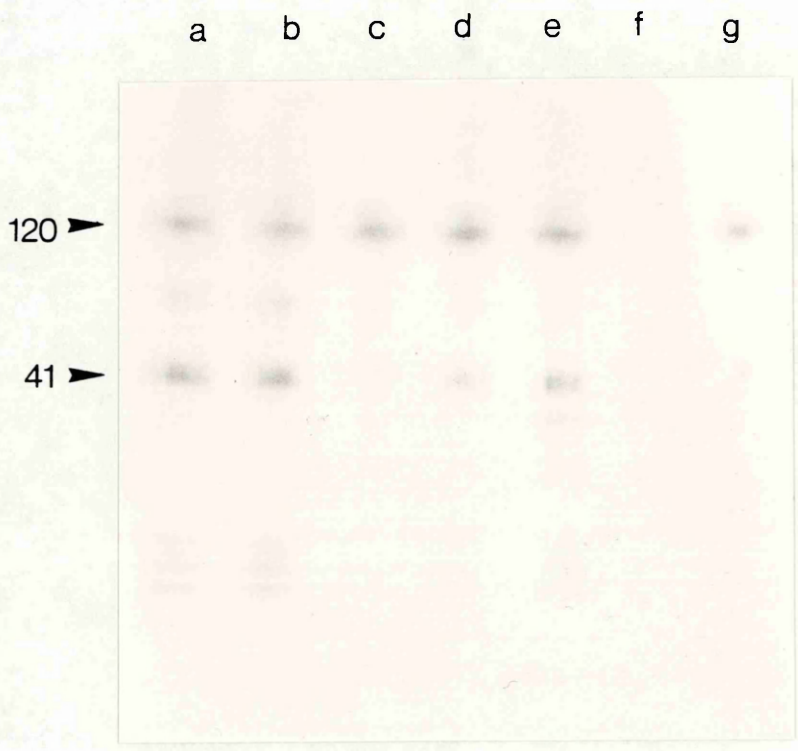



**Figure 7.1**

Radioimmunoprecipitaitions (RIPAs) of sera with $^{14}$C-glusosamine labelled FIV-8-infected P51 cells. Lanes a to e show the RIPAs of serum taken at week 15 from the 5 cats inoculated with the inactivated cell vaccine, lane f shows the RIPA of serum taken at week 15 from an unvaccinated control cat and lane g shows the RIPA taken from a cat 9 months post experimental infection with FIV-P.

were viraemic. By 6 weeks post-challenge, all 5 vaccinated cats and 4 of the 5 controls were viraemic.

### 7.3.2. Partial purification of gp120

The gp120 content at the various stages of purification are shown by immunoblot analysis in figure 7.2. The immunoblot illustrates that gp120 can be purified successfully from the core proteins. Using this method, however, the efficiency of recovery was low and therefore it was not possible to obtain a sufficient quantity of purified envelope protein for incorporation into an ISCOM vaccine.

## 7.4 DISCUSSION

Since the efficiency of recovery of gp120 is low using gel filtration and affinity chromatography, it appears to be difficult to achieve sufficient amounts for incorporation into ISCOMs using this system. As attempts to obtain large quantities of recombinant FIV envelope glycoprotein have not yet been successful, this study used inactivated FIV-infected cells as a vaccine containing significant amounts of gp120. In this way it was possible to compare the responses of cats to inoculation with a vaccine containing predominantly core proteins with the responses to a vaccine containing both core and envelope proteins.

In SIV, various degrees of protection have ben achieved with different inactivated whole virus vaccines [35,36,37]. Desrosiers et al [35] used purified, detergent disrupted virus in muramyl dipeptide adjuvant. Two of six vaccinated monkeys were protected from viraemia after challenge with 200-1000 infectious units and 5 of the 6 were protected from disease.

In contrast, Murphey-Corb et al [36] used formalin-inactivated gradient purified virus in muramyl dipeptide adjuvant. Eight of nine vaccinated monkeys were protected from challenge with 10

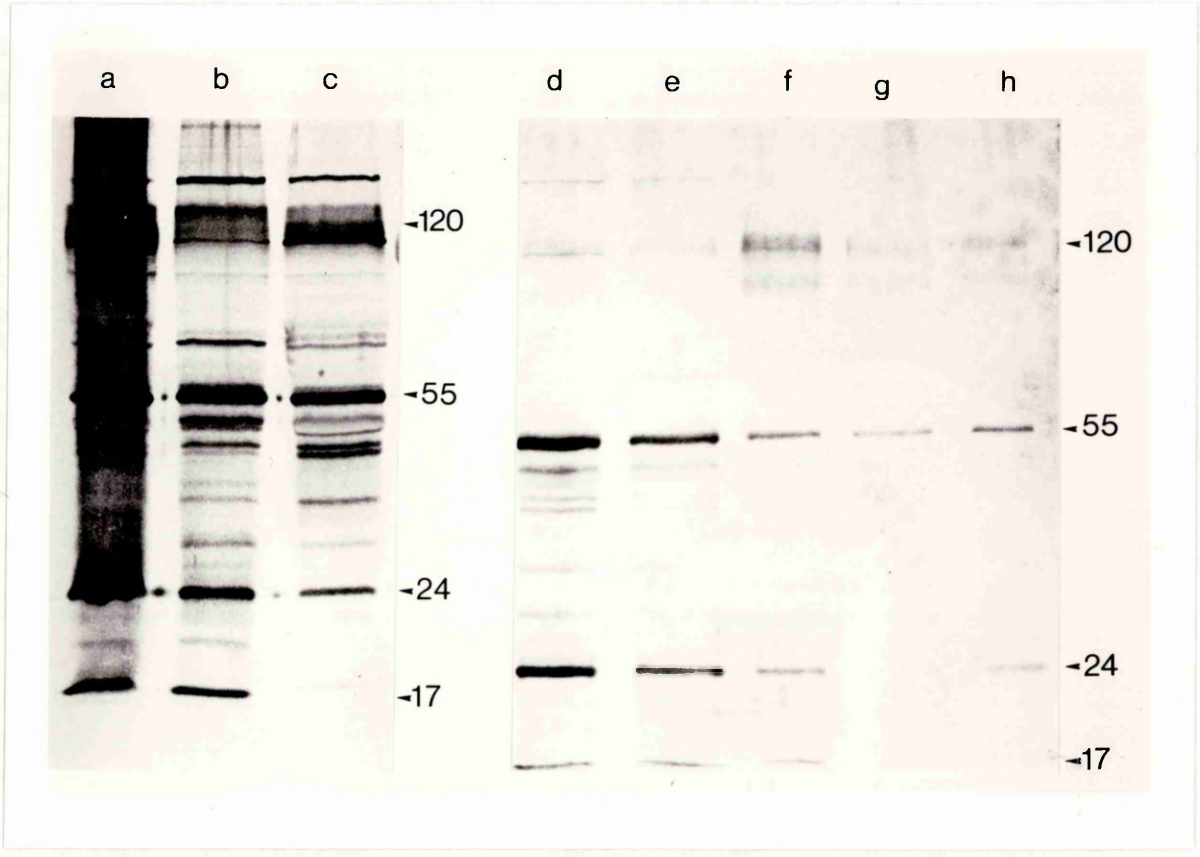



**Figure 7.2**

Immunoblot analysis of samples taken at various stages in the partial purification of gp120. Lane a shows the crude lysate; lanes b and c the two fractions collected from the FPLC apparatus; lane d the fraction which did not bind lentil-lectin; lane e the material which was washed from the lentil lectin column, lane f the material eluted with alph-methyl mannoside, lane g the material which was washed from the column and lane h the material obtained when the lentil lectin was boiled in reducing sample buffer.

infectious units of SIV. The remaining animal showed a delay in developing disease.

In a third study, Gardner et al [37] used a beta-propiolactone inactivated virus vaccine. The monkeys were challenged with 10 infectious units of SIV. There was protection in all 3 animals inoculated with vaccine in muramyl dipeptide adjuvant, in 1 of the 2 inoculated with vaccine in incomplete Freund's adjuvant and in 1 of 3 inoculated with vaccine and no adjuvant.

The differences between these observed protective efficacies may be explained by diferent immunisation schemes, choice of adjuvants or the route and dose of challenge. In all cases, the challenge virus was the same isolate of SIV as had been used in the vaccine preparation. More recent studies have shown protection against heterologous challenge with SIV (J. Stott and M. Cranage, personal communications).

In this study, a vaccine consisting of fixed, inactivated cells was produced which contained sufficient FIV envelope protein to induce anti-env antibodies. Furthermore, neutralising antibody was detectable in 4 of the 5 vaccinated cats prior to challenge. In spite of this, however, the vaccinated cats became viraemic after challenge. Indeed, as in the passive immunisation experiment and the vaccination experiment using FIV-ISCOMs, it appeared that after inoculation with the cell vaccine there was enhancement of infection in the immunised cats, the vaccinated cats becoming viraemic sooner after challenge than the controls.

There are several possible reasons which would explain the enhancement of FIV infection in this study whilst others have reported protection [54]. Similar discrepancies have been recorded with FeLV [111]. It has been shown that vaccination with FeLV envelope glycoprotein induces a protective immune response in the cat [112]. However, in one study, cats vaccinated with purified FeLV gp70 or gp85 developed antibodies against both gp70 and whole FeLV detectable by ELISA, but these antibodies did

not neutralise the virus in vitro [111]. Also, the cats were more susceptible to infection with virulent FeLV compared to unvaccinated controls. It is possible (both in this FeLV experiment and in the FIV cell vaccine experiment described in this chapter) that non-neutralising antibodies formed complexes with the challenge virus, increasing its uptake via $F_c$ receptors on monocytes, macrophages and activated T-cells. This has been demonstrated with West Nile virus [113], dengue virus [114] and HIV [27,99,115]. Alternatively, complement components which bind the antigen-antibody complex and interact with complement receptors on cells, may result in enhanced infection of the cells with virus. This type of enhancement has been demonstrated with both HIV and SIV [116,117].

It is possible that the use of different adjuvants could account for the differences in protection seen in this study (using Quil A) and in the study described by Yamamoto et al [54] (using threonyl muramyl dipeptide or a combination of Freund's complete and incomplete adjuvants). Quil A may activate the immune system, leading to an increased expression of $F_c$ receptors as T-cells became activated and hence an increase in $F_c$ receptor-mediated virus uptake. This would, however, be unlikely to explain the enhancement of infection by the FIV-ISCOM vaccination as there were no detectable anti-<u>env</u> antibodies produced and therefore no means of opsonising the virus for $F_c$ receptor-mediated uptake. It has been shown that activated T-cells are more susceptible than resting T-cells to FIV infection in vitro (M. Hosie and B. Willett, unpublished observation) and therefore activation of the immune system in vivo by vaccination may simply lead to an enhancement of infection because more susceptible cells are available.

Also, the inactivation procedure may be important in maintaining the antigenicity of the viral proteins so that neutralising antibody-inducing epitopes are available in the vaccine preparation. Virus-enhancing and virus-neutralising epitopes have already been identified on the FeLV envelope glycoprotein [118].

Therefore it will be important to identify epitopes on FIV which are responsible for inducing enhancing antibodies so that they can be excluded from potential vaccines.

## 8. GENERAL DISCUSSION

The studies described in this thesis demonstrated the value of FIV as a model for HIV and used FIV in experiments towards the development of an effective vaccine. The similarities between FIV and HIV were established by the epidemiological study of the prevalence of FIV and FeLV in cats in the UK as well as the examination of the serological response to FIV infection.

The epidemiological study, described in chapter 3, demonstrated that FIV is a significant cause of disease in the cat. The clinical signs of sick cats with FIV infection were analagous to those seen in HIV infection, namely non-specific signs such as lethargy, anorexia, weight loss, pyrexia and lymphadenopathy. The cats which were experimentally infected with FIV for the calculation of the $ID_{50}$ (section 6.3.1) were examined histopathologically approximately 6 months after infection. Examination of these tissues revealed lesions in the lymphoid tissues, kidney and lung which were strikingly similar to the lesions reported in early AIDS patients (W Jarrett, personal communication).

The data from the epidemiological survey were analysed to determine those groups of cats most at risk of FIV infection in order to identify possible routes of transmission. Also, the prevalence of infection in multi-cat households was investigated to determine how readily transmission occurred between in-contact cats. The data revealed that free-roaming male cats are most at risk of infection and therefore biting was thought to be an important route of transmission. This route of infection is analagous to HIV infection via intravenous drug use. Neither the epidemiological study nor examination of kittens born to seropositive dams have shown evidence of transmission of infection to kittens, in utero, via colostrum or via milk [119]. In utero transmission, which occurs in HIV infection, is much less common in FIV infection. This may be because the feline placenta does not permit the intimate association which occurs

between the maternal and foetal circulations in the human placenta. Sexual transmission of FIV has not been documented although it is possible to infect cats per vagina with FIV (D Harbour, personal communication).

The development of the serological response to FIV infection, described in chapter 4, was shown to parallel that of HIV infection, with antibodies developing first against the core protein p24, followed by antibodies against the other core proteins p17 and p10, the core precursor p55 and the envelope glycoprotein gp120. The use of immunoblotting identified indeterminate sera with reactivity to one or two viral proteins as had been seen in earlier studies with HIV (section 4.4). Furthermore, the serological studies demonstrated that FIV infection, like HIV, was persistent.

The ultimate aim of these studies was to use FIV as a model for the development of a vaccine against HIV infection. Firstly, a challenge system was established to test the efficacy of candidate vaccines. Section 6.3.1 describes the experiment to calculate the $ID_{50}$ of FIV-8 which was subsequently used in challenge experiments. Cats were monitored for evidence of infection by virus isolation (section 5.1.3.2) and immunoblotting (section 4.3.2). Secondly, attempts were made to protect cats from infection by passive immunisation with antibody purified from FIV-8-infected cats. As this treatment did not confer protection in two recipient cats, it was concluded that either the antibodies in persistently infected cats are not protective, that the neutralising titres achieved were inadequate or that cell mediated rather than humoral immunity has a vital role in protection against infection. Finally, it was important to identify which viral components were necessary to induce a protective immune response. It was intended that three vaccine formulations would be compared; one containing FIV core proteins, another containing envelope proteins and a third with both core and envelope proteins.

Although ultimately a vaccine would be likely to contain recombinant proteins, the sources of immunogens which were available during this study were either purified virus or virus-infected cells. As purified virus was a poor source of envelope proteins (section 4.3.2), it was thought that virus infected cells would be the best source of gp120 for use in vaccines. Various cell lines were examined for the ability to support the growth of FIV-8 and FIV-P, as described in chapter 5.

There initially appeared to be a correlation between the ability of cells to support the growth of FIV-8 and the expression of the fCD4 marker. Thus, P51 and Q201 cells were more susceptible to FIV infection than peripheral blood T-cells and the cytopathic effect induced in P51 and Q201 cells was very marked. As P51 and Q201 cells were predominantly fCD4+ whereas peripheral blood T-cells contained both fCD4+ and fCD8+ cells, the role of fCD4 as the receptor for FIV was investigated. When attempts were made to correlate the presence of fCD4 on various cell lines with their ability to support the growth of FIV, however, the correlation was incomplete. Cell lines which were fCD4- (F422 and T3) supported the growth of FIV and cells which were fCD4+ (3201) could only be infected with difficulty.

Although FIV-8 grew well in Q201 cells, these were IL-2 dependent and were therefore expensive to grow in large cultures. Also, fresh uninfected cells were required to supplement infected cultures as the virus infection led to death of the susceptible cells. Other cell lines which supported the growth of FIV-8 were F422 and T3. These cell lines were IL-2 independent and grew rapidly in culture. Both lines, however, were productively infected with FeLV and were therefore thought to be unsuitable for the production of immunogens for vaccination. Similarly, P51 cells had been shown to produce FeLV.

3201 cells had the advantage of being IL-2 independent and producing no FeLV. Despite expressing high levels of fCD4 on their surface, however, they could not be readily or reproducibly

infected with FIV-8 (section 5.2.4). CRFK fibroblast cells have been used by others to produce large amounts of virus [50] but, like the 3201 cells, they could only be infected with FIV-8 with difficulty (although they were very readily infected with FIV derived from 3201 cells). Hence it appears that virus which is adapted for growth in cell lines such as 3201 and CRFK is different to field virus and may not, therefore, be ideal for the production of immunogens for vaccination studies. This hypothesis is supported by the finding that primary field isolates of FIV cannot be made directly onto CRFK cells; T-cells are required for the initial isolation and these must then be co-cultivated with CRFK cells. Even then, only a proportion of isolates will replicate in CRFK cells (K Siebelink, personal communication).

Hence, the cells which were chosen to produce FIV-8 for the vaccination studies were normal cat peripheral blood T-cells, normal cat thymocytes or Q201 cells. Despite requiring IL-2 and large numbers of fresh, susceptible cells, it was thought that the virus produced by these cells might be closer to field virus compared to that produced by a cell line which could only become infected after long-term co-cultivation.

Two candidate vaccines were tested in cats. The first was an ISCOM vaccine containing purified FIV-8 and is described in chapter 6. This vaccine was shown to contain mostly core proteins and a small amount of envelope protein. The cats made antibodies against the core proteins but not against the envelope proteins. When they were challenged with homologous virus they became viraemic, demonstrating that high levels of anti-core antibodies are not protective. Attempts were made to purify gp120 in order to produce a vaccine containing envelope protein but insufficient material was obtained by the methods used. Therefore, the second vaccine was a fixed, inactivated cell vaccine and is described in chapter 7. It was hoped that this vaccine preparation would contain substantially more envelope protein than the ISCOM vaccine since infected cells retain more gp120 than purified virus. The cats inoculated with this preparation developed

antibodies against both core and envelope proteins but had only low neutralising antibody titres and were not protected upon challenge.

Recent reports of the successful protection of monkeys against SIV infection [35,36,37] and of cats against FIV infection [54] are encouraging in two respects. Firstly they demonstrate that vaccination against lentiviruses is feasible. Secondly, they highlight the importance of animal models in studies towards HIV vaccination.

There are two important features which an animal model for HIV should ideally possess. Firstly, infection with the virus should be biologically similar to infection with HIV. Both FIV and SIV behave like HIV. All three viruses share a similar tropism for T-lymphocytes and macrophages and a decline in CD4 numbers is seen with the progression of all three infections [23,120,49]. Infection of rhesus and cynomologous macaques with SIV and infection of cats with FIV produces a disease resembling HIV pathologically. Secondly, large numbers of animals should be available for vaccine trials. The advantage of animal models is that vaccines can be tested for their ability to induce protection from challenge. Protection tests are feasible in all animal model systems. The advantage of the feline and simian systems is that protection against disease can be determined. It is possible that vaccination will not protect against infection, but that the onset of clinical signs may be delayed or prevented.

The chimpanzee (Pan troglodytes), gibbon ape (Hylobates spp) and rabbit are the only animals which can be persistently infected with HIV-1 but none has shown signs of disease. It appears that the chimpanzee may be a HIV-1 host adapted species which controls HIV infection by an unidentified species-specific cellular mechanism which compartmentalises the virus [121]. An animal model using chimpanzees would be hampered by the expense and restricted availability of this endangered species. Nevertheless, chimpanzees remain likely to be used for vaccine testing prior to

human trials. Mice with severe combined immunodeficiency (SCID mice) have been reconstituted with human lymphoid tissue [122] and can be infected with HIV, but with no signs of disease [123]. The value of the rabbit and SCID mice models remains to be demonstrated.

Under experimental conditions, using virus adapted to growth in simian cells, HIV-2 can be used to infect rhesus monkeys, baboons and mangabeys [123,124]. Although these animals became viraemic and seroconverted, no disease developed. Therefore these models would only be of use in vaccine trials for protection against infection, not disease.

The ungulate lentiviruses (VV, CAEV, EIAV and BIV) have several features in common with HIV. They induce chronic diseases after long latent periods, cause viraemia in the presence of a weak neutralising antibody response, infect cells of the monocyte/macrophage lineage and undergo extensive genetic mutation and antigenic drift. The diseases caused by the ungulate lentiviruses (in contrast to HIV, FIV and SIV) are not immunosuppressive. The progressive neurological signs which are caused by VV and CAEV may, however, have a similar pathogenesis to the encephalopathies associated with HIV infection. Therefore the ungulate lentiviruses are useful models to study certain aspects of the pathogenesis of infection and in vaccine evaluation.

From this account of the animal model systems it is clear that SIV is remarkably similar to HIV. Furthermore, there is striking nucleotide homology between HIV-1, HIV-2 and SIV, suggesting that the simian and human immunodeficiency viruses arose from a common ancestor in recent evolutionary history. Genetically FIV has no close relative, but the organisation of its small ORFs is more closely related to VV than to HIV (J Neil, personal communication). Despite the structural data indicating that FIV is more closely related to the ungulate lentiviruses than the primate lentiviruses, in all other aspects FIV resembles the

primate immunodeficiency viruses. The pathogenesis and response to infection with FIV are indistinguishable from the response to HIV.

At present the simian model has been developed further than the feline model. Although there are relatively few immunological reagents available to characterise the immune response of the cat, these are gradually becoming available. The feline model has an advantage over the simian model as there as large numbers of cats are naturally infected with FIV and these animals can provide useful material for study. Furthermore, for investigations under controlled conditions (such as vaccination experiments) experimental cats may be housed more cheaply than monkeys.

The animal challenge experiments which have so far been successful have used inactivated whole virus, either in the form of purified virus or virus-infected cells [35,36,37,54]. It is important to identify which viral antigens are capable of eliciting protective immunity. To date, the structural components associated with the viral envelope of HIV have been studied most extensively.

The envelope is likely to be an important immunogen as it is known that HIV gp120 binds to the host cell receptor CD4 [23,24,25,26] and therefore an immune response interfering with the gp120-CD4 interaction should prevent infection. Furthermore, the principal neutralising epitope of HIV is situated within the third hypervariable region, the V3 loop of gp120 (amino acids 296-331) [106].

The presence of antibodies against the V3 loop in sera of HIV-infected mothers and their uninfected children was significant. This suggested that anti-V3 loop antibodies were protective, preventing intrauterine transmission of HIV [125]. Berman et al [126] demonstrated that chimpanzees inoculated with recombinant gp120 were protected from infection by HIV-1 whereas those

inoculated with gp160 were not. Neutralisation in vitro did not correlate with protection in vivo as high neutralising antibody titres were found in both groups. It may, however, be significant that at the time of challenge, the group inoculated with gp120 had higher titres to the V3 loop compared to the group inoculated with gp160.

Another neutralistion epitope on the envelope is situated within the transmembrane component, gp41 (amino acids 735-752). This epitope is highly conserved and has been shown to be a potent immunogen in the rabbit [127]. Two characteristics of HIV-1 are important obstacles to to an effective vaccine. Firstly there is much genomic variability, especially in env, which could permit the virus to escape the host immune responses and lead to increased pathogenicity as disease advances [128,129]. Secondly HIV can be transmitted between cells without the release of free virus. This feature might be expected to make virus destruction difficult by means of antibody alone.

Although the relative contributions of cellular and humoral immunity in HIV-1 infection have not yet been defined, humoral immunity alone has not been protective in passive immunisation experiments in the chimpanzee [105,114] or in the cat (section 6.3.5). It is therefore presumed that induction of strong T-lymphocyte immunity will be required for successful vaccination against HIV-1. Cytotoxic T-cells (CTL) eliminate virus-infected cells. The T-cell receptor on the CTL recognises viral protein fragments in combination with a major histocompatibility complex antigen molecule on the surface of the target cell. HIV-1-specific CTL are detectable in both peripheral blood and tissues of infected people and are aimed at multiple viral proteins [130]. The precise epitopes recognised by these CTL are currently being defined.

A successful vaccine should elicit protective CTL which are able to destroy HIV-infected cells only. HIV-infected cells in vivo shed gp120 which can bind CD4 on the surface of uninfected cells

[131]. The envelope protein may then be taken up, processed and presented on the surface of the cell, resulting in lysis by CTL with possible deleterious effects [132].

Unlike neutralising epitopes which are present only on the structural proteins coating the virus particle, CTL epitopes can be present in virtually any structural or non-structural viral protein. The internal viral proteins of HIV-1 are far less heterogeneous than the envelope protein and may therefore be better candidates for vaccination. There have been reports of gag-specific CTL, as well as pol-specific, vif-specific and nef-specific CTL [130]. It is likely that other regulatory proteins will also be shown to be CTL targets. However, the results of the FIV-ISCOM vaccination experiment (section 6.3.6) demonstrated that FIV gag products alone were not sufficient for protection against FIV infection.

There are several candidate HIV vacines currently undergoing clinical trials in humans [133]. Phase 1 clinical trials in healthy volunteers assess safety and immunogenicity. Detailed immunological analyses are made to characterise the elicited immunity and to check the absence of deleterious effect, such as immunosuppressive HIV components in the vaccine and the induction of autoimmune reactivity [134] or enhancing antibodies [99,114]. Vaccines currently undergoing phase 1 trials include a live vaccinia recombinant expressing gp160 [135], a subunit gp160 vaccine produced in a baculovirus expression system [136], a combination of these [137], a gp120 vaccine produced in yeast [138] and synthetic peptides corresponding to part of the core protein p17 [139]. All of these preparations were well tolerated and induced specific anti-HIV immune responses [138] but potential CTL responses generated by these vaccination protocols remain to be fully characterised.

Although tissue culture-derived material may be used experimentally in animal models to determine which components of the virus provide protection, recombinant molecular approaches

will be necessary to generate a sufficient quantity of safe immunogens for use in man. Various systems have been used to express genes coding for HIV antigens. The large DNA viruses, such as vaccinia and adenoviruses, have been used as vectors for the generation of recombinant vaccines. Upon infection of the host, these viruses can replicate, amplify the foreign protein and efficiently present the protein to the immune system.

Vaccinia virus has been used successfully in vaccines against rabies and rinderpest [140] as well as in HIV vaccination experiments. There is, however, concern about the possible development of post-vaccinal encephalitis in immunocompromised humans. Secondly, there is concern that individuals who have previously been vaccinated as part of the smallpox erradication programme will possess a pre-existing immunity to vaccinia which may limit viral proliferation and antigen production. Adenovirus vaccines have been demonstrated to be safe and efficacious, giving rise to asymptomatic adenovirus infection in humans and inducing immunity against adenoviral respiratory disease [139]. Sequences encoding gp120 have been inserted into adenovirus vectors but there is concern that severe infections may develop in immunocompromised individuals.

Hybrid polioviruses may also be used for antigen expression and delivery. The Sabin type 1 vaccine strain of poliovirus is probably the safest and most successful modified live vaccine virus used in humans. The poliovirus chimaera system allows the selective expression of defined regions of HIV proteins in an immunoprominent position and therefore enables specific immune responses to be induced [127].

The expression vector system plays a major role in the post-translational modification of the expressed protein. The recombinant clones expressed in gram-negative bacteria such as Escherichia coli or in yeasts are denatured and non- or incorrectly glycosylated respectively. Since the HIV envelope glycoproteins have been implicated in CD4 binding and syncytium

formation [23,24,25], it would appear that glycosylation may be an important consideration in the design of a recombinant vaccine. A mammalian system, chinese hamster ovary cells, has been used to express recombinant gp120 which resembles native, viral gp120 in glycosylation [141].

As most of the typical eukaryotic processes of protein post-translational modification also occur in insect cells (an exception is the inability of insect cells to make complex-type glycans and to add sialyic acid), insect cells infected with recombinant baculoviruses have provided a valuable source of expressed proteins [142]. Baculovirus expression of the complete HIV gag gene resulted in the synthesis and release of enveloped particles containing unprocessed gag gene products [143].

While the production of gp120 in mammalian cells provides a closer molecular replica of the native viral antigen, it is possible that other expression systems may be used to produce useful, although conformationally incorect, forms of the protein. The production of the envelope glycoprotein in bacteria and yeast has the advantage of producing significantly higher quantities of antigen than mammalian cells. A 200 amino acid portion from the carboxy-terminal region of HIV gp120 which was produced in Escherichia coli induced the production of neutralising antibodies [144]. This finding suggested that antigen conformation was not important for the induction of neutralising antibodies. However, other portions of gp120 produced in bacteria did not induce neutralising antibodies. In addition, in contrast to results for recombinant mammalian-derived gp120, antibodies directed against the bacterial antigen were completely type-specific [145]. Hence native gp120 may induce the formation of a higher degree of cross-neutralising antibodies compared to the denatured gp120 produced in bacteria.

Other potential HIV vaccine candidates are synthetic peptides. An important factor in the presentation of peptides is the presence of a helper T-cell epitope as well as the B-cell epitope against

which antibody is to be directed. There is considerable evidence to support the concept that T-cells recognise a complex formed between major histocompatibility complex molecules on the host cell surface and the peptides derived from processed antigen. The helper T-cell epitopes on an antigen must be able to bind to the class II molecules on the host cell surface and subsequently interact with the T-cell receptor. It was previously assumed that it was necessary for a peptide to be attached to a carrier molecule for it to function as an antigen but it is now known that a peptide can elicit antibodies if it possesses a helper T-cell epitope suitable for the host species. Alternatively a synthetic peptide or fusion construct may be produced which combines peptide epitopes recognised by T-cells and neutralising epitopes [146].

Antigens produced by recombinant DNA technology will, in general, be monomers. It is becoming increasingly apparent that to be optimally immunogenic, antigens should be presented in several copies. For example, when expressed as a dimer or tetramer on beta-galactosidase or as a mutimer on the hepatitis B core, a peptide corresponding to a 20 amino acid sequence of a foot-and-mouth disease virus protein induced a response in guinea pigs which was two-fold greater than that induced by the peptide alone [147]. The use of the core antigen of hepatitis B in humans has several advantages. The protein subunit is a 21 kilodalton polypeptide which spontaneously assembles into characteristic 27nm particles [148]. The core particle can be expressed in a wide range of systems, including bacterial, yeast, mammalian or via vaccinia virus and baculovirus. It is highly immunogenic as a result of its polymeric nature, the presence of a number of well-defined helper T-cell epitopes and its ablility to function as a T-cell independent antigen. This system, however, cannot be tested in cats.

Another carrier system is the Ty-virus-like particle (Ty-VLP). The yeast retrotransposon Ty encodes a set of proteins which are assembled into virus-like particles. The Ty particle DNA can

tolerate the insertion of a wide range of additional protein sequence, allowing full-length proteins to be accomodated [149]. Hybrid Ty-VLPs can be purified easily, using the physical properties of the particle. Ty-VLPs containing the V3 loop of HIV gp120 induced high titres of neutralising antibodies in rabbits and an HIV-specific T-cell proliferative response in mice after immunisation [150].

Several other carrier systems have been developed. Multimeric structures such as micelles and liposomes have been used. However, by incorporating an adjuvant into the antigen complex, even greater resposes are induced. The only adjuvants authorised for use in human vaccines are aluminium hydroxide and aluminium phosphate. These are, however, inferior to laboratory adjuvants (such as Freund's complete adjuvant) and do not consistently elicit cell mediated immunity [151]. Therefore it is important to develop adjuvants which are as effective as Freund's complete adjuvant but which do not have its side-effect of inducing granulomas.

In the Syntex formulation the threonyl analogue of muramyl dipeptide is used with squalene [147]. This is a stable adjuvant and it induces the production of cytokines which potentiate the vaccine response [147]. But perhaps the greatest advances have been made with immunostimulating complexes (ISCOMs) in which the glycoside Quil A (an adjuvant which is used in conventional vaccines) is incorporated into a complex together with the antigen, cholesterol and phosphatidyl choline [151]. Because the adjuvant is bound to the antigen, it can be used at drastically reduced concentrations. Quil A is a semipurified product from a crude saponin extract of the bark from the tree Quillaja saponaria Molina. It forms micelles which have regions accessible for hydrophobic interaction with amphipathic molecules such as membrane proteins. Membrane proteins from more than 20 different viruses have been isolated and incorporated into ISCOMs [152]. It has been shown that immunisation of cats with ISCOMs containing the membrane protein of FeLV induces protection [112].

Furthermore, a single subcutaneous immunisation of mice with ISCOMs containing recombinant HIV envelope protein induced cytotoxic T-cells [153].

In this thesis the strategy for FIV vaccine experiments was to identify the viral proteins important for vaccination by constructing vaccines containing core proteins alone and core and envelope proteins together. Protection against FIV infection has only been demonstrated recently by others using inactivated whole virus or inactivated virus-infected cells [54]. Neither of the vaccines described in this thesis gave protection against infection. The cats immunised with the FIV-ISCOM vaccine (described in chapter 6) did not develop antibodies against the envelope protein, suggesting that env proteins may be the major determinants of immunity. However, some of the cats in the study described by Yamamoto et al [54] had anti-env antibodies and were not protected. Similarly the cats inoculated with the inactivated cell vaccine described in chapter 7 developed anti-env antibodies and were not protected. The levels of neutralising antibodies in these cats was low (section 7.3.1) and therefore future experiments will aim to achieve high levels of neutralising antibody prior to challenge.

This study highlights the importance of avoiding induction of sub-neutralising concentrations of antibodies as these may cause an enhancement of infection. In the vaccine experiments described in chapters 6 and 7 it appears that the low levels of antibody which were induced may have enhanced infection. Although the necessary in vitro studies have not yet been carried out for FIV, in other virus systems, such as West Nile virus [112] and dengue virus [113], it has been shown that the binding of antibody-virus complexes to cellular $F_c$ receptors facilitates the attachment of virus particles to target cells. In vitro studies using HIV demonstrated that $F_c$ receptors induced by cytomegalovirus allowed immune complexes of HIV to infect fibroblasts which were otherwise not permissive to HIV infection [27]. This finding suggested that antibody-mediated HIV infection of monocytes,

macrophages and fibroblasts could occur in vivo. Enhancement of retroviral infection after vaccination was recorded in cats inoculated with purified FeLV envelope protein [111]. The cats developed antibodies against the envelope protein but these did not neutralise the virus in vitro and the cats were shown to be more susceptible to infection with FeLV compared to non-vaccinated cats.

In chapter 5 the monoclonal antibody vpg15 was shown to recognise the FIV receptor. If the preliminary evidence that vpg15 binds the low affinity $F_c$ receptor is substantiated, it is possible that the enhancement which was seen after vaccination with FIV-ISCOMs and the inactivated cell vaccine was not antibody-dependent. As the expression of the low affinity $F_c$ receptor is increased in activated T-cells, it may be that the immunisation procedure itself resulted in an activation of the immune system, increased expression of the FIV receptor and hence an enhancement of challenge in the vaccinated cats compared to the unvaccinated controls. It will therefore be important in future experiments to include control cats which have been inoculated with a mock preparation in adjuvant. In this way it will be possible to test this hypothesis for the enhancement. Future vaccines will aim to induce significantly greater levels of neutralising antibodies with minimal activation of the immune system. With this consideration, it may be important to compare the effects of different adjuvants on the expression of the FIV receptor.

Future FIV vaccine experiments will aim to identify epitopes responsible for inducing neutralising and enhancing antibodies. Recombinant DNA technology will be used to produce defined regions of FIV proteins. This would allow a more systematic approach to be used to determine the viral components required for immunity. Recently, ISCOMs containing recombinant FIV p24 have been inoculated into cats (G.Reid and O. Jarrett, personal communication). The responses of the vaccinated cats were similar to the responses made by the cats vaccinated with the FIV-ISCOM described in chapter 6. High titres of anti-p24 antibodies were

induced but there was enhancement of infection, the vaccinated cats becoming infected sooner than the controls.

Other useful recombinant vaccines would contain gp120 as this is the protein most likely to induce neutralising antibodies. It would be useful to compare gp120 produced in various expression systems to determine whether or not it is necessary to have the correct pattern of glycosylation or the correct protein conformation for the induction of protective immunity. Similarly it would be interesting to compare different delivery systems and adjuvants to directly compare their efficacies in the feline system.

As information becomes available for HIV, it is possible to extrapolate for FIV the regions of gp120 which may be useful in vaccine experiments. For example the analagous region to the principle neutralising determinant within the V3 loop of HIV can be identified in FIV. Hence vaccination with the peptide encoded by this region may be expected to induce cross-neutralising antibodies, as has been described for HIV [154]. Eventually, when sequences for other B-cell, as well as helper T-cell and CTL epitopes have been characterised, these too will be expressed and used in FIV vaccination experiments.

It is therefore clear that this study demonstrates that the animal models are required to identify which proteins, and then which epitopes, are necessary for immunity. The animal models could also be used to compare different systems for antigen presentation and to determine the relative efficacies of various adjuvants. The studies described in this thesis illustrate the potential value of the feline model which will be valuable in achieving an understanding of those mechanisms which are important for successful vaccination against HIV.

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