STUDIES OF THE PATHOGENESIS OF FELINE IMMUNODEFICIENCY VIRUS

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Abstract

Feline immunodeficiency virus (FIV) is a significant worldwide pathogen of the domestic cat. The disease is characterised by early acute phase pyrexia 6-8 weeks post infection, followed by an indefinite period of asymptomatic infection, which in many infected cats is followed by progression to the immunodeficiency phase. The primary target of FIV is the $CD4^+$ T lymphocyte with depletion of this population characterising progression to the acquired immunodeficiency syndrome like (AIDS-like) stage. To date, only limited success has been achieved in the development of an effective vaccine, most success occurring with laboratory-adapted isolates such as FIV-Petaluma (FIV-PET), whereas primary isolates such as FIV-Glasgow8 (FIV-GL8) resist vaccine-induced protection. Indeed, due to their ability to induce higher proviral DNA loads, higher viral RNA loads, inversion of the CD4:CD8 T lymphocyte ratio and this resistance to vaccine protection has led to the hypothesis that these isolates are more pathogenic. FIV, like HIV-1, uses the chemokine CXCR4 as a coreceptor for infection of cells *in vitro* and studies using the CXCR4 antagonist AMD3100 have demonstrated that laboratory-adapted isolates have a greater affinity for CXCR4 than primary isolates. However, it is unknown whether FIV, like HIV-1, changes phenotype and undergoes receptor switching as disease progresses.

These findings call into question the relevance of these laboratory-adapted isolates to vaccine development. The virulence and phenotype of field isolates is poorly documented and it is unclear whether isolates fall into two specific groups typified by the prototype viruses FIV-GL8 and FIV-PET or whether a range of biological phenotypes exist. Furthermore, it was unknown whether receptor usage and cell tropism *in vitro* could be correlated with virulence *in vivo*.

The project had three aims, namely-

(i) to investigate the *in vitro* cell tropism of a range of field isolates from cats at different stages of disease and compare their phenotype with the well-characterised prototype viruses FIV-PET and FIV-GL8.

(ii) to study the pathogenicity of these viruses *in vivo* in order to examine any correlation between virulence *in vivo* and tropism *in vitro*.

(iii) to look at the role of the *env* gene in the pathogenicity of FIV.

In vitro studies of cell tropism revealed that isolates from cats in the terminal stage of disease had a greater ability to utilise CXCR4 than isolates from cats displaying no clinical signs. *In vivo*, these symptomatic isolates, with greater CXCR4-tropism *in vitro*, displayed less virulence when compared with isolates from asymptomatic cats.

Chimaeras were made by inserting the *env* genes of an isolate from the asymptomatic or terminal disease stages into a FIV-G8Mya backbone, allowing comparison of the cell tropism and receptor usage of these genes and the study of their phenotype with regard to virulence *in vivo*. The *env* genes from FIV-PET and the symptomatic isolate ($F0827H_s$) had a greater affinity to utilise CXCR4 for cell entry *in vitro* and this correlated with reduced virulence *in vivo* when compared to the asymptomatic isolate *env* and FIV-G8Mya.

These studies highlight a trend where tropism *in vitro* can be correlated with virulence *in vivo*. Furthermore, the study indicated that viruses from asymptomatic cats (with a lesser ability to utilise CXCR4) have increased virulence. As these are the agents most likely to be transmitted in the field by the apparently healthy cat, vaccine development should focus on this population of viruses.

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Author's Declaration

This presentation is entirely the product of my own efforts except where specifically stated in the text and in the acknowledgements section. This thesis has not been previously submitted for the award of a degree to any university. The following publication contains work included in this thesis:

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Hayley Haining

June 2004

Dedication

To Willie

and to Seb.

With all my love

Hayley

Definitions

AIDS	Acquired immunodeficiency syndrome
AMD3100	CXCR4 antagonist
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CAEV	Caprine encephalitis virus
CA	Capsid
CD	Cluster determinant
ConA	Concanavalin A
CrFK	Crandell feline kidney cells
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
EIAV	Equine infectious anaemia virus
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent antibody cell sorting
FIV	Feline immunodeficiency virus
FTLV	Feline T-lymphotropic virus
FVU	Feline virus unit
HIV	Human immunodeficiency virus
HSPG	Heparan sulphate proteoglycans
IIC	Inactivated infected cell
IL-2	Interleukin-2
IN	Integrase
kD	kiloDaltons
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MA	Matrix
NC	Nucleocapsid
NDCL	Nondomestic cat lentivirus
NSI	Nonsyncytium inducing
PBA	Phosphate buffered saline with azide
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction

PR	Protease
PWM	Pokeweed mitogen
QVI	Quantitative virus isolation
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDF-1α	Stromal cell derived factor 1α
SE	Standard error
SHIV	Simian-human immunodeficiency virus (chimaeric)
SI	Syncytium inducing
SIV	Simian immunodeficiency virus
SU	Surface glycoprotein
TM	Transmembrane protein
VI	Virus isolation
V-MV	Visna-Maedi virus
WIV	Whole inactivated virus

Chapter One

INTRODUCTION

1.1. The discovery of feline immunodeficiency virus

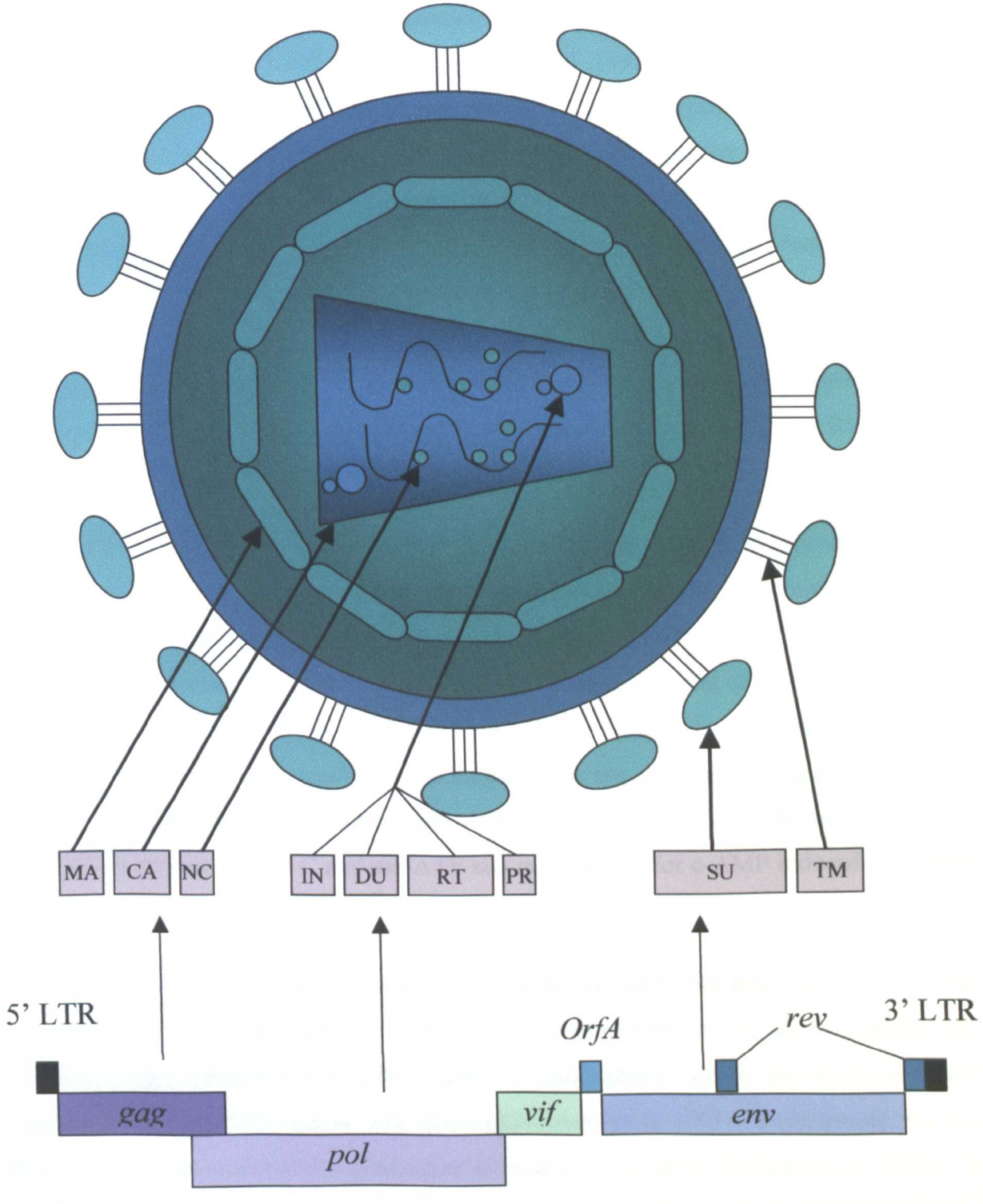
Feline immunodeficiency virus (FIV), previously known as feline T-lymphotropic lentivirus (FTLV), was first isolated in 1986 from a specific pathogen free cat that had been inoculated with either blood or plasma of a cat from a rescue colony in California which was showing clinical signs of an illness that resembled acquired immunodeficiency syndrome (AIDS) (Pedersen et al. 1987). This virus had the characteristics of a lentivirus. FIV causes a lifelong infection in domestic cats which, after an indefinite period of asymptomatic disease, may progress to an AIDS-like phase, similar to that seen with human immunodeficiency virus (HIV).

Serological studies have been conducted by many groups and have shown FIV to be distributed worldwide with the prevalence of infection varying from 1-15% in healthy cats and 3-44% in sick cats, reviewed by Miyazawa and Mikami (Miyazawa and Mikami 1993). Differences in population density and varying husbandry conditions may contribute to the large variations recorded amongst different studies. Although the virus was first isolated in 1986 (Pedersen et al. 1987), examination of archival samples found evidence of the virus as early as 1968 (Gruffyd-Jones et al. 1988) and phylogenetic studies indicate that the virus is very ancient (Talbott et al. 1989; Shelton et al. 1990a; Olmsted et al. 1992).

1.2. The retroviruses

Lentiviruses are single stranded RNA viruses with a diploid genome, which is organised in a way characteristic of all retroviruses with the *gag*, *pol* and *env* genes always occurring in that order (see Figure 1.a.). The *gag* gene encodes the internal structural proteins, nucleocapsid (NC), capsid (CA or p24), the major core protein p24 and matrix (MA) proteins (Yamamoto et al. 1988; Talbott et al. 1989). The *pol* gene encodes proteins with enzymatic functions: protease (PR), reverse transcriptase (RT), integrase (IN) and dUTPase. The RT is a DNA polymerase that has several functions including synthesis of DNA from a mRNA template, synthesis of DNA from a DNA template and ribonuclease activity. Like other lentiviruses, FIV RT depends on Mg^{2+} and operates at identical molar concentrations to primate lentiviruses (Yamamoto et al. 1988). The IN is responsible for

Figure 1.a. Schematic representation of the virion and genome organisation.



The *gag* gene encodes the following proteins, MA=matrix protein, CA=capsid, NC=nuclear capsid. The *pol* gene, IN=integrase, DU=dUTPase, RT=reverse transcriptase, PR=protease. *Env* encodes SU=surface glycoprotein, TM=transmembrane protein

integrating the viral DNA at any point in the host genome (Madigan et al. 2000). Both the *gag* and *pol* genes of FIV are highly conserved areas of the genome (Talbott et al. 1989).

The *env* gene encodes the envelope glycoprotein (Env), gp120, which comprises two parts, the surface glycoprotein (SU) and the transmembrane (TM) protein. The two proteins have masses of 95kD and 40kD respectively (Stephens et al. 1991). The *env* gene has several variable regions and viral isolates can be classified into subtypes on the basis of the nucleotide sequence of this region (Sodora et al. 1994). The sequence diversity of the *env* gene is organised in clusters and nine hypervariable regions (V1-V9) have been defined (Pancino et al. 1993b). The first two, V1 and V2, are found in the leader sequence and do not encode the mature Env protein due to proteolytic processing (Verschoor et al. 1993), V3 to V6 are in the SU and V7 to V9 are in the TM. The Env protein has been shown to be a principal determinant for virus/cell interactions, fusogenicity and cell tropism as well as containing a major immunodominant domain (Lombardi et al. 1993; de Ronde et al. 1994; Pancino et al. 1995).

The FIV genome in its proviral form is flanked by long terminal repeats (LTR) of approximately 350bp (Talbott et al. 1989; Olmsted et al. 1989b) that control and direct DNA and RNA synthesis via two TATA boxes, which are promoter sites for transcription. One of these is similar to that of HIV and the other identical to that of caprine encephalitis virus (CAEV), visna-maedi virus (V-MV) and equine infectious anaemia virus (EIAV) (Sparger et al. 1992). Enhancer/promoter binding sites are also present, namely AP-1, AP-4, ATF-1 and EBP20. AP-1 has been shown to be required for T cell activation responses mediated by protein kinase C and the ATF site is the target for c-AMP induced responses by protein kinase A (Sparger et al. 1992).

Lentiviruses are more complex than the simple retroviruses like feline leukaemia virus since they have accessory genes. The accessory genes of FIV are *vif*, which controls cell-free infectivity (Tomanaga et al. 1992), and *rev*, which participates in the stabilisation and transport of incompletely spliced viral RNA (Kiyomasu et al. 1991). Furthermore, *rev* has been shown to be essential for productive infection of the virus (Phillips et al. 1992). A further gene called *OrfA* or *Orf2* which lies between *vif* and *env* is required for the productive infection of T lymphocytes (Waters et al. 1996) and facilitates the transactivation of FIV (de Parseval and Elder 1999).

1.3. Evolution and the phylogenetic analysis of FIV

Phylogenetic analysis of the *env* genes revealed five subtypes (Sodora et al. 1994; Kakinuma et al. 1995; Pecoraro et al. 1996) and with evidence of recombination in the FIV *env* gene occurring in natural infection there is the opportunity for great genetic diversity (Carpenter et al. 1998).

FIV diverged early in the evolution of lentiviruses, shortly after the divergence of primate and non-primate lentiviruses (Talbott et al. 1989), but is most closely related to the ungulate lentiviruses EIAV, CAEV and particularly V-MV (Olmsted et al. 1989b). As well as in domestic cats, FIV strains can be found in many other wild felid species (nondomestic cat lentiviruses, NDCL) such as the puma (*Puma concolor;* FIV-Pco) and the lion (*Panthera leo;* FIV-Ple) (Olmsted et al. 1992; Brown et al. 1993; Carpenter and O'Brien 1995; Carpenter et al. 1996). Similar to simian immunodeficiency virus (SIV), infection of the natural host of NDCL in nondomestic felids with their natural strains of NDCL does not appear to cause disease and seropositivity has not been correlated with the occurrence of disease (Olmsted et al. 1992). The degree of *pol* sequence diversity between FIV-Pco, FIV-Ple and FIV is 20-30%, which suggests that these viruses have coexisted with their respective feline hosts since the divergence of the felid species, allowing attenuation of the virus due to immunological adaptation and host selection (Olmsted et al. 1992; Carpenter and O'Brien 1995; Carpenter et al. 1996). Evidence of interspecies transmission has been found in African lions and in Tsushima cats in Japan (Spencer et al. 1992; Nishimura et al. 1999; Bull et al. 2002). Previous studies have shown NDCLs to be infectious for domestic cats (Olmsted et al. 1992; Carpenter et al. 1996; Vandewoude et al. 1997).

1.4. Natural infection

The source of FIV is the persistently viraemic cat. Epidemiological studies have shown that in-contact animals have an infection rate of 21-48% (Pedersen et al. 1987; Ishida et al. 1988; Hosie et al. 1989; Hopper et al. 1991). Since FIV is most prevalent in male, free roaming cats and can be isolated from the saliva of FIV-infected cats (Yamamoto et al. 1989) it seems that biting is the most likely mode of transmission

FIV-infected cats reaching the later stages of infection can present with multiple infections, both chronic and opportunistic. The commonest problems are vague signs of malaise

including, lethargy, inappetance, weight loss, pyrexia and lymphadenopathy, and more specifically gingivitis, diarrhoea, rhinitis, upper respiratory tract infections, ocular discharge and neurological signs (Yamamoto et al. 1989). Opportunistic infections and multiple simultaneous infections may occur. Haematological abnormalities also occur with cats often having cytopenias (the most common being anaemia, neutropenia, lymphopenia) and monocytosis although some cats may have lymphocytosis (Hopper 1989; Shelton et al. 1990b). Concurrent chronic infections are major factors in the haematological disorders, summarised by Callanan (Callanan 1995).

1.5. Immunodeficiency

Immune system dysfunction occurs as the disease progresses, with a steady reduction in $CD4^+$ T lymphocytes and changes in the $CD4^+$ and $CD8^+$ T lymphocyte ratios. Reduction of the CD4:CD8 ratio or inversion of the ratio has been documented (Novotney et al. 1990; Hoffman-Fezer et al. 1992; Lawrence et al. 1992). Expansion of the $CD8\alpha^+\beta^{low}$ T lymphocyte subset post infection has been documented (Lehmann et al. 1992; Willett et al. 1993) and is thought to contribute to the lymphocyte inversion seen. While the role of these cells is still unclear they are thought to be activated T cells (Bucci et al. 1998b). There is reduced responsiveness of peripheral blood mononuclear cells to mitogens such as pokeweed mitogen (PWM), Concanavalin A (Con A), lipopolysaccharide (LPS) and to stimulation by interleukin-2 (IL-2) (Lin et al. 1990; Siebelink et al. 1990; Barlough et al. 1991).

1.6. Clinical signs of FIV in experimental infection

Experimental infection may be achieved by many routes of inoculation including: intraperitoneal (Pedersen et al. 1987; Yamamoto et al. 1988), intravenous (Yamamoto et al. 1988), intramuscular, subcutaneous (Siebelink et al. 1990) and intrathecal (Dow et al. 1990). In addition, cats can be infected by oral, nasal (Callanan 1995), vaginal and rectal administration (Bishop et al. 1992). Successful infection has been achieved using whole blood, plasma, cell-free virus and cell-associated virus (Pedersen 1993).

Within 6 weeks post infection (p.i.) generalised lymphadenopathy usually occurs, brought about by follicular hyperplasia, paracortical activation and expansion, accompanied by increased numbers of plasma cells due to initiation of the humoral response. Around this time mantle zone attenuation and invagination occurs, follicles become irregular and some

become markedly enlarged due to fusion with neighbouring follicles (Callanan et al. 1993). There are also increased numbers of germinal centre $CD8^+$ cells, sinus lymphocytosis and B cell reactions (Callanan et al. 1993; Parodi et al. 1994). By 8-12 months p.i. involution of the lymph nodes first becomes apparent, and hyalinised follicles appear (Callanan et al. 1993). Often coinciding with generalised lymphadenopathy are transient neutropenia, lymphopenia, pyrexia, dullness and anorexia (Pedersen et al. 1987; Yamamoto et al. 1988; Yamamoto et al. 1989; Moraillon et al. 1992; Mandell et al. 1992; Callanan et al. 1992b). This is termed the acute phase of infection and cats generally recover, becoming free of clinical signs but persistently viraemic. Healthy, persistently viraemic cats are said to be in the asymptomatic phase, which may last for variable periods. During this time haematological parameters are generally unremarkable but by eighteen months p.i. a gradual decline in $CD4^+$ T lymphocytes occurs (Ackley et al. 1990; Barlough et al. 1991; Willett et al. 1993) sometimes with a concurrent increase in $CD8^+$ T lymphocytes (Ackley et al. 1990).

1.7. The clinical staging for FIV

It has been proposed that the disease conditions associated with FIV infection can be classified into five recognisable clinical disease stages (Ishida and Tomoda 1990), analogous to the clinical stages found in HIV infection.

1. The acute phase characterised by pyrexia and generalised lymphadenopathy 42-56 days post infection as reviewed by Pedersen (Pedersen 1993).

2. The asymptomatic phase (Yamamoto et al. 1988) which has been found to vary in duration with age at infection (George et al. 1993).

3. Persistent generalised lymphadenopathy where cats are often presented to the veterinary surgeon for unthriftiness and vague nonspecific clinical signs, reviewed by Pedersen (Pedersen 1993).

4. AIDS-related complex – 50% of cats present with signs similar to HIV in humans (Ishida and Tomoda 1990).

5. AIDS – less than 10% of FIV-infected cats are presented in the terminal AIDS-like stage, as many are euthanased due to the very poor prognosis.

6. Sometimes a sixth group is included which comprises miscellaneous diseases associated with FIV (Pedersen 1993), e.g. neoplasia has been recorded in naturally infected (Alexander et al. 1989; Shelton et al. 1990a; Rosenberg et al. 1991; Hutson et al. 1991; Buracco et al. 1992; Barr et al. 1993) and experimentally infected FIV-positive cats (Callanan et al. 1992a; Poli et al. 1994). The mechanism whereby FIV initiates malignancy is still unclear but one report describes a monoclonally integrated FIV provirus within tumour DNA (Beatty et al. 1998). However, viral gene expression could not be detected which indicated that expression was not required in order to maintain the transformed phenotype (Beatty et al. 1998). There is no doubt, however, that cats with FIV have an increased risk of developing lymphoma (Hutson et al. 1991; Poli et al. 1994; English et al. 1994; Callanan et al. 1996).

1.8. Tropism *in vivo*

Upon infection, the principal target for FIV is the $CD4^+$ T lymphocyte (English et al. 1993; Beebe et al. 1994; Dean et al. 1996) and this subpopulation decreases rapidly as the disease progresses to the immune deficiency stage (Ackley et al. 1990; Torten et al. 1991; Hoffman-Fezer et al. 1992). Infection of macrophages occurs (Brunner and Pedersen 1989) and macrophage-tropic variants are thought to predominate during the asymptomatic phase (Beebe et al. 1994). The prevalence of macrophage-tropic variants was shown to increase by 40-95% at the time of the early acute illness (Beebe et al. 1994), possibly due to the decreasing $CD4^+$ T lymphocyte population forcing selection of macrophage-tropic variants. Macrophage-tropic strains are thought to be necessary for the persistence of the infection and also dissemination of the virus within the body. Macrophages may play an important role in viral dissemination since viral infection is less cytopathic in macrophages compared to $CD4^+$ T cells (Beebe et al. 1994). Circulating macrophages were found not to express viral antigen until adherence occurred *in vitro* (Dow 1999), suggesting that monocyte infection in the peripheral blood might occur at a level higher than previously thought, since monocytes might express viral antigen only after maturation into tissue macrophages, which occurs on adhering to a substrate, e.g. extracellular matrix (Dow 1999).

FIV infects $CD8^+$ T cells (Brown et al. 1991) and B cells (English et al. 1993) as well as $CD4^+$ T cells. The virus is found increasingly in B cells during the progression from the

asymptomatic to chronic stage of infection (English et al. 1993; Dean et al. 1996). The $CD8^+$ T lymphocyte population is not depleted following infection and is sometimes expanded, contributing to a decrease in the $CD4^+$:$CD8^+$ T cell ratio (Torten et al. 1991; Hoffman-Fezer et al. 1992; Willett et al. 1993). The expanded $CD8^+$ population expressed lower amounts of the CD8 protein on its surface and was designated $CD8^{low}$ (Willett et al. 1993), later defined further as $CD8\alpha^+\beta^{low}$ (Shimojima et al. 1998a; Shimojima et al. 1998b).

Neuronal cells are also susceptible to FIV infection, with astrocytes being the most susceptible to infection and syncytium formation. Microglia are also susceptible and can remain persistently and productively infected (Dow et al. 1992).

1.9. Tropism *in vitro*

FIV isolates can be cultured *in vitro,* with isolates having different capacities for replication in different cell lines. Laboratory-adapted isolates are defined as having been passaged extensively *in vitro*, whereas primary isolates have been minimally passaged *in vitro*, infection being limited to PBMCs or highly susceptible IL-2 dependent cell lines, e.g. Mya-1 cells. Primary isolates can be grown in mitogen-activated PBMCs, macrophages, T-lymphoblastoid cell lines such as thymocytes, the IL-2-dependent cell line Mya-1, and the IL-2 independent cell line 3201 (Miyazawa et al. 1990; Tochikura et al. 1990). In these cells the virus causes cytopathic effects in the form of cell death and syncytium formation, with or without cell lysis. Some isolates have been shown to have a broader cell tropism, having the ability to infect also cells of the Crandell feline kidney (CrFK) cell line. Clones of the FIV-Petaluma (FIV-PET) isolate, namely FIV-14 and FIV-34TF10, can infect CrFK cells (Olmsted et al. 1989a) in which they can produce a persistent, noncytopathic infection (unpublished data, Retrovirus Research Laboratory, Glasgow University Veterinary School).

By inserting the *env* gene from variant clones into a FIV-PET backbone, the ability to infect CrFK cell lines was shown to be associated with an increase in charge of the hypervariable region 3 (V3) of SU (Verschoor et al. 1995). This difference in charge was considered to be the result of a glutamate to lysine (E to K) mutation at position 407 or 409. A concurrent glycine to arginine (G to R) mutation at position 397 was found to result in improved replication kinetics, similar to the original clone. Similarly, an increase in charge that was related to changes in tropism has been described in HIV isolates (de

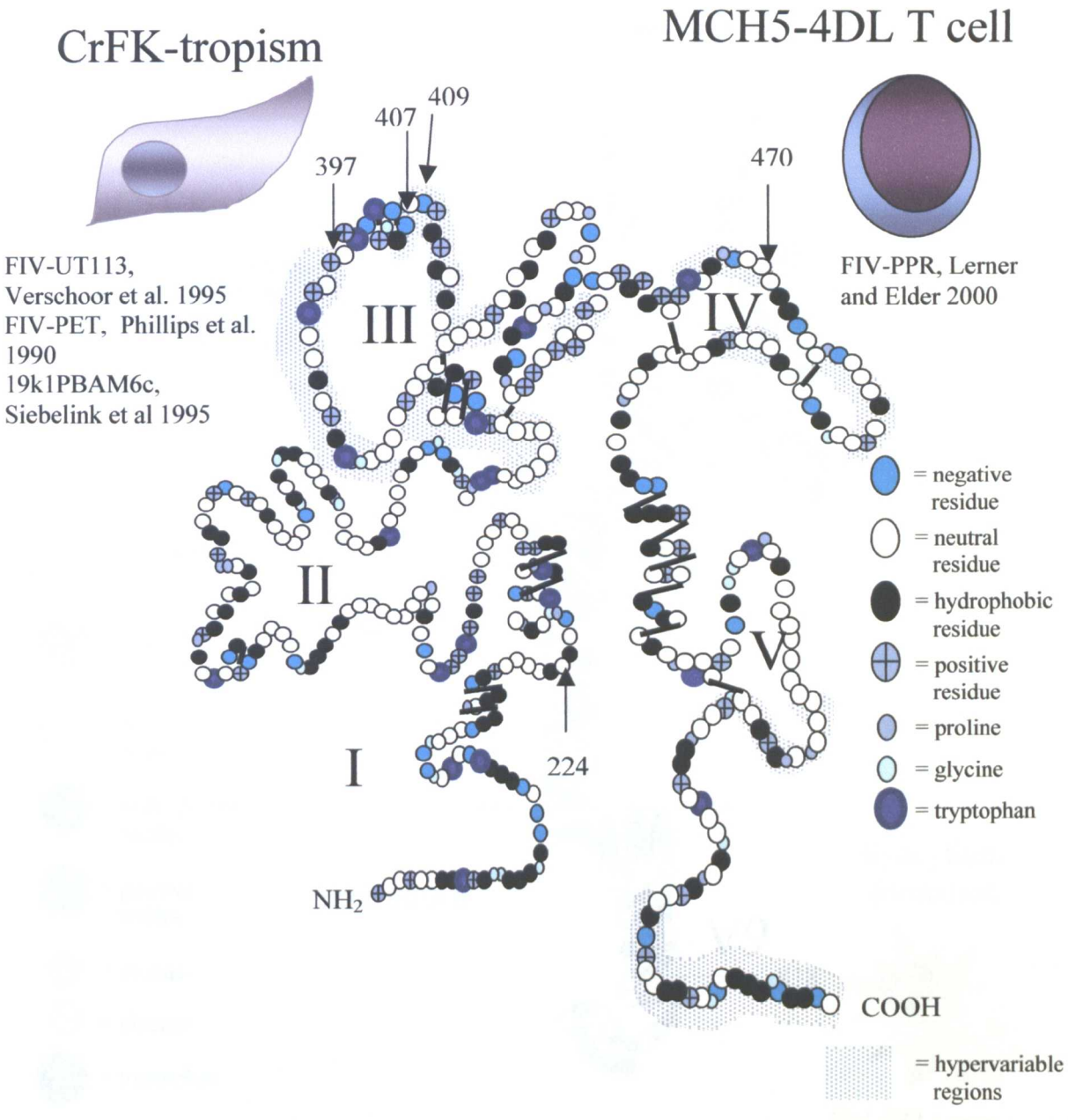
Jong 1992). Several regions of the *env* gene other than V3 also contain determinants of tropism. CrFK-tropism was the result of a methionine to threonine (M to T) mutation in the TM of the envelope glycoprotein of FIV-UT113 (Vahlenkamp et al. 1997). Expanded host cell tropism of a clone of FIV-PPR after passage in IL-2 independent T cells (MCH5-4DL) was shown to be associated with 3 mutations; glutamine to proline in the second constant domain of SU, threonine to proline in the V4 hypervariable loop of SU, and a premature stop codon in the cytoplasmic tail of the transmembrane protein which was found to be responsible for syncytium formation (Lerner and Elder 2000) (see Figure 1.b. and 1.c.).

Other lentiviruses, such as SIV, HIV-1, HIV-2 and EIAV, have been found to expand host cell tropism by truncation of the cytoplasmic domain of SU by a premature stop codon (Lerner et al. 2000). These truncations have been shown to increase growth kinetics, envelope fusogenicity and cytopathogenicity *in vitro*. Similarly, the FIV *orf2* gene encodes a protein that allows infection of peripheral blood lymphocytes, as seen in the clone FIV-PPR (Phillips et al. 1990). In contrast, the Petaluma clone 34TF10, which has a premature stop codon in this region, was severely restricted to adherent monolayer cell lines and replicated poorly *in vivo*. Repair of the *orf2* allowed the 34TF10 clone to replicate in T lymphocytes (Waters et al. 1996). Hence the role of *orf2* in tropism remains unclear.

1.10. FIV infection of human cell lines

There is evidence that some FIV isolates may have the ability to infect human cells. For example, FIV chimaeras with the Env protein of FIV-PET infected the human lymphoblastoid cell line MOLT-4 and, although productive infection was not achieved, provirus was detectable within the cells (Ikeda et al. 1996). Infectious virus was also produced when an infectious molecular clone of the TM1 strain was transfected into the non-lymphoid human cell line HeLa (Miyazawa et al. 1992). More recently FIV isolates V1-CSF and FIV-PET were reported to infect human PBMCs productively by cell-free infection, although infection was inefficient (Johnston and Powers 1999). FIV has been proffered as a useful tool in human gene therapy as an efficient retroviral vector (Poeschla et al. 1998). Although no human has been reported as becoming infected with FIV (Pedersen 1993; Butera et al. 2000) or even seroconverting, great caution should be exercised in selecting vector strains owing to the great range in tropism and ability to adapt

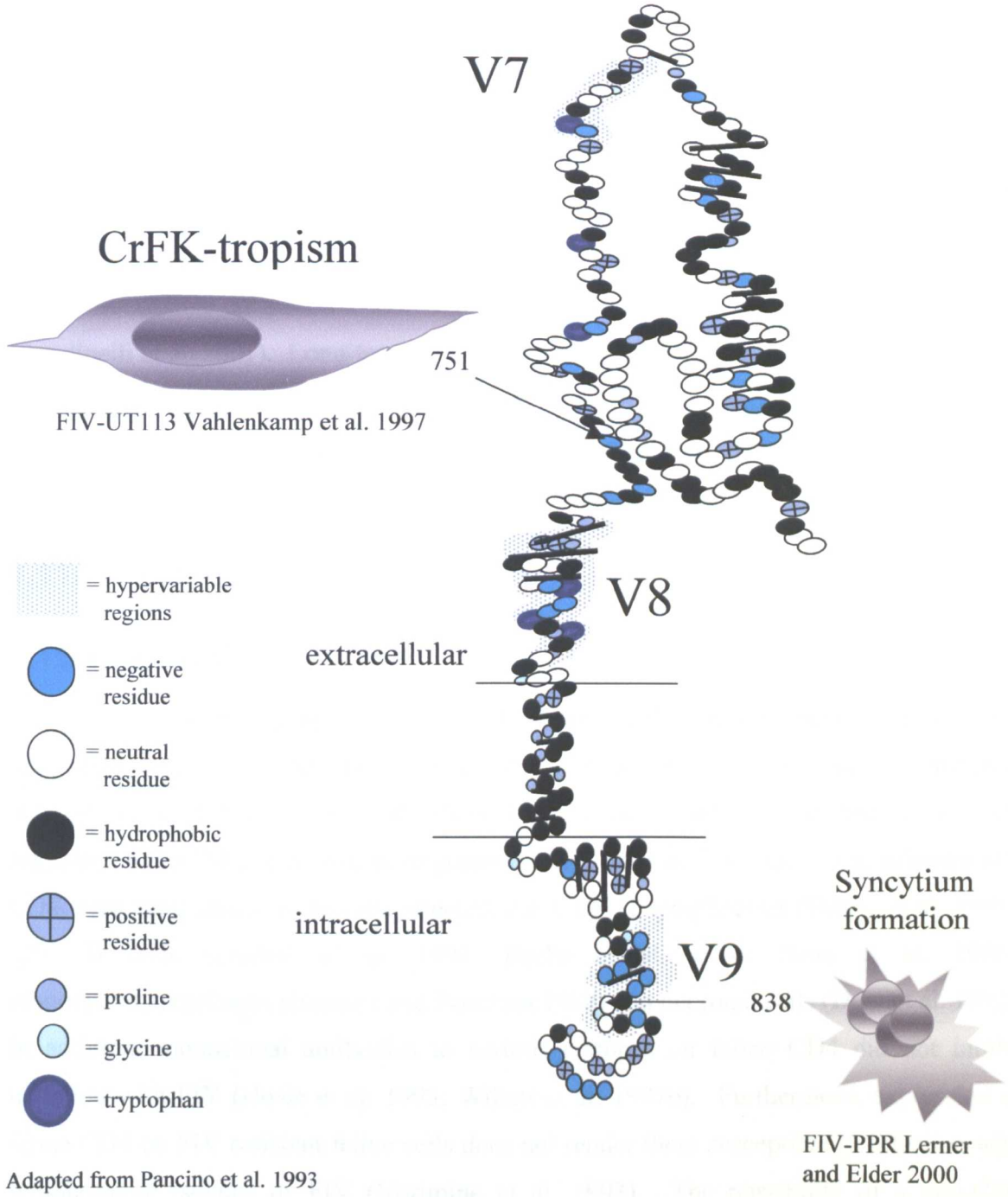
Figure 1.b. Schematic representation of the SU of the Env protein.



Adapted from Pancino et al. 1993

Amino acids are represented by coloured circles (see key). Black arrows indicate sites where mutations occur, leading to expanded tropism. Roman numerals I – V indicate variable loops. CrFK-tropism can be brought about by mutations at sites 407 or 409 glutamate lysine mutation (E to K) or at site 751 within the TM by a methionine to threonine (M to T). Mutations at 224 (glutamine to proline, (E to P) and 470 threonine to proline, (T to P) were responsible for growth in the IL2 independent T cell line MCH5-4DL by FIV-PPR.

Figure 1.c. Schematic representation of the TM of the Env protein.



Amino acids are represented by coloured circles (see key). Black arrows indicate sites where mutations occur, leading to expanded tropism. CrFK-tropism can be brought about by mutations at sites 407 or 409 glutamate lysine mutation (E to K) or at site 751 within the TM by a methionine to threonine (M to T). Mutations at 224 (glutamine to proline, (E to P) and 470 threonine to proline, (T to P) were responsible for growth in the IL2 independent T cell line MCH5-4DL by FIV-PPR. Truncation of the cytoplasmic tail from residue 838 of this clone was responsible for syncytium formation.

displayed by FIV. From this evidence it can be seen that isolates of FIV have evolved many ways in which to broaden or improve their infective and replicative capacity within numerous cell lines. The poor proof reading capabilities of RT leads to this high rate of mutation and contributes to the virus having the ability to evade the immune system. Primary isolates of FIV have broad resistance to antibody-mediated neutralisation, which is thought to contribute to the persistence of these viruses and may cause failure of experimental vaccines. Reversion to broad neutralisation resistance arose in cats inoculated with a tissue culture-adapted strain (FIV-PET) and a lysine to asparagine or glutamine change at position 481 was found to be a key determinant in reversion (Bendinelli et al. 2001). Long-term viral revertants exhibited a serine to asparagine change at 557 in the fifth variable region (V5), a mutation which is thought to collaborate with other mutations in the maintenance of neutralisation resistance. However, neither of these mutations was reported to cause a change in cell tropism (Bendinelli et al. 2001).

1.11. Cell Receptors

1.11.1. The CD4 molecule

CD4 is the principal receptor for entry of primate lentiviruses (Dalgleish et al. 1984; Klatzmann et al. 1984) and $CD4^+$ T lymphocytes are the principal target for FIV infection (English et al. 1993; Beebe et al. 1994; Dean et al. 1996). These findings initially suggested that CD4 might have an important role in entry of FIV. However, subsequently $CD4^-$ cells were shown to become infected, e.g. $CD8^+$ T lymphocytes (Brown et al. 1991), IgG^+ B cells (English et al. 1993; Beebe et al. 1994; Dean et al. 1996), monocyte/macrophages (Brunner and Pedersen 1989) and neuronal cells (Dow et al. 1992). In addition, monoclonal antibodies to various epitopes on feline CD4 did not inhibit infection with FIV (Hosie et al. 1993; Willett et al. 1997b). Furthermore, expression of feline CD4 on FIV resistant feline cells does not render them susceptible to infection with lymphotropic isolates of FIV (Norimine et al. 1993). The possibility of a non-CD4 receptor was further highlighted when the anti-feline CD9 monoclonal antibody vpg15, blocked infection of FIV susceptible cells (Hosie et al. 1993), implicating CD9 as a putative receptor for FIV entry. Subsequently, however, the blocking properties of vpg15 were found to act at a post-entry stage (de Parseval et al. 1997; Willett et al. 1997b).

1.11.2. The chemokine receptor CXCR4

The discovery of the seven transmembrane domain receptor "fusin" as a coreceptor for HIV-1 entry (Feng et al. 1996) led to the investigation of its use by FIV. Fusin, or CXCR4 as it is now known, is a G-protein coupled receptor. The name CXC comes from the positioning of the first two highly conserved cysteine residues of the amino acid sequence. The CXCR4 receptor is essential for mammals (Ma et al. 1998; Zou et al. 1998) as the deletion of this gene in embryos is lethal. CXCR4 is found on the surface of many cells such as endothelial and epithelial cells. It plays a major role in haematopoiesis, organogenesis, B cell maturation and T cell trafficking, and is also a potent lymphocyte/monocyte chemoattractant (Murdoch 2000). The ligand for CXCR4 is the chemokine, stromal cell derived factor-1 (SDF-1), a relationship that is thought to be monogamous (Ma et al. 1998).

Usage of CXCR4 by FIV, was indicated in a similar fashion to HIV-1, by inhibition assays against cell culture-adapted strains of FIV, conducted using the anti-human CXCR4 antibody 12G5 (Willett et al. 1997a). Antibody 12G5 led to a dose-dependent inhibition of fusion between the human cell line HeLa (expressing CXCR4) and persistently FIV-infected CrFK cells (viral envelope glycoproteins protrude from the infected cells and fuse with the CXCR4 of the HeLa cells). SDF-1α, the natural ligand for CXCR4, was shown to block infection of CrFK cells by a cell culture-adapted strain of FIV, but not the infection of the IL-2 dependent T cell line Mya-1 (Hosie et al. 1998a), which suggested not only that CXCR4 mediated the entry of cell culture-adapted strains, but also that there might be an alternative mode of viral entry in cell lines such as Mya-1. As well as a dose-dependent inhibition, enhancement was observed in FIV infection of CrFK cells after prolonged incubation with SDF-1α (Hosie et al. 1998a). This was considered to be due to upregulation of CXCR4 expression on the cell surface leading to increased viral uptake.

Further studies, using the bicyclam derivative AMD3100 that binds CXCR4, demonstrated that a dose-dependent inhibition of infection by laboratory-adapted strains of FIV could be achieved. Blocking was achieved in CrFK cells and feline thymocytes at AMD3100 concentrations of 14ng/ml and 62 ng/ml respectively (Egberink et al. 1999). The same studies demonstrated that primary isolates also used CXCR4, as infection of feline thymocytes was blocked in a dose-dependent manner at inhibitory concentrations ranging between 21 ng/ml to 68 ng/ml for the 6 primary isolates studied. Furthermore, it was

shown that the blocking effect of AMD3100 acted at an envelope-mediated stage since blocking of a pseudotyped virus expressing a primary isolate envelope glycoprotein was inhibited, whereas there was no inhibition of infection by pseudotypes expressing the G-protein of vesicular stomatitis virus (Richardson et al. 1999).

Feline CXCR4 was cloned from the mRNA of cells of the T lymphoblastoid cell line Mya-1 (and of CrFK cells) and was shown to have 94.9% homology, at the amino acid level, with human CXCR4 (Willett et al. 1997c). Ectopic expression of feline CXCR4 on the human cell line U87 allowed fusion with CrFK cells infected with FIV-GL8. That neither CD4 nor CD9 were receptors for FIV was confirmed when U87 cells expressing CD4 or CD9 did not support fusion (Willett et al. 1997c). A further study using rat/human chimeras of CXCR4 demonstrated that the second extracellular loop (EC) loop of CXCR4 was the principle determinant for fusion, while the first and third loops were important for conformational presentation of the second EC loop or were directly involved themselves (Willett et al. 1998).

1.11.3. Involvement of another (co)receptor

HIV-1 isolates fall into two biological groups. They were first classified by their *in vitro* growth properties as 'rapid-high' or 'slow-low' depending on their replication rates (Åsjo et al. 1986) or as syncytium-inducing (SI) or nonsyncytium-inducing (NSI) (Åsjo et al. 1986; Tersmette et al. 1988) and also macrophage-tropic or T-lymphotropic (Gartner et al. 1986). Studies on the usage of the chemokine receptor CCR5 by macrophage-tropic isolates of FIV have provided no evidence of a role for CCR5. Incubation of CrFK or Mya-1 cells with the β-chemokines, MIP-1α, MIP-1β and RANTES did not block infection with FIV-PET (Hosie et al. 1998a). More recently, however, a study comparing the isolates FIV-PPR and FIV-PPR_{glial} demonstrated the possibility of CC chemokine receptor usage, as the β-chemokine RANTES inhibited infection of T cells by FIV-PPR by 20-40% (Lerner and Elder 2000). Therefore, FIV may use a CC chemokine as a method of entry.

Further evidence of non-CXCR4 receptor usage by FIV was highlighted by a study using a fusion protein consisting of the Fc domain of human immunoglobulin G1 with the variable domain being represented by the SU (gp95) of either FIV-PPR (primary isolate) or FIV-34TF10 (laboratory-adapted isolate). It was shown that the SU of the gp95 of FIV-PPR bound only to primary feline T lymphocyte cell lines whereas the SU of the gp95 of FIV-

34TF10 bound to all cell lines tested. SDF-1α, RANTES, MIP-1 and heparin all failed to inhibit binding of gp95 to primary T cells, implicating a non-CXCR4 receptor (Richardson et al. 1999). Further studies to elucidate the receptor usage of FIV have found that a 43kD non-CXCR4 molecule, as well as heparan sulphate proteoglycans (HSPG), is used but that the receptor used is dependent on cell type (de Parseval and Elder 2001; Elder and de Parseval 2002). For example, FIV-PPR infection is restricted to cells expressing the 43kD molecule, such as IL-2 dependent cells and PBMCs, whereas the FIV-PET clone 34TF10 can use CXCR4, the 43kD molecule and HSPGs. On primary T cells, 34TF10 was shown to use the 43kD molecule but on non-primary T cells e.g. 3201 cells, the virus was shown to bind directly to CXCR4. This strain was shown to have further diversity as it was able to infect adherent cell lines by binding to HSPGs and then CXCR4 (Elder and de Parseval 2002). Further studies in the same report showed that similar to HIV, DC-SIGN can transmit FIV to other cells, again highlighting the usefulness of FIV as a model for HIV infection and AIDS.

1.12. Immunopathogenesis

1.12.1. The lymphocyte subpopulations

Perturbation of the immune system is best examined in the experimentally infected cat, as the duration of infection and the dose and characteristics of the inoculum are known. $CD4^+$ T cell cytopenia is common to both natural and experimental infection but is generally seen later in natural infection, concurrent with the AIDS-like stage, whereas it is recorded earlier in experimental infection (Walker et al. 1994; Diehl et al. 1996; Walker et al. 1996). However, this decrease in $CD4^+$ T lymphocytes is not solely responsible for immunodeficiency (Pedersen 1993). Although CD4 levels and plasma virus load were thought to be inversely correlated (Diehl et al. 1995a), studies have shown that they may be affected by route of infection and viral strain (Diehl et al. 1995b; Burkhard et al. 2002). Indeed, studies of SIV infection have shown that receptor usage affects the $CD4^+$ T cell population, as inoculation of CCR5- or CXCR4-type viruses have quite different effects (Harouse et al. 1999).

Early experimental infection is characterised by an expansion of the $CD8^+$ T lymphocyte population which coincides with reduced viral replication (Willett et al. 1993). This population of cells expresses only low amounts of the CD8 β chain ($CD8\beta^{low}$) (Willett et

al. 1993; Shimojima et al. 1998a; Bucci et al. 1998b; Shimojima et al. 1998b; Gebhard et al. 1999; Orandle et al. 2000). These cells have been shown to be noncytolytic $CD8^+$ T cells which produce soluble factors that suppress FIV replication (Jeng et al. 1996; Hohdatsu et al. 1998; Flynn et al. 1999; Choi et al. 2000) and, in some cases suppression or clearance of the virus occurs without seroconversion (Bucci et al. 1998a). The exact role of $CD8\beta^{low}$ lymphocytes is unclear as there is evidence that they are not solely responsible for the antiviral activity (Flynn et al. 1999; Crawford et al. 2001) and their numbers increase as virulent FIV variants emerge (Gebhard et al. 1999; Hosie et al. 2000; Hosie et al. 2002). This increase in $CD8\alpha^+\beta^{low}$ lymphocytes can be detected as early as one week p.i. in the peripheral and mesenteric lymph nodes and the blood (Flynn et al. 2002) and has been correlated to a reduction in viral detection (Crawford et al. 2001).

1.12.2. The cell mediated immune response

The cell mediated immune response is detectable before the humoral response (Song et al. 1992; Beatty et al. 1996; Flynn et al. 1996). Cell mediated immunity is seen as early as 2 weeks p.i. (Beatty et al. 1996) in peripheral blood (Song et al. 1995). The level of cytotoxic T lymphocyte activity is lower in chronically infected cats (Li et al. 1995) than in acutely infected cats (Beatty et al. 1996). Also the distribution of CTL changes, activity being concentrated in the lymph nodes and spleen during the asymptomatic stages, i.e. the major sites for viral replication at this time (Beebe et al. 1994; Flynn et al. 1996). The CTL response in naturally infected cats tends to be directed towards Gag proteins of the virus (Flynn et al. 2002) whereas in cats vaccinated with whole inactivated virus (WIV) that were protected from infection the CTL response has been shown to be predominantly directed towards Env proteins (Flynn et al. 1996).

1.12.3. The humoral immune response

Seroconversion can be detected as early as 3-5 weeks p.i. (Yamamoto et al. 1988; Rimmelzwaan et al. 1994; Callanan et al. 1996; Burkhard et al. 2002) and antibodies are directed to both Gag and Env (Hosie and Jarrett 1990; Egberink et al. 1990). There are conflicting reports of which antibodies appear first, perhaps reflecting strain differences or the result of varying sensitivity of the assays used (Burkhard and Dean 2003). Virus neutralising antibodies arise 6 weeks p.i. (Yamamoto 1999) and levels continue to increase over the first 6-8 months (Inoshima et al. 1996). The third hypervariable region of the Env is the principal immunodominant domain for neutralising antibody (Lombardi et al. 1993;

de Ronde et al. 1994) but the V4 and V5 regions have also been implicated (Siebelink et al. 1993).

The extent to which these immune responses participate in protection against infection with FIV is unknown, but it has been suggested that humoral immunity is less important than cell mediated immunity and the noncytolytic immune response (Flynn et al. 2000).

1.13. Vaccination

1.13.1. Early vaccines

Since the discovery of HIV in 1983 (Barré-Sinoussi et al. 1983), efforts have been directed toward the production of a vaccine. The discovery of FIV in 1987 (Pedersen et al. 1987), with its many similarities to HIV, has led to its employment as a vaccine model for HIV. The first successful vaccines against FIV were WIV or inactivated infected cell (IIC) vaccines made from the FL-4 cell line, with over 90% protection being achieved against homologous challenge with FIV-PET and the closely related FIV-DIXON (Yamamoto et al. 1991). Similarly, protection was achieved using the same vaccine but challenge inoculum was grown in an unrelated cell line. However, protection was not achieved against the heterologous virus FIV-GL8 (Hosie et al. 1995). The resistance to vaccine-induced protection demonstrated by the primary isolate FIV-GL8, along with induction of the $CD8\alpha^{+}\beta^{low}$ T lymphocyte population (Willett et al. 1993) as well as higher proviral DNA loads and greater reduction of the CD4:CD8 T lymphocyte ratio (Hosie et al. 2002), led to the hypothesis that FIV-GL8 is a more virulent strain of FIV compared to FIV-PET.

1.13.2. Subunit vaccines

Due to the inherent risks involved with WIV vaccines, particularly for HIV, much research has been conducted in the field of subunit vaccines. Immunogens that have been tested include recombinant FIV proteins expressed in mammalian cells using vectors such as baculovirus, vaccinia virus, and *E.coli* in prokaryotic cell lines. The majority of subunit vaccines have been based on the Env protein (Lutz et al. 1995; Siebelink et al. 1995c; Hosie et al. 1996a). Smaller subunits using only the V3 region of the Env protein have also been tested (Lombardi et al. 1994; Flynn et al. 1995), as well as the p24 protein (Hosie et al. 1992), but little success has been achieved using these immunogens. At best, suppression of viral load was achieved with affinity purified Env, although all the vaccinates became infected (Hosie et al. 1996a). Furthermore, enhancement of infection,

where viral loads have been higher or the time to viral isolation shorter, has been reported following immunisation with several of these subunit vaccines (Hosie et al. 1992; Lombardi et al. 1994; Siebelink et al. 1995c).

1.13.3. DNA vaccines

The advent of DNA vaccination appeared promising as this system would allow the mass production of vaccines, which would be economical and affordable for third world countries where HIV is very prevalent. DNA vaccination presents further advantages: vaccines may be produced for pathogens which are problematic to grow in culture; they contain inherent immunostimulatory sequences, namely CpG motifs; they may induce both humoral and cell mediated immune responses; and there is no evidence for integration into the host genome (reviewed by Dunham (Dunham 2002)). DNA vaccination against FIV infection has given some promising results to date. Vaccines have included proviral DNA with various deletions within the genome, mostly in the integrase or reverse transcriptase genes (Hosie et al. 1998b; Hosie et al. 2000; Dunham et al. 2002), the *vif* gene (Lockridge et al. 2000) or the AP-1 binding site (Kohmoto et al. 1998). Also an Env construct in a minimalistic immunogenic defined gene expression (MIDGE) system has been tested (Borretti et al. 2000; Leutenegger et al. 2000). Partial protection was reported in two trials using the construct FIV-PPR-Δ*vif* (Leutenegger et al. 2000) or the surface protein plus part of the transmembrane protein of FIV-$_{Zurich2}$ (FIV-Z2) (Borretti et al. 2000) when cats were challenged with homologous virus strains. Similar to the subunit vaccines, enhancement of infection was documented when either wild type *env* or *env* genes with mutations in the principal immunodominant domain was used as vaccine immunogens (Richardson et al. 1997). In studies where cats were challenged with heterologous strains, no protection was seen. Comparison of vaccine trials is difficult due to the great variation in vaccine production, challenge strains, adjuvants employed and the routes of immunisation and challenge.

The most success has been achieved with WIV vaccines derived from either the subtype A FIV-PET (Yamamoto et al. 1991; Yamamoto et al. 1993; Hosie et al. 1995) or the subtype B FIV-M2 (Matteucci et al. 2000a) but again these vaccines were only successful against homologous challenge. A similar situation occurs in IIC vaccines where a degree of success has been reported in trials investigating mucosal transmission, which is pertinent to HIV infection (Stokes et al. 1999; Finerty et al. 2002).

1.13.4. The future of vaccines

A successful FIV vaccine should protect against homologous and heterologous challenge. To date, only a single vaccine has become commercially available (Fel-O-Vax FIV, Fort Dodge). This vaccine is a WIV vaccine using subtype A FIV-PET and subtype B FIV-Shizuoka (FIV-SHI) and was shown to protect 100% of vaccinates challenged with FIV-PET and 80% of vaccinates challenged with FIV-Bangston (FIV-BANG) (Pu et al. 2001). These results are highly promising but the vaccine has yet to be tested under field conditions where challenge will be in the form of virus populations or quasispecies that arise during natural infection. Only one trial has been conducted under natural conditions of challenge. Cats in a rescue shelter were vaccinated with an IIC vaccine based on the subtype B strain M2 (Matteucci et al. 2000b), and all 12/12 immunised cats were protected while 5/14 of control, unvaccinated cats became infected. Although these results were promising, the duration of vaccine immunity using the same vaccine was reported to be short (Matteucci et al. 1997), so that repeated immunisation may be necessary to conserve immunity. Whether this vaccine would protect against challenge with virulent subtype A viruses remains unknown. The vaccine was tested in Italy where subtype B viruses predominate and it has been suggested that these viruses are more ancient than subtype A strains and therefore more host adapted and less virulent (Bachmann et al. 1997; Pistello et al. 1997).

1.14. Does tropism *in vitro* correlate with pathogenicity *in vivo*?

Since the discovery that chemokine receptors are essential for HIV-1 entry into cells and may be involved in the evolution of disease, studies of receptor utilisation have led to a greater understanding of the pathogenesis of HIV and AIDS. The error-prone RT leads very quickly to the formation of a quasispecies population within the infected individual. The development of viruses with broad genetic variability can lead to differing phenotypes, of which cell tropism, replication rates and syncytium inducing (SI) capacities have been studied (Cheng-Mayer et al. 1988; Tersmette et al. 1988; Tersmette et al. 1989a; Connor et al. 1993a).

Tropism on MT-2 cells has been correlated with disease stage (Connor et al. 1993a) with asymptomatic individuals having mostly macrophage-tropic, slowly replicating NSI HIV-1 variants. During disease progression more T cell-tropic, rapidly replicating variants appear, although only 50% of patients with disease progression have viruses of the SI

phenotype. HIV-1 infection is established by macrophage-tropic isolates, most of which use the chemokine receptor CCR5. Some isolates may have dual tropism for both CXCR4 and CCR5 and small numbers may be restricted to CXCR4 alone. With disease progression, SI variants often emerge, which display broader coreceptor usage *in vitro* (using CCR2b, CCR3, CCR8, STRL33 and V28 (de Roda Husman and Schuitemaker 1998). The reason for this selection of NSI macrophage-tropic variants is unclear. However, the main receptors *in vivo* are still thought to be CXCR4 and CCR5 (Zhang and Moore 1999).

It is thought that CXCR4 viruses are rarely transmitted for several reasons. For example, CCR5 viruses predominate in most infected individuals, extensive SDF-1 expression on mucosal surfaces may act as a barrier (Agace et al. 2000) and there is down regulation of CXCR4 within the gut associated lymphoid tissue (Harouse et al. 1999). The finding that individuals homozygous for a 32bp deletion in the CCR5 gene (Δ32/Δ32 CCR5 homozygotes) which leads to a truncated CCR5 protein that fails to reach the cell surface, are highly resistant to HIV-1 infection via sexual, blood contact or mother to child transmission further support the hypothesis that CXCR4 viruses are rarely transmitted (Clapham and McKnight 2002). Contrary to these findings, inoculation of predominantly SI variants was reported to result in persistence of the SI virus population in the recipient and inoculation of a predominantly NSI population into another recipient resulted in amplification of SI viruses before seroconversion, followed by suppression at seroconversion (Cornelissen et al. 1995). This suggested that SI variants were suppressed by an immune mediated mechanism (Lathey et al. 1997).

The sequences associated with change in phenotype between SI and NSI viruses have been mapped to the V1/V2 and V3 regions of *env* (Groenik et al. 1993) and involve increases in charge in the V3 region and length and charge of the V2 region (Fouchier et al. 1992; Groenik et al. 1993; Fouchier et al. 1995). Similarly, the switch from CCR5 usage to CXCR4 usage is accompanied by an increased positive charge in the V3 loop (de Jong et al. 1992).

1.15. Relevance of receptor usage for vaccine studies

Most promising FIV vaccines tested to date have only been effective against homologous or laboratory-adapted viruses. The commercially available vaccine, Fel-O-Vax (Fort Dodge Animal Health) does not induce sterilising immunity against infection with the

primary isolate FIV-GL8 (Dunham, S.P., personal communication) although viral burdens may be reduced following challenge. As yet, little is known about the potential impact of these vaccines as the characteristics and nature of the population of viruses in the field is not well defined.

Two prototypic FIV isolates have been extensively characterised in this laboratory; namely, the laboratory-adapted FIV-PET and the primary isolate FIV-GL8. Unlike FIV-PET, *in vivo* FIV-GL8 establishes high viral loads, inversion of the CD4:CD8 ratio and is resistant to vaccine-induced protection. *In vitro,* FIV-GL8 appears to require an additional component to CXCR4 in order to enter host cells. It is unknown whether FIV-PET and FIV-GL8 represent two distinct phenotypes of FIV or whether isolates display a range of biological behaviour. Furthermore, the relationship between tropism and chemokine receptor usage *in vitro* and biological behaviour *in vivo* is still unclear as is whether FIV, like HIV, undergoes a switch in phenotype and receptor usage which correlates with disease progression.

Most vaccines have to date targeted laboratory-adapted isolates like the CXCR4-tropic FIV-PET. If such viruses are mostly isolated from cats in the terminal stage of disease, then they are less likely to be transmitted, since terminally ill cats have a decreased tendency to roam or display normal territorial behaviour. Therefore, the first aim of this project was to establish the phenotype of primary FIV isolates made from field cats compared at different stages of infection and compare them with FIV-PET and FIV-GL8 to determine whether the phenotype was different from cats at different stages of infection. The human terminology "symptomatic" and "asymptomatic" was adopted for convenience and to aid comparison with HIV. The infected cats were classified as 1. terminally ill (symptomatic), 2. responders that had displayed clinical signs but responded to treatment, 3. asymptomatic cats that had remained disease free since diagnosis (asymptomatic). The isolates were examined *in vitro* to investigate the role of chemokine receptors and host cell tropism in the asymptomatic and terminal phases of the disease. After thorough characterisation *in vitro*, isolates were selected from groups 1 and 3 and were inoculated into specific pathogen free kittens to examine their pathogenicity *in vivo*. Pathogenicity was assessed by measuring plasma viral load and proviral loads in PBMCs and tissues. Lymphocyte subpopulation changes were examined by fluorescent antibody cell sorting (FACS). To elucidate the role of Env in the pathogenesis of FIV, chimaeric viruses were constructed using a FIV-GL8$_{MYA}$ backbone into which a selection of field isolates and

prototypic virus *env* genes were inserted. The resultant clones were subjected to similar *in vitro* assays and *in vivo* study to determine whether different pathogenic behaviour could be attributed to *env*.

Chapter Two

MATERIALS AND METHODS

2.1. Animals

Specific pathogen free (SPF) kittens were obtained from Biolabs, Eire, at 10 weeks of age. A two-week acclimatisation period was allowed for all kittens before the commencement of each study. The cats were maintained in a facility that is exceptional in the provision of environmental enrichment to ensure animal welfare of the highest standard.

2.2. Blood samples

Blood samples at each time point were collected into EDTA. Whole blood was used for routine haematological analysis and fluorescent antibody cell sorting (FACS). Plasma was harvested by centrifugation at 2000 rpm for 10 minutes. The plasma was aliquoted and stored at -70°C. The blood cells were then resuspended in RPMI with no additives and suspended over 3ml Ficoll-paque Plus (Amersham Biosciences, Buckinghamshire, U.K.) in a 15ml Falcon tube (Becton and Dickinson, France), then centrifuged at 2000 rpm for 10 minutes with the centrifuge brake off. While erythrocytes and platelets sink below the Ficoll, the mononuclear cells collect at the interface from where they may be harvested. The harvested cells were washed twice in RPMI with no additives and counted using white cell counting fluid (1% glacial acetic acid plus crystal violet to colour).

2.3. Collection of samples at post-mortem examination

Cats were anaesthetised using a premedicant, xylazine 2% solution (Rompun, Bayer, Bury St. Edmonds, U.K.) at 1.1mg/kg followed by ketamine (Ketaset, Fort Dodge Animal Health, Southampton, U.K.) 20 minutes later at 22mg/kg. Both were administered by the i.m. route.

Blood was collected under full anaesthesia by intracardiac puncture into 50ml Falcon tubes (Becton and Dickinson, France) containing 25ml Alsever's solution (Scottish Diagnostics, Edinburgh, U.K.), and lithium heparin and EDTA blood tubes were used for the collection of plasma and whole blood for analysis by FACS. Mononuclear cells were harvested using Ficoll-paque Plus as described. Before collection of tissues, the cats were euthanased by

intracardiac injection of pentobarbitone sodium (Euthatal, Merial Animal Health Ltd., U.K.).

Lymph nodes, spleen, thymus and femur bones were colleted in 50ml Falcon tubes containing 25ml culture medium (RPMI supplemented with 100μg/ml streptomycin and 100 IU/ml penicillin).

Cells were harvested from lymph nodes, thymus and spleen by dissecting the tissue roughly in culture medium, using sterile scalpel blades. The medium was pipetted into a 50ml Falcon tube. This was repeated until the medium remained clear, indicating that most cells had been harvested from the tissue. The cells were then washed twice in culture medium. The cells were counted using white cell counting fluid, which lyses any remaining red cells. Cell pellets containing at least 10^6 cells were washed twice in PBS and then stored at -70°C until required.

2.4. Cell lines and culture media

The lymphoblastoid cell line Mya-1 (Miyazawa et al. 1989) was grown in RPMI 1640 medium (Gibco Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 100μg/ml streptomycin, 100 IU/ml penicillin, 5 x 10^{-5} M 2-mercapto-ethanol, 2mM L-glutamine (complete RPMI medium) and 100 IU/ml human recombinant interleukin-2 (IL-2).

The CrFK(HO6T1) (clone CrFK ID10 cells transfected with the *ras* gene by Dr. D. Spandidos, National Hellenic Research Foundation, Athens, Greece) cells were grown in Dulbecco's modification of Eagle's medium (DMEM) (Gibco Life Technologies, Paisley, UK) supplemented with 10% FBS, 2mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin (complete DMEM medium).

The feline fibroblast cell line AH927, transduced with a retroviral vector expressing CXCR4 (AH927 FX4E) (Willett et al. 1998) was cultured in complete DMEM medium and selected with 400μg/ml Geneticin 418.

Peripheral blood monocyte/macrophages were isolated from blood from SPF cats into EDTA. The mononuclear cell population was separated by Ficoll-paque Plus centrifugation and then cultured in 48-well plates (Becton and Dickinson, France), precoated with feline affinity purified IgG (Sigma-Aldrich, U.K.) at 10^6/well in 500μl of

DMEM containing 2mM glutamine, 10% FBS, 100 IU/ml penicillin, 100μg/ml streptomycin and 50μM 2-ME (complete macrophage medium) and supplemented with 1000 IU/ml recombinant human granulocyte-macrophage-colony stimulating factor (rhGM-CSF) (R and D systems Europe Ltd., Oxon, U.K.) and incubated at 37°C for 60 minutes. The plates were washed three times with phosphate buffered saline (PBS) to remove nonadherent cells, fresh medium was added and the plates were incubated for 24-48 hours. The cells were then washed in PBS before being incubated with the virus overnight. The following day the virus was washed from the cells with PBS and fresh medium added and then the cultures were incubated for three days before being tested for viral replication using an RT activity assay (Cavidi Tech, Sweden).

All cell lines were incubated at 37°C in a 5% CO_2 incubator.

2.5. Viruses

2.5.1. Viral stocks

Viruses were collected from samples submitted to the Feline Virus Unit at the University of Glasgow. Mononuclear cells were separated from EDTA anti-coagulated blood by Ficoll-paque Plus centrifugation. The cells were washed and then set up in cultures of 10^6 PBMC and 2 x 10^6 Mya-1 cells in 5ml of complete RPMI plus IL-2. Once a cytopathic effect was evident, cultures were expanded by adding 10^7 Mya-1 cells and suspended in 20 ml complete RPMI plus IL-2. The cultures were tested for p24 production by FIV antigen ELISA (IDEXX, Portland ME) and once positive, supernatants were passed through a 0.45μm filter and cell pellets washed twice in PBS. Both were stored at -70°C until required.

The four viruses used extensively throughout the studies came from cats F0425H, F0556H, F0795H and F0827H. Cat F0425H was a 12 year old male presenting with anorexia, dullness and unilateral iritis and cat F0556H was a 10 year old female presenting with severe gingivitis and oral ulceration. Both cats responded well to treatment and continued to improve and remained free of clinical signs. Cat F0795H was a 15 year old male neuter presenting with dysphagia, retching, gingivitis and weight loss and cat F0827H was an 11 year old female presenting with anorexia, weight loss, jaundice and an abdominal mass. Both of these cats failed to respond to treatment and were euthanased due to their deteriorating condition. The viruses from cats F0425H and F0556H were classified as

asymptomatic isolates and the viruses from cats F0795H and F0827H were classified as symptomatic or terminal isolates.

2.5.2. Viral titration

To titrate each viral stock, 2 x 10^5 Mya-1 cells were suspended in 250µl of complete RPMI 1640 medium with IL-2. Virus was diluted fivefold from 1:10 to 1:3,906,250. Virus (50µl of each dilution) was added in triplicate and incubated in a humid incubator at 37°C for 60 minutes. The cells were then washed twice with RPMI 1640 medium supplemented with 5% FBS, resuspended in 500µl of complete RPMI 1640 medium supplemented with IL-2 and incubated in 48-well plates at 37°C for 7 days.

The cultures were harvested after 7 days. The culture fluids were collected and p24 production was measured by the enzyme linked immunosorbent assay (ELISA), Petcheck FIV antigen ELISA (IDEXX Corp., Portland, Maine, USA). Subsequently, 50% tissue culture infectious doses ($TCID_{50}$) were calculated using the Karber formula:

–log the highest concentration – (sum proportion of wells positive – 0.5).log dilution factor = $TCID_{50}$

Once titrated, each virus stock was tested for CXCR4 receptor usage and affinity by conducting a series of AMD3100 blocking assays.

2.5.3. Virus isolation

At intervals during the *in vivo* studies, the presence of virus in PBMCs was detected by culturing 10^6 PBMCs with 2 x 10^6 Mya-1 cells in 5ml of complete RPMI plus IL-2. The cultures were tested for p24 production by FIV antigen ELISA (IDEXX). Once positive, the cells were harvested, washed twice in PBS, pelleted and stored at -70°C. Supernatants were passed through a 0.45µm sterile filter, aliquoted and also stored at -70°C.

2.5.4. Quantitative viral isolation

The infectious viral burden was measured post infection in both *in vivo* studies by the method described by Meers (Meers et al. 1992). PBMCs were seeded into wells of a 96-well plate in 3-fold decreasing numbers (1 x 10^4, 3 x 10^3, 1 x 10^3, 3 x 10^2, 1 x 10^2, 30, 10, 3, 1, 0). 50µl of a suspension of Mya-1 cells at 10^6 cells/ml were added to each of the wells to a final volume of 200µl. 50µl of complete RPMI medium supplemented with IL-2

was added to each well on day 4 p.i. Samples were tested on day 7 or 10 post infection for the presence of FIV p24 by ELISA (IDEXX, Portland, ME). Results were calculated using the Reed and Muench formula (Reed and Muench 1937).

2.6. Preparation of virus for inoculation

Viruses for inoculation were diluted to the correct concentration in RPMI medium containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and kept on ice prior to use.

2.7. Preparation of DNA

DNA was harvested from PBMCs using a DNA kit (Qiagen, Hilden, Germany) following the manufacturers instructions (based on the principle first described by Boom (Boom et al. 1990)), and stored at –20°C. DNA yield was measured by spectrophotometry at a wavelength of 260nm (Sambrook et al. 1969) and calculated thus:

Concentration of DNA μg/μl = optical density (260nm) x 50 x dilution factor/1000

2.8. Preparation of RNA

Cell-free plasma was treated with reagents from a QIAamp viral RNA mini kit (Quiagen), following the manufacturers instructions, using the principle first described by Boom (Boom et al. 1990) (Qiagen, Hilden, Germany).

2.9. Statistical analysis

All results were analysed using the software packages SigmaPlot 2001 for windows version 7.0 (Copyright© 1986-2001 SPSS Inc.), and SigmaStat for Windows 2.03 (Copyright© 1992-1997 SPSS Inc.). Student's t-test was used to compare two groups. Variation between more than two groups was analysed using the analysis of variance method (ANOVA). Where an effect was evident, the groups were then subjected to an All Pairwise Multiple Comparison Procedure (Tukey test) and p values were used to indicate where a statistical difference was present between groups. p values <0.05 were regarded as statistically significant and <0.01 as highly significant.

Chapter Three

IN VITRO TROPISM OF PRIMARY ISOLATES OF FIV

3.1. Introduction

As discussed in Chapter 1, the similarities to HIV make FIV a suitable and practical model for vaccination trials. Protection following vaccination has been achieved using a WIV vaccine derived from the Petaluma strain (FIV-PET), challenging with either the homologous isolate or the closely related FIV-DIXON (Yamamoto et al. 1991; Yamamoto et al. 1993) but not the virulent Glasgow-8 (FIV-GL8) isolate (Hosie et al. 1995). Likewise, protection was achieved with a DNA vaccine using a *vif*-deleted mutant of the strain FIV-PPR, challenging with the homologous wild type virus FIV-PPR (Lockridge et al. 2000). In an extension of the original studies (Yamamoto et al. 1991), a dual subtype whole inactivated virus (WIV) vaccine has been shown to induce broad spectrum cellular and humoral immunity leading to protection against FIV-BANG and FIV-PET (Pu et al. 2001). Each of these viruses establishes a relatively low viral load. Therefore, there are some concerns that the efficacy of vaccines that have been studied and tested only under laboratory conditions remains unknown in the field, as no protection has been achieved against more virulent isolates such as FIV-GL8, which is considered a representative primary field isolate. To date only one vaccine trial under field conditions has been reported. This provided evidence for protection induced by a cell-associated FIV-M2 (FIV subtype B) strain fixed with paraformaldehyde (Matteucci et al. 2000b).

The range of physiological and genetic properties possessed by isolates in the field is not well understood. The isolates FIV-GL8 and FIV-PET have been studied for many years and possess markedly different properties when studied *in vivo*, under SPF conditions, and *in vitro*. *In vitro* studies show that FIV-PET has a broader cell tropism, infecting PBMCs, thymocytes, IL-2 dependent T lymphocytes and also the fibroblast cell line CrFK (Phillips et al. 1990). Growth of the more virulent FIV-GL8, which has been minimally passaged *in vitro* is restricted to PBMCs, thymocytes and IL-2 dependent T lymphocytes. Consequently, FIV-GL8 is regarded as a primary isolate. Infection of CrFK cells by FIV-GL8 is only achieved after "adaptation" following cocultivation with infected Mya-1 cells. The ability to infect CrFK cells was shown to correlate with an E to K mutation in the third hypervariable region of the envelope glycoprotein resulting in an increased charge of the

V3 loop (Verschoor et al. 1995; Siebelink et al. 1995b). Having undergone this mutation, isolates appear to have a greater affinity for the chemokine receptor CXCR4 (Willett et al. 1998). A similar mutation occurs in HIV during the progression to the AIDS stage that is correlated with a switch in coreceptor usage from the chemokine receptor CCR5 to CXCR4 (Callanan et al. 1996). Therefore, the analogous substitution in the FIV loop was predicted to be involved in CXCR4 binding (Willett et al. 1997a).

In vivo, FIV-PET infection leads to the establishment of low viral and proviral loads in the blood, and CD4:CD8 lymphocyte ratios are maintained. It has been proposed that this relative lack of virulence is the reason why vaccine induced protection has been achieved against challenge with this virus. In contrast, FIV-GL8 infection induces high viral and proviral loads, inversion of the CD4:CD8 lymphocyte ratio (Hosie et al. 2000), and in some cases expansion of the CD8 T lymphocyte population (Willett et al. 1993). This isolate is resistant to vaccine-induced immunity. It is unknown whether FIV-PET and FIV-GL8 represent distinct groups of FIV isolates or whether they fall within a population possessing a wide range of physiological and genetic properties. The aim of the studies in this chapter was to investigate the *in vitro* properties of a panel of isolates derived from samples submitted to the Feline Virus Unit (F.V.U.) diagnostic virology laboratory at the University of Glasgow Veterinary School.

A panel of 45 isolates was gathered from submissions to the FVU. Following a telephone follow-up survey, the isolates were classified into three categories; 1. animals which were euthanased due to terminal illness (symptomatic-s), 2. animals which responded to treatment but had recurrent clinical signs (responders-r) and 3. animals which had remained free of clinical signs since FIV was diagnosed (asymptomatic-as). Isolates were subjected to a range of tropism studies: 1. AMD blocking assays (n=15), 2. tropism on CrFK cells (n=17), 3. tropism on AH927 cells expressing CXCR4 (n=29). Using these assays it was hoped that the isolates could be grouped according to their physiological properties and to determine whether FIV-GL8 and FIV-PET are representative of these groups or form outlying strains. Due to limited data for the responder group viruses, studies of them were discontinued early in the course of the project.

3.2. Materials and Methods

3.2.1. Cell lines and culture conditions.

Culture conditions for the cell lines Mya-1, CrFK(HO6T1), AH927 FX4E are described in Chapter 2.

3.2.2. Assays

3.2.2.1. Viral titrations

Initially the viruses were titrated using the T-lymphoblastoid cell line Mya-1 as described in Section 2.5.2.

3.2.2.2. AMD3100 Blocking assays

2 x 10^5 cells were incubated in triplicate with 250µl complete RPMI medium containing fivefold dilutions of AMD3100 from 10µg/ml to 16ng/ml. The cells were incubated at 37°C for 60 minutes. 50µl of FIV (100 $TCID_{50}$) was added to each tube, and then incubated for 60 minutes. The cells were then washed twice using RPMI with 5% FBS. The cells were then resuspended in 500µl of complete RPMI 1640 containing IL-2 and incubated in a 48-well plate. On day 7 the p24 production was measured by ELISA (FIV p24 antigen ELISA, IDEXX).

3.2.2.3. Tropism on CrFK(HO6T1) cells

2 x 10^6 Mya-1 cells were incubated overnight with 1ml of each stock in a total volume of 5ml in T25 tissue culture flasks. The following day the cells were washed, resuspended in fresh medium and cultured until syncytium formation was evident, when culture fluids were tested for FIV p24 by ELISA.

When infected Mya cultures were established, CrFK(HO6T1) cells were set up overnight at 2 x 10^5 cells per T25 tissue culture flask (2 flasks per isolate). The following day the CrFK(HO6T1) cells were incubated with 2ml cell-free culture fluid from infected Mya-1 cells or were cocultivated with 2ml of infected Mya-1 cell culture and 3ml of complete DMEM medium. The cultures were incubated for two days before washing and subculturing the CrFK(HO6T1) cells. Following positive p24 ELISA results, cell pellets

were frozen as a source of DNA for amplifying *env*, and the cultures were maintained and tested weekly to determine if persistently infected cell lines could be generated.

3.2.2.4. CXCR4 usage AH927 FX4E assays

The feline fibroblast cell line AH927 is resistant to infection by FIV but when transduced with a retroviral vector expressing CXCR4 (AH927 FX4E) the cells become susceptible to infection with FIV-PET (Willett et al. 1998). Therefore, the AH927 FX4E cell line was used to determine CXCR4 usage by the panel of field isolates.

10^4 AH927 FX4E cells were seeded per well of a 48-well plate 500µl of complete DMEM medium and were incubated overnight in a 37°C humid incubator. The following day, the medium was aspirated gently and 250µl of virus stock was added to each well and incubated for one hour. Each well was then washed twice with phosphate buffered saline (PBS). 500µl of fresh culture fluid was added and the cultures were incubated in a humid incubator for 10 days, sampling 25 µl of culture fluid on days 4, 7 and 10. Culture fluids were stored at -70°C until assayed for RT activity using the Lenti-RT activity assay (Cavidi Tech, Sweden).

3.2.2.5. AH927 FX4E assays

Cell pellets were harvested for DNA preparation 24 hours after viral infection of AH927 FX4E cells in order to determine the usage of CXCR4 for viral entry. Viral DNA was detected by PCR using primers designed for the *gag* region of the FIV genome 5'-GGG ATT AGA CAC TAG GCC ATC TA-3' and 5'-GAC CAG GTT TTC CAC ATT TAT TA-3' to amplify a 871bp fragment. A control cellular DNA fragment was amplified using primers designed for the β-actin gene 5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3' and 5'-CGT CAT CCT GCT TGC TGA TCC ACA TCT GC-3'. Reactions were denatured at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute and extension at 72°C for 1 minute with a final extension of 10 minutes at 72°C. HiFidelity reaction mix (Roche) was used as per manufacturers instructions, on a GeneAmp 9700 thermo-cycler (Perkin Elmer). The PCR product was identified following resolution on a 1% agarose electrophoresis gel containing ethidium bromide.

3.3. Statistical Analysis

Results were analysed as described in Section 2.9. Highly significant results with $p<0.01$ are denoted by (**) and results which are significant, $p<0.05$, denoted by (*).

3.4. Results

3.4.1. A proportion of FIV isolates can infect CrFK cells.

It is known that some isolates may become adapted for growth in the CrFK cell line (Phillips et al. 1990). Therefore, we tested our panel of field isolates from asymptomatic and symptomatic cats to determine whether isolates from either of these categories more readily adapted for growth in CrFK cells. None of 8 isolates from asymptomatic and 2/9 isolates from symptomatic cats were shown to replicate in CrFK(HO6T1) cells following cell-free infection. These proportions were not significantly different (see Figure 3.a. and Table 3.a.). Similarly, although higher proportions of isolates (5/8 asymptomatic and 6/8 symptomatic) replicated in CrFK(HO6T1)s following cocultivation with infected Mya-1 cells, no significant differences were observed. Three asymptomatic isolates ($F0291H_{as}$, $F0513H_{as}$, $F1412H_{as}$) and two symptomatic isolates ($F559H_s$ and $F1128_s$) failed to produce a persistent infection. $F0835H_s$, although consistently positive throughout the study, always gave low positive readings, just above the assay cut-off when measured by p24 FIV antigen ELISA (see Figure 3.b. and Table 3.b.). In conclusion, the CrFK(HO6T1)-tropism demonstrated by our isolates from different stages of disease are not significantly different.

3.4.2. Cell-free infection of the CrFK cell line CrFK(HO6T1) cells was observed with only 2/17 isolates tested.

Productive infection of CRFK(HO6T1) cells was observed following cell-free infection with two isolates from symptomatic cats. $F0795H_s$ produced a positive FIVp24 ELISA reading on day 17 post infection (p.i.) (Table 3.a.) but did not result in a persistent infection. $F0827H_s$ was positive for FIVp24 by ELISA from day 31 onwards (see Figure 3.a., Table 3.a.), suggesting that adaptation had occurred in this isolate. Mya cell infection with the latter isolate was not significantly blocked by 400ng/ml AMD3100 (see Table 3.c.), indicating that perhaps $F0827H_s$ bound to the coreceptor with a higher affinity compared to $F0795H_s$.

Table 3.a. Cell-free infection of HO6T1 cells.

Isolate	7 days p.i.	14 days p.i.	17 days p.i.	21 days p.i.	24 days p.i.	28 days p.i.	31 days p.i.	35 days p.i.
Recurrent clinical signs								
F0135H_r	0.24	0.13	nd	0.1	nd	0.23	nd	0.23
Asymptomatic isolates								
F0291H_{as}	0.2	0.14	nd	0.09	nd	0.21	nd	0.21
F0359H_{as}	0.3	0.13	nd	0.1	nd	0.23	nd	0.23
F0418H_{as}	0.16	0.15	nd	0.28	nd	0.20	nd	0.18
F0425H_{as}	0.19	0.14	nd	0.28	nd	0.20	nd	0.19
F0513H_{as}	0.14	0.18	nd	0.27	nd	0.23	nd	0.23
F0556H_{as}	0.17	0.14	nd	0.32	nd	0.21	nd	0.18
F1412H_{as}	0.29	0.13	nd	0.08	nd	0.25	nd	0.20
Symptomatic isolates								
F0559H_s	0.18	0.1	nd	0.08	nd	0.25	nd	0.25
F0795H_s	1.21	0.15	1.04	0.13	0.1	0.11	0.13	0.22
F0827H_s	0.78	0.14	0.13	0.23	0.21	0.22	1.46	2.59
F0835H_s	0.11	0.05	0.1	0.1	0.07	0.1	0.11	0.21
F0894H_s	0.86	0.12	0.16	0.17	0.09	0.13	0.12	0.22
F0973H_s	0.05	0.05	0.14	0.11	0.06	0.15	0.2	0.18
F1029H_s	0.23	0.12	nd	0.11	nd	0.24	nd	0.24
F1115H_s	0.19	0.11	nd	0.1	nd	0.27	nd	0.27
F1128H_s	0.18	0.09	nd	0.18	nd	0.25	nd	0.25

FIV p24 read at optical density A650nm.

nd = not done.

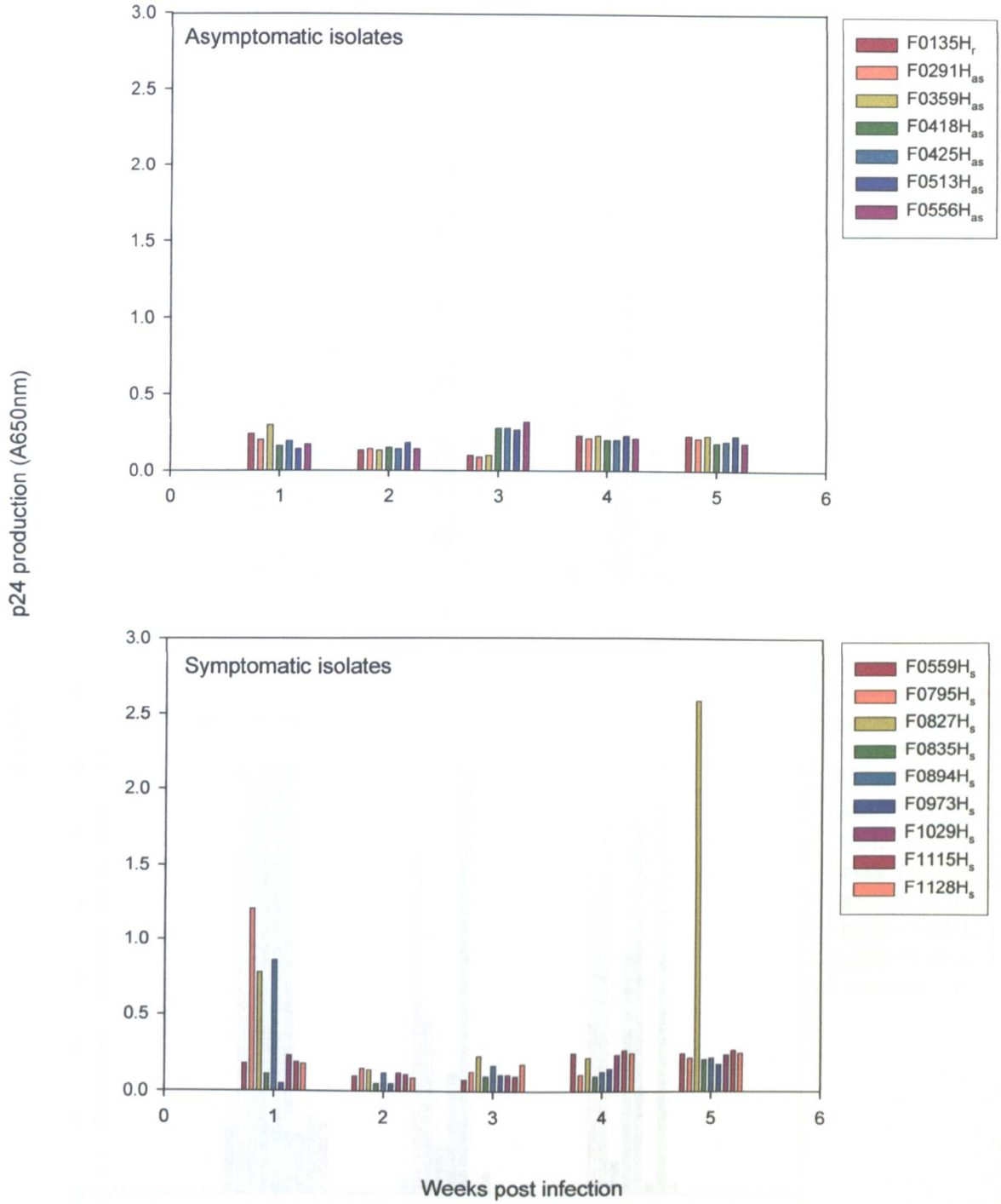
Table 3.b. Cocultivation of HO6T1 cells with infected Mya-1 cells.

Isolate	7 days p.i.	14 days p.i.	17 days p.i.	21 days p.i.	24 days p.i.	28 days p.i.	31 days p.i.	35 days p.i.
Recurrent clinical signs								
F0135H_r	1.49	0.65	nd	4.68	nd	6	nd	4.54
Asymptomatic isolates								
F0291H_{as}	2.2	0.17	nd	0.1	nd	0.18	nd	0.24
F0359H_{as}	2.05	0.3	nd	1.36	nd	5.59	nd	4.98
F0418H_{as}	4.07	6	nd	5.58	nd	nd	nd	0.24
F0425H_{as}	1.85	6	nd	5.58	nd	nd	nd	nd
F0513H_{as}	3.1	0.31	nd	0.37	nd	nd	nd	nd
F0556H_{as}	1.2	4.09	nd	6	nd	nd	nd	nd
F1412H_{as}	1.59	0.2	nd	0.24	nd	0.17	nd	nd
Symptomatic isolates								
F0559H_s	0.94	0.2	nd	0.1	nd	0.25	nd	0.25
F0795H_s	6	4.79	nd	4.64	nd	nd	nd	nd
F0827H_s	6	2.43	nd	6	nd	nd	nd	nd
F0835H_s	6	0.68	nd	0.46	nd	nd	nd	nd
F0894H_s	6	1.71	nd	3.45	nd	nd	nd	nd
F0973H_s	6	6	nd	5.24	nd	nd	nd	nd
F1029H_s	0.83	0.12	nd	0.18	nd	0.16	nd	0.23
F1115H_s	1.13	0.3	nd	6	nd	6	nd	6
F1128H_s	0.98	0.14	nd	0.13	nd	0.18	nd	0.71

FIV p24 read at optical density A650nm.

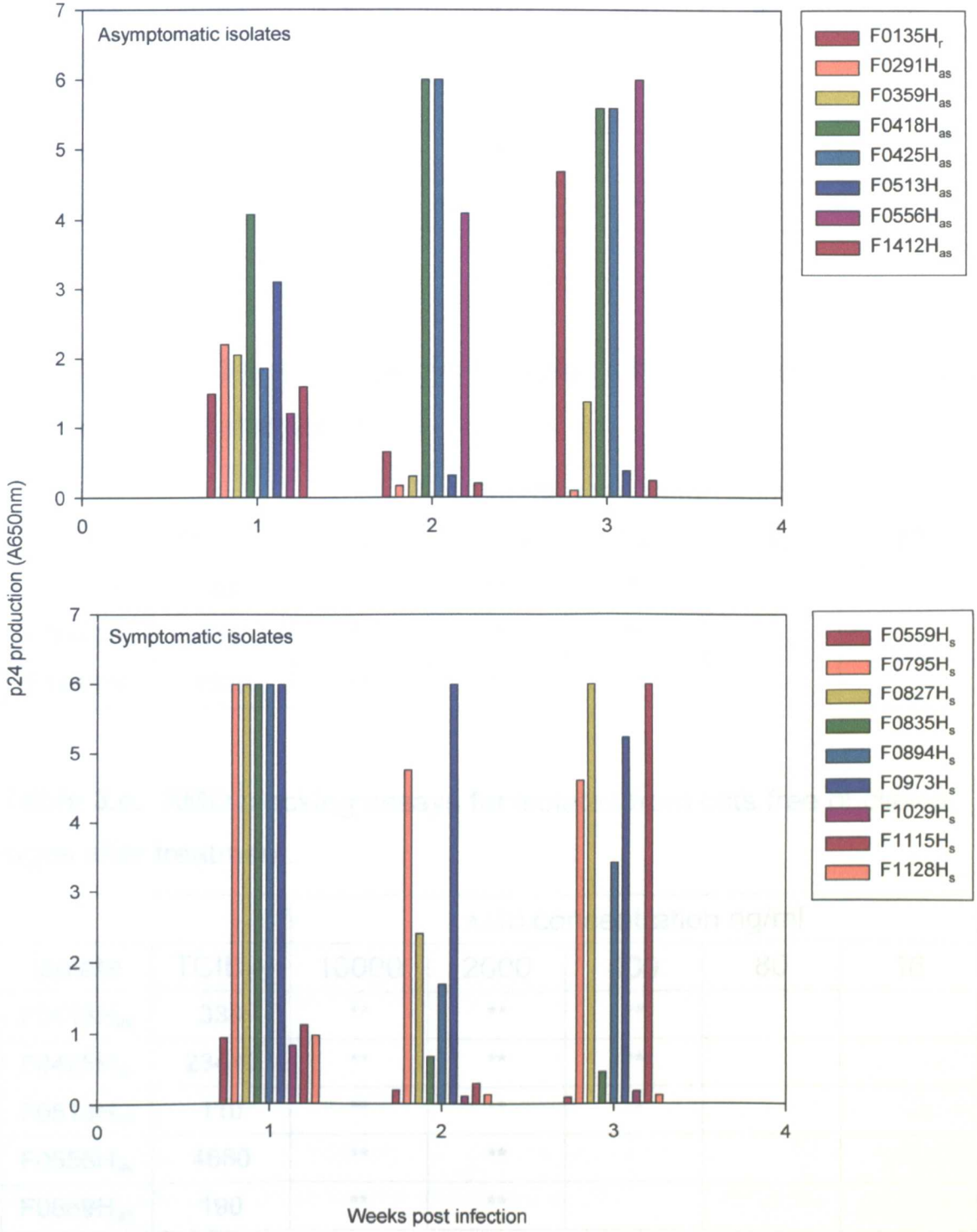
nd = not done.

Figure 3.a. Cell-free infection of CrFK(HO6T1) cells.



Cell-free viral supernatant was incubated with CrFK(HO6T1) cells for 12 hours. The cultures were washed and incubated with fresh medium at 37°C in a CO_2 incubator, subculturing twice weekly. p24 production was measured by FIV p24 antigen ELISA (Idexx). None of the asymptomatic, but 2 of the symptomatic isolates (F0795H$_s$ (not shown) and F0827H$_s$) resulted in productive infection.

Figure 3.b. Cocultivation of CrFK(HO6T1) cells with infected Mya-1 cells.



Infected Mya-1 cells were cocultivated with CrFK(HO6T1) cells for 12 hours. The cultures were washed and incubated with fresh medium at 37°C in a CO_2 incubator, subculturing twice weekly. p24 production was measured by FIV p24 antigen ELISA (Idexx). No significant difference between groups was noted with respect to the ability to infect CrFK(HO6T1)s by cocultivation.

Table 3.c. AMD blocking assays for isolates from cats in terminal stage of disease.

		AMD concentration ng/ml				
Isolate	$TCID_{50}$	10000	2000	400	80	16
$F0795H_s$	4680	**	**	**		
$F0827H_s$	8130	**	**			
$F0835H_s$	40	**	**	**		
$F0894H_s$	4790	**	**	**		
$F0973H_s$	70	**	**	*		

Table 3.d. AMD blocking assays for isolates from cats with recurrence of clinical signs after treatment.

		AMD concentration ng/ml				
Isolate	$TCID_{50}$	10000	2000	400	80	16
$F0135H_r$	40	**	**	*		
$F0667H_r$	110	**	**	**		
$F1844H_r$	1620	**	**	**		

Table 3.e. AMD blocking assays for isolates from cats free of clinical signs after treatment.

		AMD concentration ng/ml				
Isolate	$TCID_{50}$	10000	2000	400	80	16
$F0418H_{as}$	330	**	**	**		
$F0425H_{as}$	23440	**	**	**		
$F0513H_{as}$	110	**	**	**		
$F0556H_{as}$	4680	**	**			
$F0669H_{as}$	190	**	**			

Mya-1 cells were infected with field isolate viral supernatants and incubated in the presence of AMD3100. The asterisks demonstrate the concentration of AMD3100, which resulted in significant blocking of Mya-1 cell infection by AMD3100. ** = $p<0.01$, * = $p<0.05$.

3.4.3. Increased use of CXCR4 by symptomatic isolates.

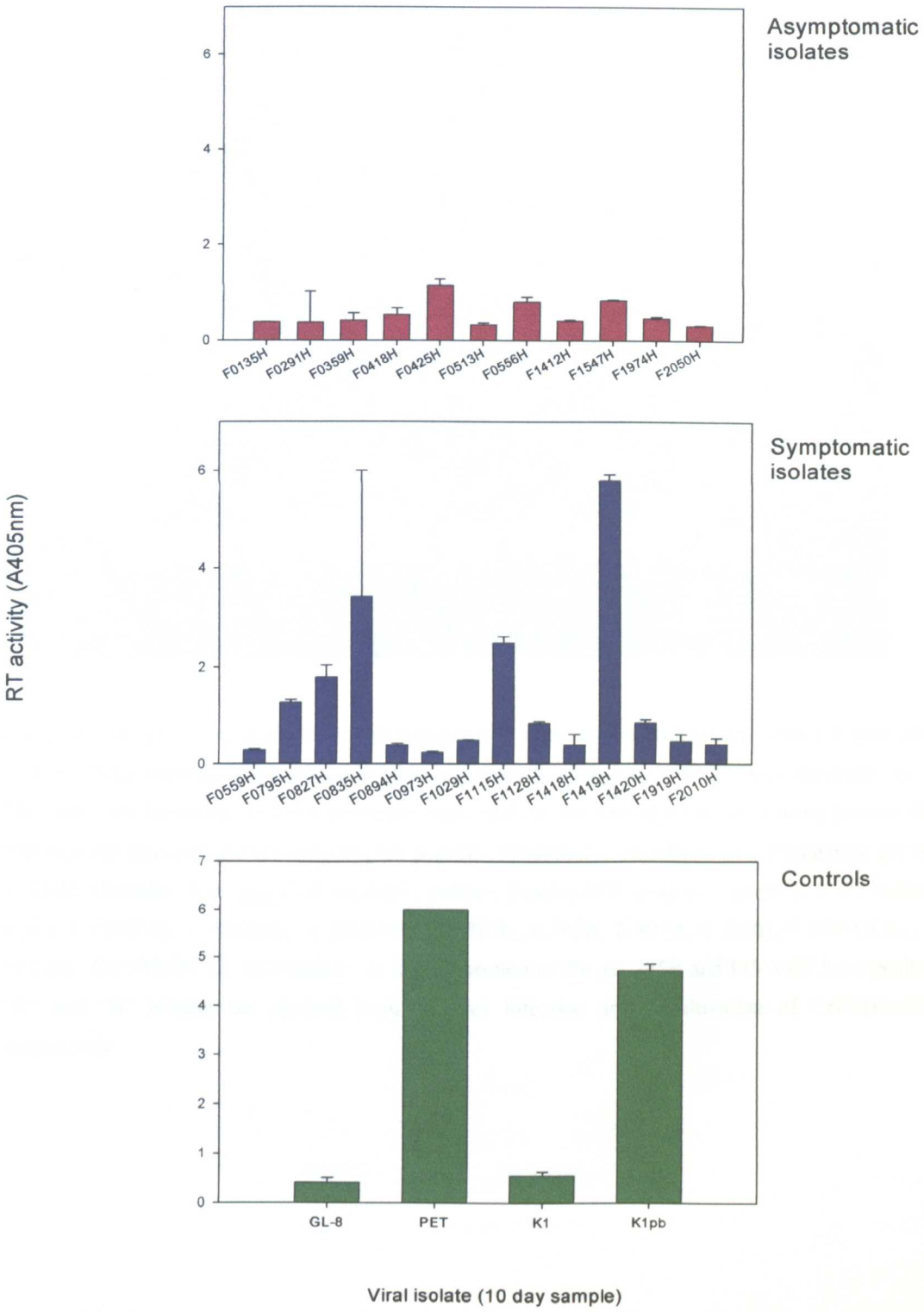
Since infection of CrFK cells is CXCR4-dependent, the ability of our isolates to utilise CXCR4 was tested. AH927 cells, which are not naturally susceptible to FIV, were transduced with a retroviral vector expressing feline CXCR4 to generate the cell line AH927 FX4E, which is susceptible to infection with laboratory-adapted isolates of FIV (Willett et al. 1998). The results of a screening assay illustrate the varying degrees to which the field isolates can utilise the chemokine receptor CXCR4 (Figure 3.c.). The field isolates were compared to the primary isolates FIV-GL8 and FIV-K1(19K1), which are not CXCR4-tropic along with the laboratory-adapted FIV-PET and chimaeric clone FIV-K1pb(19K1pbam6c) (a K1 backbone with a CrFK-tropic *env* of FIV-AM6c) which are known to be CXCR4-tropic (Phillips et al. 1990; Siebelink et al. 1995b). A trend was evident, with a higher proportion (9/14) of the symptomatic isolates utilising CXCR4 and inducing higher levels of RT activity than the asymptomatic isolates (5/11). The primary isolate FIV-GL8 and the majority of the isolates from asymptomatic cats did not establish infection. Of the symptomatic isolates that were able to infect AH927 FX4E cells, only F0795H_s and F0827H_s were able to infect CrFK cells by cell-free infection, confirming that cell-free infection of CrFK cells is mediated by the chemokine receptor CXCR4.

Semi-quantitative AH927 FX4E entry assays using PCR to detect viral Gag proteins confirmed the efficiency with which FIV-PET uses CXCR4 (see Figure 3.d.). The progeny virus F0795H_s which was harvested following cocultivation of infected Mya-1 cells with CrFK(HO6T1) cells, also efficiently used CXCR4. Faint product bands were evident in lanes 2, 3, 4, 5, 7, 8, and 9. However, these may have been attributable to either virus particles remaining adherent to the cells after washing or to very small amounts of virus that successfully infected these cells.

3.4.4. Inhibition of infection by AMD 3100 varied between the isolates

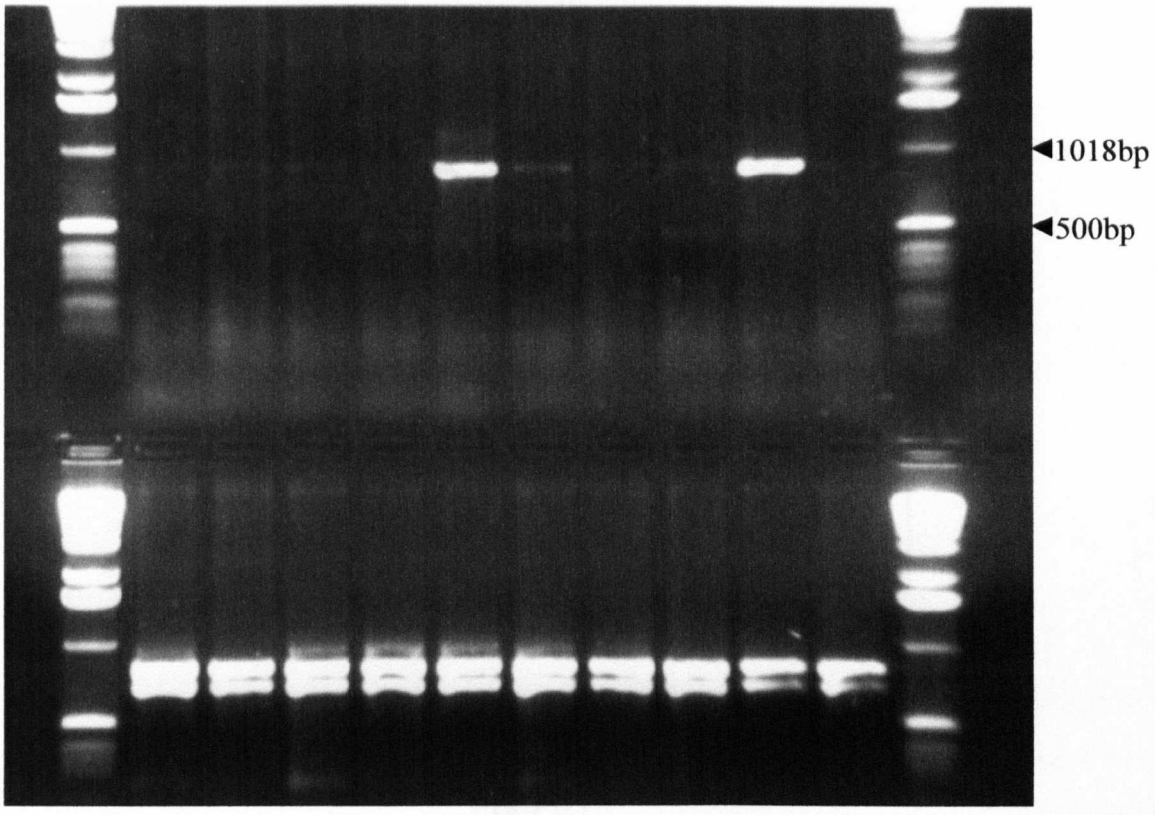
The bicyclam AMD3100, a CXCR4 antagonist, has been used to demonstrate CXCR4 usage by different isolates (Egberink et al. 1999; Richardson et al. 1999). AMD3100 blocking assays demonstrate marked differences in CXCR4 affinity between the isolates FIV-GL8 and FIV-PET (see Figure 3.e.). FIV-PET infection of Mya-1 cells was only inhibited significantly ($p \leq 0.01$) by very high concentrations of the selective CXCR4 antagonist AMD3100 (10μg/ml). In contrast, infection of Mya-1 cells by FIV-GL8 was

Figure 3.c. Cell-free infection of AH927 FX4E cells.



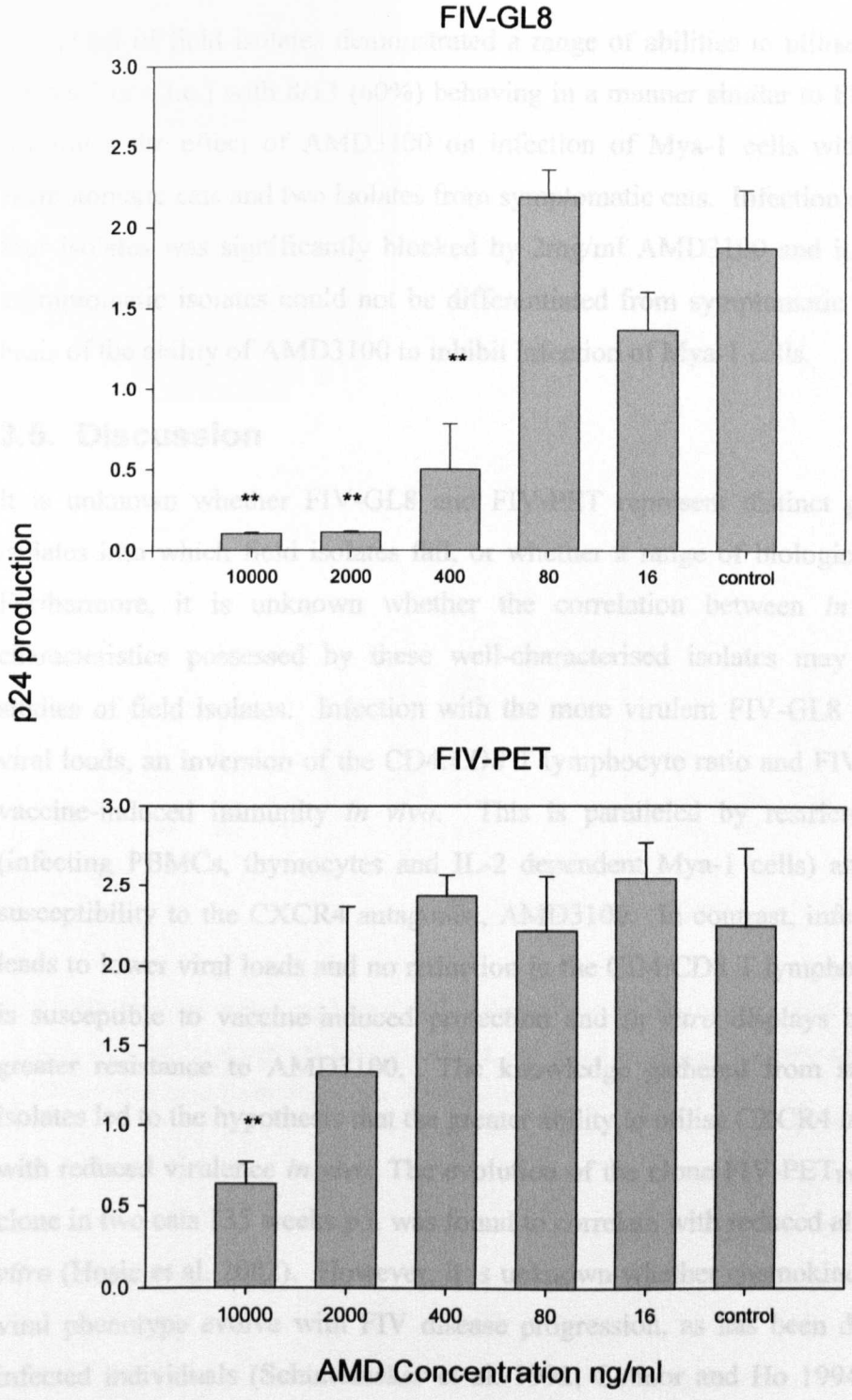
250µl of virus stock was incubated with AH927FX4E for 1 hour at 37°C. The cells were then washed and incubated at 37°C before testing for viral replication by RT activity assay.

Figure 3.d. AH927 FX4E entry assay.



AH927 FX4E cells were incubated for 60 minutes with virus supernatant or mock infected with DMEM as a control. After washing twice with PBS, the cells were incubated at 37°C in a CO_2 incubator for 24 hours. The cells were harvested for DNA preparation and analysis for viral DNA by PCR using primers for the *gag* region of the provirus and the cellular gene β-actin. Product was visualised on a 1% agarose gel containing ethidium bromide. Top: gag PCR products, bottom: β-actin PCR product. Lanes: 1. 1 Kb ladder (Gibco, U.K.), 2. F0425H$_{ac}$, 3. F0556H$_{ac}$, 4. F0795H, 5. F0827H$_{ac}$, 6.795B, 7. 827A, 8. 827B, 9. FIV-GL8$_{414}$, 10. FIV-PET,11. CONTROL 12. 1Kb ladder. In the top section of the gel 795B and FIV-PET have positive bands. "A" and "B" isolates are derived from cell-free infection and cocultivation of CrFK(HO6T1) cells, respectively.

Figure 3.e. AMD blocking assays for prototype viruses.



AMD3100 blocking assays for both the prototype viruses to demonstrate the affinity for CXCR4 possessed by FIV-GL8 (top) and FIV-PET (bottom).

** highlight the AMD3100 concentration when significant block to infection of Mya-1 cells was produced.

inhibited significantly ($p \leq 0.01$) by very low concentrations of AMD3100 (400ng/ml), consistent with the latter isolate having a lower affinity for CXCR4.

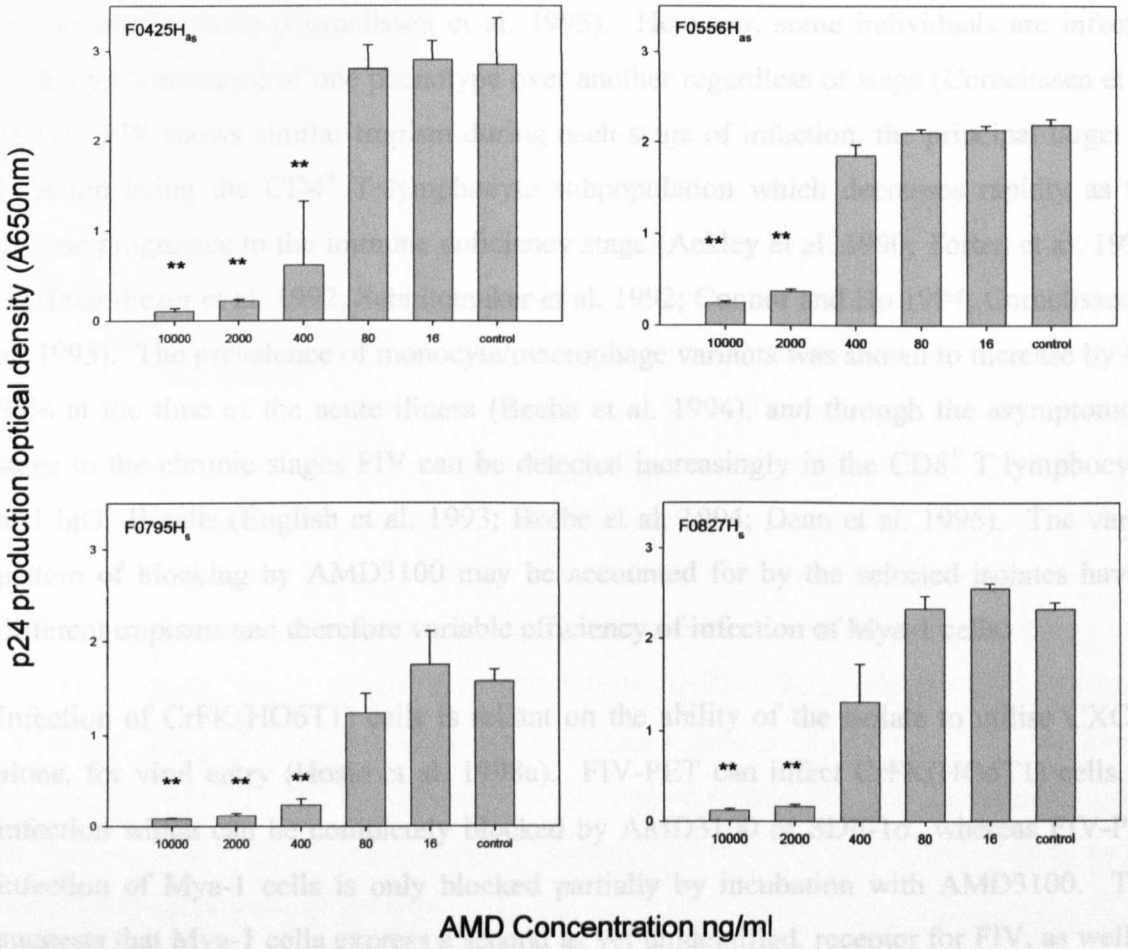
The panel of field isolates demonstrated a range of abilities to utilise CXCR4 alone (see Tables 3.c. - 3.e.) with 8/13 (60%) behaving in a manner similar to FIV-GL8. Figure 3.f. illustrates the effect of AMD3100 on infection of Mya-1 cells with two isolates from asymptomatic cats and two isolates from symptomatic cats. Infection of Mya-1 cells by all four isolates was significantly blocked by 2mg/ml AMD3100 and it was concluded that asymptomatic isolates could not be differentiated from symptomatic isolates only on the basis of the ability of AMD3100 to inhibit infection of Mya-1 cells.

3.5. Discussion

It is unknown whether FIV-GL8 and FIV-PET represent distinct groups of FIV viral isolates into which field isolates fall, or whether a range of biological phenotypes exist. Furthermore, it is unknown whether the correlation between *in vitro* and *in vivo* characteristics possessed by these well-characterised isolates may be extrapolated to studies of field isolates. Infection with the more virulent FIV-GL8 isolate induces high viral loads, an inversion of the CD4:CD8 T lymphocyte ratio and FIV-GL8 is resistant to vaccine-induced immunity *in vivo*. This is paralleled by restricted tropism *in vitro* (infecting PBMCs, thymocytes and IL-2 dependent Mya-1 cells) and a high degree of susceptibility to the CXCR4 antagonist, AMD3100. In contrast, infection with FIV-PET leads to lower viral loads and no reduction in the CD4:CD8 T lymphocyte ratio; FIV-PET is susceptible to vaccine-induced protection and *in vitro* displays broader tropism and greater resistance to AMD3100. The knowledge gathered from studies of these two isolates led to the hypothesis that the greater ability to utilise CXCR4 *in vitro* may correlate with reduced virulence *in vivo*. The evolution of the clone FIV-PET_{F14} to a more virulent clone in two cats 135 weeks p.i. was found to correlate with reduced affinity for CXCR4 *in vitro* (Hosie et al. 2002). However, it is unknown whether chemokine receptor usage and viral phenotype evolve with FIV disease progression, as has been documented in HIV-infected individuals (Schuitemaker et al. 1992; Connor and Ho 1994; Cornelissen et al. 1995).

AMD blocking assays conducted on the panel of field isolates revealed a varied pattern of CXCR4 affinity. This variation may reflect different tropisms between isolates because of mixed virus populations. It is known that the viral phenotype within HIV infected

Figure 3.f. AMD blocking assays for field isolates.



AMD3100 blocking assays on Mya-1 cells demonstrate the affinity for CXCR4 possessed by the field isolates. No significant patterns were associated with stage of disease. Concentrations of AMD3100 where significant blocking of infection occurred are shown with ** ($p<0.01$).

individuals is dynamic throughout the course of infection. Lymphotropic virus predominates early in infection before seroconversion and during the decline to the AIDS-phase but monocytotropic forms predominate from the acute stage and through the asymptomatic phase (Cornelissen et al. 1995). However, some individuals are infected with a predominance of one phenotype over another regardless of stage (Cornelissen et al. 1995). FIV shows similar tropism during each stage of infection, the principal target on infection being the $CD4^+$ T lymphocyte subpopulation which decreases rapidly as the disease progresses to the immune deficiency stage (Ackley et al. 1990; Torten et al. 1991; Hoffman-Fezer et al. 1992; Schuitemaker et al. 1992; Connor and Ho 1994; Cornelissen et al. 1995). The prevalence of monocyte/macrophage variants was shown to increase by 40-95% at the time of the acute illness (Beebe et al. 1994), and through the asymptomatic stage to the chronic stages FIV can be detected increasingly in the $CD8^+$ T lymphocytes and IgG^+ B cells (English et al. 1993; Beebe et al. 1994; Dean et al. 1996). The varied pattern of blocking by AMD3100 may be accounted for by the selected isolates having different tropisms and therefore variable efficiency of infection of Mya-1 cells.

Infection of CrFK(HO6T1) cells is reliant on the ability of the isolate to utilise CXCR4 alone, for viral entry (Hosie et al. 1998a). FIV-PET can infect CrFK(HO6T1) cells, an infection which can be completely blocked by AMD3100 or SDF-1α, whereas FIV-PET infection of Mya-1 cells is only blocked partially by incubation with AMD3100. This suggests that Mya-1 cells express a second as yet unidentified, receptor for FIV, as well as CXCR4 (Hosie et al. 1998b; de Parseval and Elder 2001). Laboratory adaptation can result in virus that has acquired the ability to utilise CXCR4 alone for infection. The laboratory-adapted isolate FIV-PET was found to contain a glutamate to lysine mutation in the third variable region of the *env* gene when infecting CrFK(HO6T1) cells. Laboratory-adaptation was found to occur in two other isolates FIV-UT113 and FIV-PBAM6c, which were able to infect CrFKs following a glutamate to lysine mutation at amino acid position 407 or 409 respectively (Verschoor et al. 1995; Siebelink et al. 1995b). Hence, tropism on CrFK(HO6T1) cells further defines the ability of an isolate to use CXCR4. Of great interest in this study were the positive FIVp24 readings recorded for isolate $F0795H_s$ on day 17 (see Table 3.a.) and isolate $F0827H_s$ which produced a persistent infection from day 31 onwards (see Table 3.a.). This suggests that these isolates may have acquired the ability to use CXCR4 during a period of laboratory adaptation, similar to that documented for FIV-PET (Phillips et al. 1990).

Using the AH927 FX4E infection assays, a panel of field isolates was screened for the ability to utilise CXCR4. A greater proportion of the symptomatic isolates demonstrated the ability to utilise CXCR4. Undiluted virus stock was used as only the ability to infect these cells was investigated. It would be interesting to investigate the role of virus concentration on efficiency of infection. $F0795H_s$ and $F0827H_s$ (both isolated from terminally ill cats) were able to use CXCR4 to infect AH927 FX4E cells and infected CrFK(HO6T1) cells without cocultivation with infected Mya-1 cells, at days 17 and 31 p.i. suggesting the existence of virus within each of the virus populations with the fitness to infect CrFK cells leading to expanded host cell tropism. In contrast to the CrFK(HO6T1) and AH927 FX4E-tropism studies, only $F0795H_s$ (cocultivated) was able to enter AH927 FX4E cells in 24 hours and replicate similarly to FIV-PET, suggesting that this virus had adapted to use CXCR4 efficiently alone. Virus from cell free infection of CrFK(HO6T1) cells by $F0795H_s$ was not available. Faint bands of PCR product were detected in other samples but it is not known if these were due to only very small amounts of virus penetrating the cells or if residual virus remained adherent to the cell membrane after washing. These results suggest that infection of AH927 FX4E cells by field isolates is inefficient compared to laboratory-adapted strains such as FIV-PET. The successful infection of AH927 cells by several isolates, both asymptomatic and symptomatic, may have arisen due to mixed populations of phenotypes occurring within each isolate, as has been described previously for HIV.

The field isolates displayed a broad range of biological behaviour *in vitro*. Isolates $F0795H_s$ and $F0827H_s$ infected CrFK(HO6T1)s without cocultivation with infected Mya-1 cells and also infected AH927 FX4E cells using CXCR4 alone. Interestingly, both of these isolates originated from cats with AIDS-like stage disease, suggesting that the CXCR4 phenotype may predominate in FIV infection as disease progresses to feline AIDS.

In these studies viruses utilising CXCR4 alone for viral entry (i.e. FIV-PET like) were isolated predominantly from terminally ill cats, whereas isolates from asymptomatic cats had a lesser ability to utilise CXCR4 alone (i.e. FIV-GL8 like). Viruses in the field are most likely to be transmitted from asymptomatic cats, which are still able to behave and interact with other cats normally. Therefore, the virus phenotype in the field with the highest transmission rate is likely to be that of the FIV-GL8 type. This has implications for future vaccines studies, as FIV-GL8 remains resistant to vaccine-induced immunity.

Chapter Four

STUDIES ON THE PATHOGENESIS OF FIELD ISOLATES OF FIV

4.1. Introduction

The experiments described in Chapter 3 indicated that viruses isolated from cats in the terminal stages of infection may be more likely to infect cells via CXCR4 alone than isolates from cats in the asymptomatic phase. During HIV infection, a similar pattern has been observed in a proportion of patients, in whom CXCR4 and T lymphocyte-tropic, SI viruses appear at the time when AIDS develops (Tersmette et al. 1989a; Schuitemaker et al. 1992; Koot et al. 1993; Connor et al. 1997). SI viruses infect T cell lines and induce syncytia in MT-2 cells (Åsjo et al. 1986; Cheng-Mayer et al. 1988; Tersmette et al. 1989a; Tersmette et al. 1989b; Koot et al. 1992). From these observations it has been suggested that SI, CXCR4-tropic viruses are more pathogenic than the CCR5-tropic viruses that predominate in the early stages of HIV infection (Schuitemaker et al. 1992; Zhu et al. 1993; Connor et al. 1993b), and may cause the rapid progression of disease in the patients from whom they were isolated. However, an alternative reason for the appearance of the CXCR4-tropic viruses is that they are a consequence of the developing immunodeficiency rather than its cause. Clearly it is not possible to distinguish between these alternatives by direct experimentation in man.

Having established that CrFK-tropic FIV isolates were present in cats in the terminal stages of infection, it was possible to test whether these viruses were more pathogenic for cats than isolates from asymptomatic cats that were not solely CXCR4-tropic. The results of these experiments might help to resolve the issue of the relevance of the CXCR4-tropic viruses in the development of both human and feline AIDS.

In this chapter, experiments are described in which cats were inoculated with four representative FIV isolates from either terminally ill cats or cats with no clinical signs in order to compare directly their pathogenicity. These were named as asymptomatic isolates or symptomatic isolates in common with human terminology. In addition, for comparison, control cats were inoculated with matched doses of the well-characterised FIV-GL8_{414}. The extent of virus replication of each isolate was determined by the measurement of viral

burden by three methods: proviral DNA in PBMCs, infectious virus released from PBMC and plasma viral RNA. At the end of the experiment, at post-mortem examination 15 weeks after infection, the proportions of cells with proviral DNA in lymph nodes, bone marrow and thymus were also determined.

4.2. Materials and Methods

4.2.1. Inoculation of cats

Ten 13 week old specific pathogen free kittens were assigned at random to five groups of two kittens. Four groups were inoculated with the field isolates and one group received the molecular clone FIV-GL8$_{414}$. Isolates F0425H$_{as}$ and F0556H$_{as}$ were from asymptomatic cats and F0795H$_{s}$ and F0827H$_{s}$ were from terminally ill (symptomatic) cats (see Table 4.a.). Each cat was inoculated with 250 tissue culture infectious doses (TCID$_{50}$) by the intra-muscular (i.m.) route (as determined by titration on Myas – see Section 2.5.2.).

4.2.2. Collection of samples

4.2.2.1. Blood samples

Blood samples were collected into EDTA on the day of inoculation and then at three-week intervals until 15 weeks post inoculation when post-mortem examinations were conducted. Routine haematological examinations were carried out on each sample. The plasma was removed and PBMCs were isolated following Ficoll-Hypaque centrifugation as described in Section 2.2. 10^6 PBMCs were washed twice in PBS, pelleted, snap-frozen in dry ice and stored at -70°C until required for proviral load analysis. The remaining PBMCs were stored frozen in liquid nitrogen for QVI.

4.2.2.2. Tissue samples

Mesenteric and peripheral lymph nodes (retropharyngeal, submandibular, axillary and popliteal) and thymus were collected post-mortem as described in Section 2.3. Pellets of 10^6 cells were washed twice in PBS and snap-frozen in dry ice and stored at -70°C until further analysed by real time PCR.

Table 4.a. Cat numbers and viral isolate received.

Cat number	Virus inoculum
A701 A702	$F0425H_{as}$ $F0425H_{as}$
A703 A704	$F0556H_{as}$ $F0556H_{as}$
A705 A706	$F0795H_{s}$ $F0795H_{s}$
A707 A708	$F0827H_{s}$ $F0827H_{s}$
A709 A710	$GL8_{414}$ $GL8_{414}$

as = asymptomatic isolate, s = symptomatic isolate.

4.2.2.3. Collection of bone marrow cells

A femur from each animal was collected post-mortem into a 50ml Falcon tube (Becton and Dickinson, France) containing RPMI supplemented with antibiotics (see Chapter 2). The ends of each femur were removed and the marrow pulp was removed by washing through with medium. The pulp was then washed twice with RPMI and pellets of approximately 10^6 cells were washed twice in PBS and snap-frozen in dry ice and stored at -70°C until required.

4.2.3. Real time RT-PCR and PCR

4.2.3.1. Detection of mismatches in the primer and probe sites

A region of the *gag* gene of each field isolate was amplified by polymerase chain reaction (PCR) using primers GAG660f (5'-GGC CAT TAA GAG ATG (CT)AG TAA-3') and GAG1837r (5'-GAC CAG GTT TTC CAC ATT TAT TA-3') and sequenced to determine the sequences in the Taqman primer and probe binding sites. The reaction was performed on a Perkin Elmer GeneAmp PCR system 9700 PCR machine. After the initial denaturation of 94°C for 3 minutes, amplification was achieved by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 60 seconds, elongation at 72°C for 60 seconds and a final elongation stage at 72°C for 10 minutes. The PCR product was checked on a 1% agarose gel before cloning into pCR®BluntII-TOPO® vector (Invitrogen, U.K.) following the manufacturer's instructions and sequenced on an ABI capillary sequencer with Big Dye Terminator 2, using the methods described by Sanger and Rosenblum (Sanger et al. 1977; Rosenblum et al. 1997) and primers M13f (5'-GTC GTG ACT GGG AAA AC-3') and M13r (5'-GTC CTT TGT CGA TAC TG-3'). Analysis of results was carried out using the Wisconsin GCG sequence analysis package. Sequence data were analysed using Seqed and BESTFIT (Smith and Waterman 1981) and sequences were compared using BLAST (Lipman and Pearson 1988) (National Centre for Biotechnology Information).

4.2.3.2. Measurement of proviral load

The proviral load of PBMCs was measured by real-time PCR. The primers used were FIV0771f (5'- AGA ACC TGG TGA TAT ACC AGA GAC-3') and FIV1081r (5'-TTG GGT CAA GTG CTA CAT ATT G-3'). The probe used with this combination was

FIV1010p (5'-FAM-TAT GCC TGT GGA GGG CCT TCC T-TAMRA-3'). The accumulation of PCR product was measured through the dual labelled Taqman probe (Heid et al. 1996). Primers FIV0771f and FIV1010p have been shown previously to detect several subtype-A viruses, namely FIV-PET, FIV-GL8 and FIV-AM6 (Klein et al. 1999). The reaction mixture contained 10mM Tris (pH 8.3), 50mM KCl, 3mM $MgCl_2$, 200 nM dATP, dCTP, dGTP, and 400 nM dUTP, 300nM of each primer, 200nM of the fluorogenic probe and 2.5 units of Taq DNA polymerase. The PCR cycle employed an initial denaturation step of 95°C for 2 minutes followed by amplification for 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The emission of fluorescence from the probe was measured on-line on the sequence detector system ABI 7700 (Perkin Elmer, Foster City, California). Standards prepared from FIV-GL8 in fourfold dilutions (copy numbers ranged from 9.5 to 155,833 copy numbers/5µl) were used in each run.

The DNA content per PCR reaction was assessed by amplifying 18S rDNA genes for each reaction using primers rDNA343f (5'-CCA TCG AAC GTC TGC CCT A-3'), rDNA409r (5'-TCA CCC GTG GTC ACC ATG-3') and probe rDNA370p (5'-FAM-CGA TGG TGG TCG CCG TGC CTA-TAMRA-3') (Klein et al. 2000). Each reaction was carried out in duplicate.

4.2.3.3. Measurement of viral RNA load

The viral load in plasma was quantified by real-time reverse transcriptase PCR (RT-PCR) using the same primers as described in proviral load section and then repeated with the 1416p system (1360f 5'-GCA GAA GCA AGA TTT GCA CCA-3', 1416p 5'FAM-TGC AGT GTA GAG CAT GGT ATC TTG AGG CA-TAMRA-3', 1437r 5'-AGG AAA ATT GGC CGC CAT A-3'). The 25µl samples contained 12.5µl of 2 x Thermoscript Reaction Mix (Platinum quantitative RT-PCRKit; Life Technologies, Karlsruhe, Germany), a 300nM concentration of each primer, a 200nM concentration of the fluorogenic probe, 0.5µl of the Thermoscript Plus/Platinum *Taq* Enzyme mix, 20U of RNaseOUT (Life Technologies), and 5µl of the sample. A reverse transcription step of 30 minutes at 60°C was followed by a denaturation step, 5 minutes at 95°C and 60 seconds at 60°C for 45 cycles.

4.3. Results

4.3.1. Proviral loads

4.3.1.1. Investigation of primer mismatches

Our aim was to compare the viral and proviral loads in cats infected with the field isolates using the primers FIV0771f and FIV1081r that were designed to be 100% homologous to the FIV subtype A isolates FIV PET (Genebank accession number M25381), FIV PPR (M36968), FIV ZURICH 1 (X57002), FIV UTRECHT 1 (X68019). Firstly, the sequences of the *gag* gene of each field isolate were determined so that any mismatches of sequence in the regions of the primers and the probe binding sites could be identified. Only F0795H_s had a mismatch in the forward primer at position 13. In the probe region only one isolate (F0556H_{as}) had a mismatch at position 21. In the reverse primer region isolates F0556H_{as} and F0795H_s had mismatches at positions 3 and 14 respectively (see Figure 4.a.).

Within the 1416p system numerous primer mismatches were found in the field isolate sequences. Isolate F0827H_s has two mismatches in the forward and reverse primers at three and seven and seven and thirteen respectively. F0795H_s has one mismatch in the forward primer at nucleotide seven and one in the reverse primer at nucleotide seven. Lastly, isolates F0425H_{as} has one mismatch in the forward primer at nucleotide one and two mismatches in the reverse primer at nucleotides seven and ten and a further mismatch in the probe at nucleotide fourteen (see Figure 4.a.). The 1010p system was thought the more reliable due to the smaller number of mismatches present within the field isolate sequences.

4.3.1.2. PBMC proviral loads

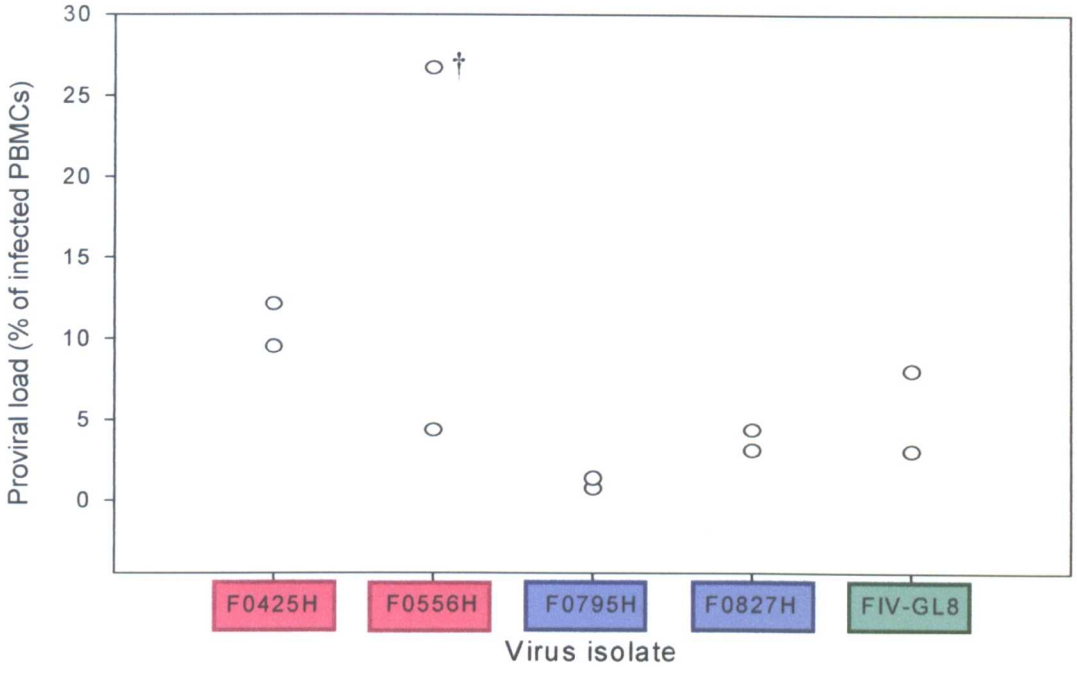
When the proviral loads were compared, it was evident that there was a trend for higher proviral loads to be recorded in the recipients of the asymptomatic isolate, with peak proviral loads ranging from 5.2% (A704) to 26.7% (A703) of cells infected (Figure 4.b.). In contrast, cats receiving symptomatic isolates recorded peak proviral loads of only 0.78% (A705) to 4.3% (A707). Proviral loads continued to increase until the end of the study at 15 weeks p.i. in 3/4 of asymptomatic recipients whereas the proviral load of cat A703 peaked at 9 weeks p.i. (see Figure 4.c.). The proviral loads of two cats inoculated with

Figure 4.a. Primer mismatches.

Primer	Sequence
FIV0771f	
FIV-GL8	5'-AGA ACC TGG TGA TAT ACC AGA GAC-3'
425	5'-AGA ACC TGG TGA TAT ACC AGA GAC-3'
556	5'-AGA ACC TGG TGA TAT ACC AGA GAC-3'
795	5'-AGA ACC TGG TGA AAT ACC AGA GAC-3'
827	5'-AGA ACC TGG TGA TAT ACC AGA GAC-3'
FIV1081r	
FIV-GL8	5'-TTG GGT CAA GTG CTA CAT ATT G-3'
425	5'-TTG GGT CAA GTG CTA CAT ATT G-3'
556	5'-TTG GGT CAA GTG CTA CAT ACT G-3'
795	5'-TTG GGT CTA GTG CTA CAT ATT G-3'
827	5'-TTG GGT CAA GTG CTA CAT ATT G-3'
FIV1010p	
FIV-GL8	5'-FAM-TAT GCC TGT GGA GGG CCT TCC T-TAMRA-3'
425	5'-FAM-TAT GCC TGT GGA GGG CCT TCC T-TAMRA-3'
556	5'-FAM-TAT GCC TGT GGA GGG CCT TCT T-TAMRA-3'
795	5'-FAM-TAT GCC TGT GGA GGG CCT TCC T-TAMRA-3'
827	5'-FAM-TAT GCC TGT GGA GGG CCT TCC T-TAMRA-3'
FIV1360f	
FIV-GL8	5'-GCA GAA GCA AGA TTT GCA CCA-3'
F0425H	5'-TCA GAA GCA AGA TTT GCA CCA-3'
F0556H	5'-GCA GAA GCA AGA TTT GCA CCA-3'
F0795H	5'-GCA GAA CCA AGA TTT GCA CCA-3'
F0827H	5'-GCT GAA CCA AGA TTT GCA CCA-3'
FIV1437r	
FIV-GL8	5'-AGG AAA ATT GGC CGC CAT A-3'
F0425H	5'-AGG AAA ATT AGC TGC CAT A-3'
F0556H	5'-AGG AAA ATT GGC CGC CAT A-3'
F0795H	5'-AGG AAA ATT GGC TGC CAT A-3'
F0827H	5'-AGG AAA GTT GGC TGC CAT A-3'
Probe 1416p	
FIV-GL8	5'-FAM-TGC AGT GTA GAG CAT GGT ATC TTG AGG CA-TAMRA-3'
F0425H	5'-FAM-TGC AGT GTA GAG CCT GGT ATC TTG AGG CA-TAMRA-3'
F0556H	5'-FAM-TGC AGT GTA GAG CAT GGT ATC TTG AGG CA-TAMRA-3'
F0795H	5'-FAM-TGC AGT GTA GAG CAT GGT ATC TTG AGG CA-TAMRA-3'
F0827H	5'-FAM-TGC AGT GTA GAG CAT GGT ATC TTG AGG CA-TAMRA-3'

Reverse primer and probe are read from the composite strand for clarity. Mismatches between primer/probe and primary isolate sequence are highlighted in red.

Figure 4.b. Peak proviral loads in PBMCs.



Cats are grouped according to isolate received.

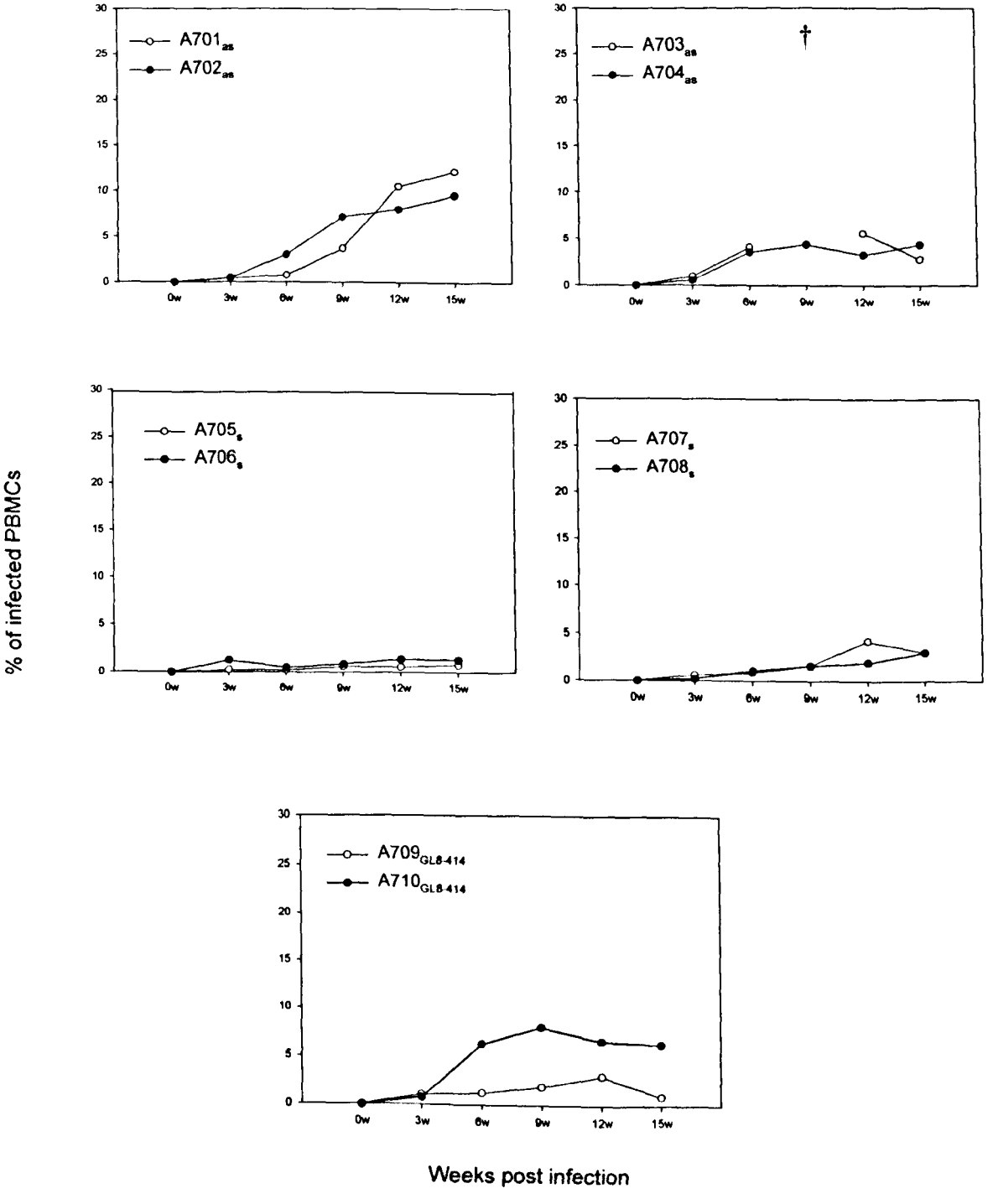
† represents data removed from A703 at 6 weeks p.i as considered unreliable.

Red = cats receiving virus from the asymptomatic phase

Blue = cats receiving virus from the symptomatic phase

Green = cats receiving virus clone $GL8_{414}$

Figure 4.c. PBMC proviral loads 0-15 weeks post infection.



PBMC proviral DNA loads were measured by real-time PCR using the primer probe FIV1010p system. Cats are grouped according to the isolate received.

† represents data removed from A703 at 6 weeks p.i as considered unreliable.

symptomatic isolates (A706 and A707) peaked at 12 weeks while those of the remaining two cats (A705 and A708) continued to increase until the end of the study (see Figure 4.c.).

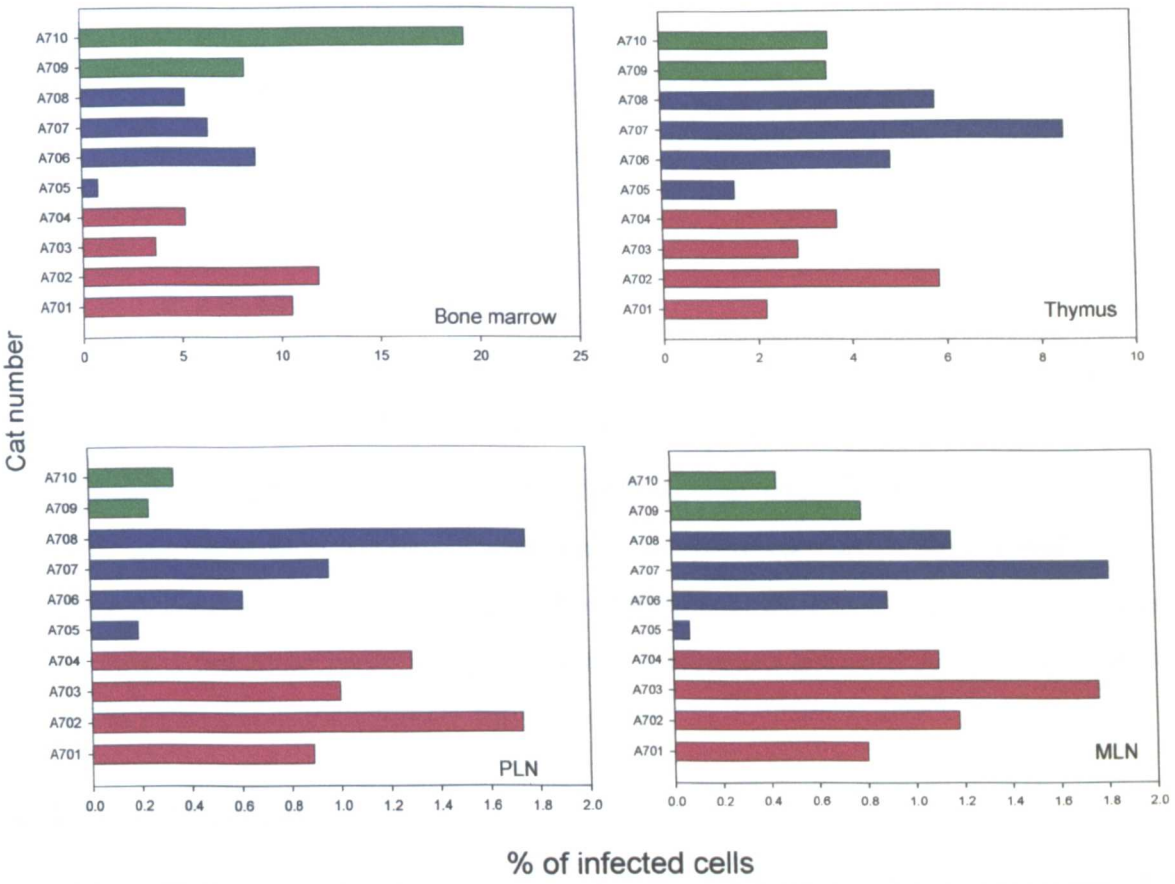
The two control cats (inoculated with the clone FIV-$GL8_{414}$) displayed markedly different peak proviral loads and rates of increase in proviral burden throughout the 15 week study (see Figure 4.c.). Cat A710 showed a marked rise in proviral burden between 3 and 9 weeks p.i. (0.7% to 8%), whereas the peak proviral load of cat A709 was only 3.06%. This pair of cats demonstrated the greatest variability between final proviral loads (see Figure 4.c.).

Following infection with isolate F0425H_{as}, cats A701 and A702 demonstrated peak proviral loads greater than 9% and the kinetics of infection were similar to each other (Figure 4.c.). Cats A703 and A704 were inoculated with isolate F0556H_{as} and displayed similar proviral burdens until 9 weeks p.i. when A703 showed a peak proviral burden of 26.7% of cells infected (see Figure 4.c.). This cat had suffered an intestinal intussusception between 3 and 6 weeks p.i. which was corrected surgically and the cat subsequently made a full recovery. It is unknown if stress factors of this kind may have influenced the proviral kinetics. By 15 weeks post infection their proviral burdens were similar (2.85% and 4.35%). Cats A705 and A706 were inoculated with isolate F0795H_s and showed the lowest peak proviral loads, both cats having less than 2% of PBMCs infected. The two cats receiving isolate F0827H_s (A707 and 708) also showed similar low proviral loads, peaking at 4.3% and 3% respectively. The peak proviral load of A703 at 26.7% was considered unreliable and was not included in the statistical analysis. The difference between the two groups was still significant even when data from cat A703 were excluded ($p=0.032$, Student's t-test).

4.3.1.3. Tissue proviral burdens

Proviral burdens of tissues at 15 weeks p.i. are presented in Figure 4.d. The proviral DNA burdens in the tissues were variable. However, cat A705 had the lowest proviral burdens in all of the tissues examined (see Figure 4.d.). There were no statistically significant differences among the proviral burdens of the groups.

Figure 4.d. Tissue proviral burdens.



Proviral DNA load within selected tissues was measured by real-time PCR. Each histogram depicts the percentage of infected cells from either bone marrow, thymus, peripheral lymph node (PLN) or mesenteric lymph node (MLN).

Red = cats receiving virus from the asymptomatic phase

Blue = cats receiving virus from the symptomatic phase

Green = cats receiving virus clone $GL8_{414}$

4.3.1.4. Lymph nodes

Proviral burden was lower within the lymph nodes compared to other tissues examined, all cats having less than 2% infected cells in both mesenteric and peripheral lymph nodes (see Figure 4.d.). Variation within the pairs of cats in each group was much smaller than other tissues sampled (mesenteric lymph node mean = 0.9% ± 0.18 SE; peripheral lymph node mean = 0.97% ± 0.17 SE).

4.3.1.5. Bone marrow

Three groups showed small variations between the pairs ($F0425H_{as}$, $F0556H_{as}$ and $F0827H_s$). The levels of infection between the two groups of cats receiving asymptomatic isolates were different, A701 and A702 both having over 10% of bone marrow cells infected compared to A703 and A704 where the proviral burden was below 6%. Both groups receiving symptomatic isolates showed similar mean proviral burdens within the bone marrow but there was a marked degree of variation between A705 and A706 (0.8% and 8.7% respectively)(see Figure 4.d.).

4.3.1.6. Thymus

Proviral burdens within the thymus were very variable between the ten cats (mean 4.2% ± 0.65 SE). A705 records the lowest burden at less than 1.5% and A707 had the highest proviral burden within the thymus (8.5%). Both of these cats received symptomatic isolates (see Figure 4.d.).

4.3.2. Viral RNA loads in the peripheral circulation

In contrast to the proviral DNA loads in PBMCs, the plasma viral RNA loads of all cats receiving field isolates were remarkably low throughout the 15 week study period (see Figure 4.e.). Small increases in viral load occurred in cat A702 at 9 weeks p.i. and cat A708 at 3 weeks p.i. with viral loads of approximately 25 000 and 31 000 virus/ml plasma respectively. Within pairs of cats inoculated with each isolate, variation was marked in the peak viral loads as well as the periods of time that elapsed before the peak burden was reached. Seven of eight cats receiving field isolates had no detectable viral RNA by 15 weeks p.i., (A707 had a viral burden of approximately 2400 virus/ml plasma at 15 weeks p.i.). The two cats receiving the clone FIV-$GL8_{414}$ had the highest viral burdens in plasma with A710 reaching a peak at approximately 190 000 virus/ml plasma at 3 weeks p.i. and

A709 reaching peak burden at 3 weeks p.i. (22 000 virus/ml plasma). Whereas proviral load tended to increase steadily throughout the period of study, viral burden would fluctuate, often being below the assay detection limit (see Figure 4.c. and Figure 4.e.).

4.3.3. Quantitative viral isolation

The levels of infectious virus at 6 weeks p.i. were measured by *in vitro* cultivation of PBMCs with Mya-1 cells (see Figure 4.f.). When asymptomatic isolate recipients were compared with symptomatic isolate recipients, the differences in infectious virus burden were significant ($p=0.023$, Student's t-test), with higher burdens in the asymptomatic isolate recipients.

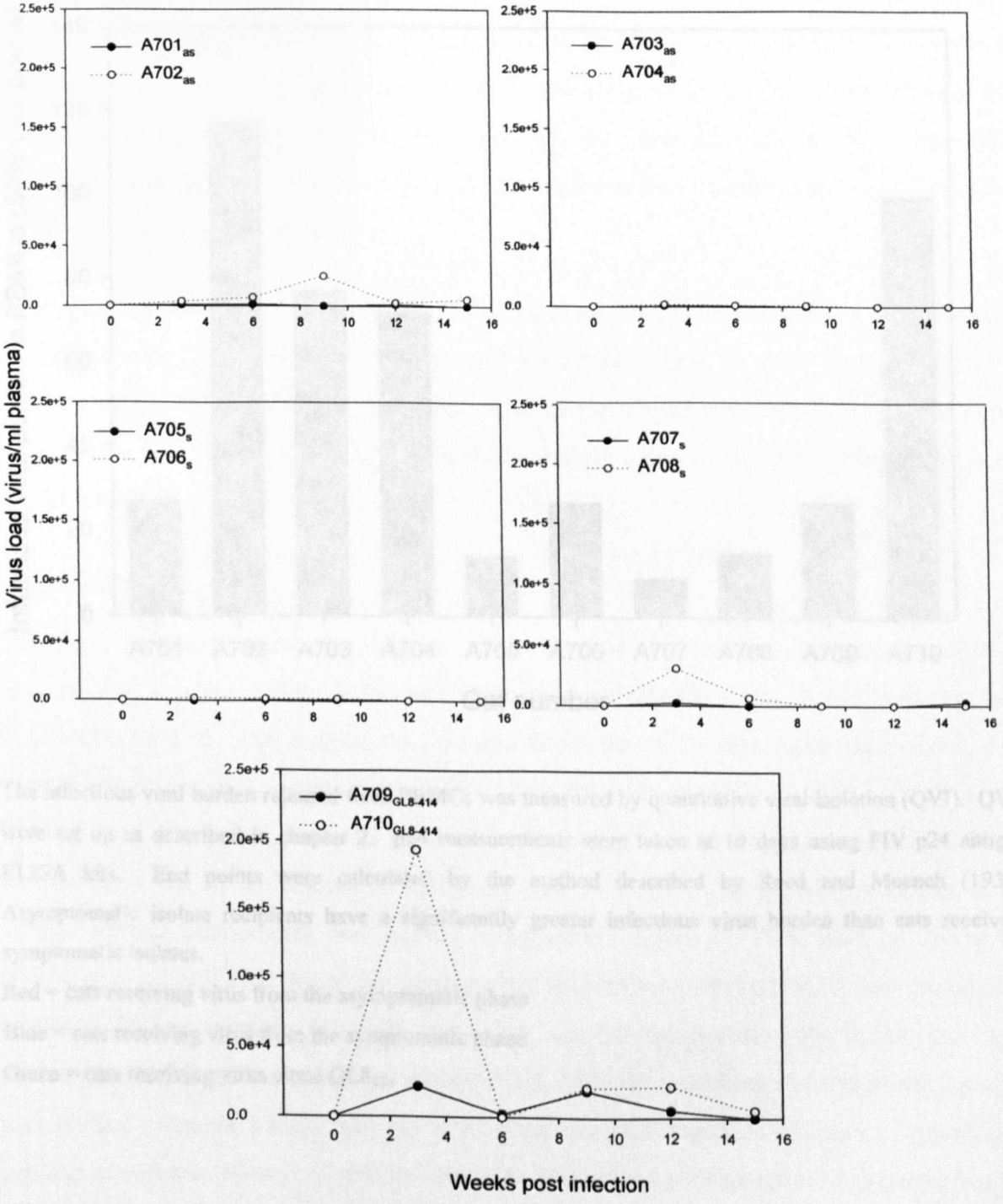
4.3.4. Correlation between proviral load and viral loads

The proviral loads recorded throughout the study were markedly higher than the viral loads for the cats inoculated with field isolates and there was no correlation between the two parameters. Cat A710 developed the higher peak viral load (3 weeks p.i.) and proviral load of the pair of cats receiving FIV-$GL8_{414}$. Interestingly, the proviral loads were similar in cats A709 and A710 for the first two time points before the load of cat A710 increased dramatically. At 3 weeks p.i. a peak viral load of 19 000 virus/ml plasma was detected in the plasma of cat A710 but this high viral burden did not coincide with an increased proviral load in PBMCs or any tissue examined post-mortem. However, a high infectious viral load was detected in cat A710 by QVI at 6 weeks p.i.

4.3.5. Confirmation of viral loads using 1416p system

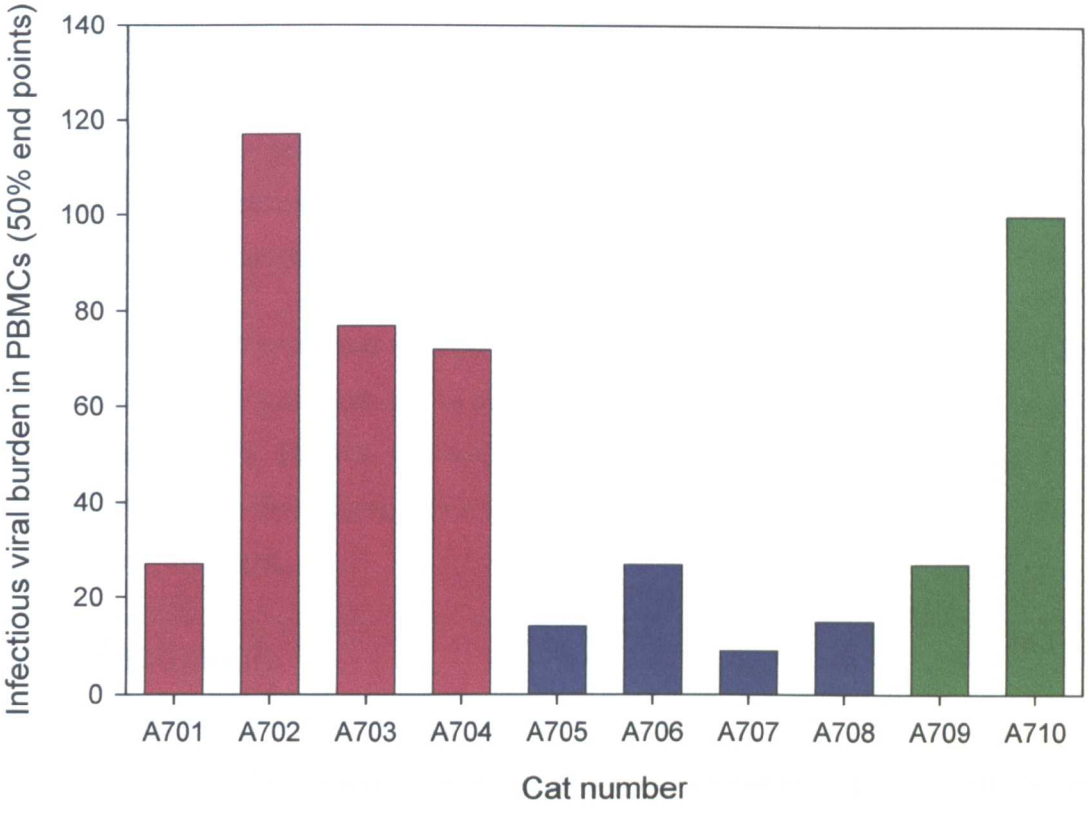
The viral RNA burden in the plasma was then measured using the 1416p system to confirm the levels recorded by the 1010p system. The differences between the peak viral loads recorded using each set of probe and primer were not significant ($p=0.186$, Student's t-test) however, the final viral loads were significantly greater using 1416p system ($p=0.031$, Student's t-test). This system also recorded higher results for the group infected with FIV-$GL8_{414}$ with cats A709 and A710 recording peak viral burdens of 73 000 and 530 000 virus/ml of plasma respectively. The peak viral burden recorded in the cats infected with field isolates was 41 000 virus/ml of plasma using the 1416p system whereas the highest viral burden recorded by the 1010p system was 24 700 virus/ml of plasma. The peak viral burdens between the asymptomatic and symptomatic groups were not significantly different when measured by either system (data not shown).

Figure 4.e. Plasma viral RNA load measured by real time RT-PCR.



Viral RNA load in plasma was measured by real-time RT-PCR using the FIV1010p system (Klein et al 1999). Cats are grouped according to inoculate received.

Figure 4.f. Quantitative viral isolation at 6 weeks post infection.



The infectious viral burden released from PBMCs was measured by quantitative viral isolation (QVI). QVIs were set up as described in chapter 2. p24 measurements were taken at 10 days using FIV p24 antigen ELISA kits. End points were calculated by the method described by Reed and Muench (1937). Asymptomatic isolate recipients have a significantly greater infectious virus burden than cats receiving symptomatic isolates.

Red = cats receiving virus from the asymptomatic phase

Blue = cats receiving virus from the symptomatic phase

Green = cats receiving virus clone $GL8_{414}$

The viral RNA loads at 6 weeks p.i. were compared with the QVIs which were also carried out at 6 weeks p.i. The infectious viral burdens in asymptomatic isolate recipients measured by QVI were significantly greater than the burdens in symptomatic recipients (see Figure 4.f.). However, the viral loads at 6 weeks p.i., measured by real-time RT-PCR, were quite different. Whereas the infectious viral burden in the symptomatic recipients was very similar with 50% endpoints under 30, the range of viral RNA loads between the groups receiving isolates F0795H_s and F0827H_s was marked (500-26800). The viral burdens in cats infected with asymptomatic isolates was less variable and ranged between 1800 and 7700 virus/ml of plasma.

Each reaction was carried out in duplicate and the coefficient of variation (CV) calculated in order to quantify the reliability of the viral load data. The CVs were then grouped into three groups, <10%, 10-30% and >30%. The 1010p system produced the most reliable results with 63% of the data having CVs <10% whereas only 47% of the data in the 1416p system had CVs <10%.

4.4. Discussion

The aim of this study was to investigate field isolates of FIV from cats at different stages of disease, ranging from terminal illness to resolution of clinical signs. Groups of two cats were infected with each isolate; two groups inoculated with asymptomatic isolates, two groups inoculated with symptomatic isolates and the fifth group inoculated with the clone FIV-$GL8_{414}$. Viral loads were measured by real-time RT- PCR using a standardised assay with primers which are known to recognise four FIV strains of subtype A (Klein et al. 1999). Primer mismatches may have a considerable impact on PCR reaction efficiency (Klein et al. 1999) and from the field isolate sequences investigated, it was noted that isolate F0556H_{as}, received by A703 and A704, had two mismatches, one in the probe and one in the reverse primer while isolate F0795H_s had one mismatch in each of the forward and reverse primers which may have reduced the PCR reaction efficiency, potentially leading to reduced values of proviral burden. However, a comparison of proviral burden found in the PBMCs is interesting as the results indicate that the rate of viral replication was greater in the cats that received asymptomatic isolates. The group sizes were small and hence caution must be exercised when analysing results statistically, but when asymptomatic and symptomatic recipients were compared, the difference in mean peak

proviral burden of the PBMCs between the two groups was significant ($p=0.03$, Student's t-test).

The role of HIV-1 proviral burden in PBMCs as a prognostic indicator is not clear and contradicting evidence has been reported so far (Aleman et al. 1999; Tetali et al. 1999; Russell et al. 2001). Pathogenic simian-human immunodeficiency viruses (SHIV) that lead to rapid depletion of $CD4^+$ T cell count and progression to an AIDS-like disease (SHIV-89.6P and SHIV-KB9) have been reported to induce higher proviral DNA loads in PBMCs during early infection of rhesus monkeys (Reimann et al. 1999), whereas nonpathogenic SHIVs were shown to induce lower proviral DNA loads. In our present study we examined only the acute phase of infection, up to 15 weeks after exposure to the virus, and therefore progression to disease was unknown. The differences between the proviral DNA burdens of cats A709 and A710 were surprising given that these cats were inoculated with the cloned virus FIV-$GL8_{414}$; in contrast, the other groups were inoculated with biological isolates and large differences between cats could be explained by the presence of mixed populations of viruses in the inocula. However, differences may be explained also by variable individual host responses to infection.

In this study, high proviral loads in PBMCs did not correlate with high proviral loads in the tissues examined 15 weeks p.i. Proviral burdens in lymph nodes were less than 2% in all cats. Contrary to the findings in our study, other workers have reported similar proviral burdens between lymph nodes and PBMCs 12-16 weeks p.i. (Dean et al. 1996). Tissue proviral burdens have also been reported to be virus strain dependent (Burkhard et al. 2002).

The viral RNA loads of cats receiving the field isolates were remarkably low, in contrast to the proviral burdens measured in the PBMCs. In studies of HIV-1 infection any correlation between proviral and viral RNA loads is still unclear and reports are contradictory (Cone et al. 1998; Aleman et al. 1999).

A possible explanation for the low viral loads detected is low primer binding efficiency. However, since the primers and probe that were used in the real-time RT-PCR (sequence mismatch in the forward primer binding site of isolate F0795H_s and isolate F0556H_{as} had one mismatched nucleotide in the probe binding site) performed with adequate efficiency in proviral measurements, we confirmed their efficiency for use in detecting the isolates

used in this study. Nucleotide mismatches were more frequent in the 1416p system. The effect of primer mismatches is not fully quantified and is thought to depend on several factors including differences in length of the amplicon. The effect of mismatches is greater when they occur nearer the 3'-end of the oligonucleotide and depend on the number of nucleotide changes. Thus two mismatches in a primer may decrease the reaction efficiency and decrease the viral load detected by up to 4 orders of magnitude (Klein et al. 2001). All of these factors would have some bearing on the detection of virus by both of the systems. The viral RNA loads measured by the two real-time RT-PCR systems detect viral burdens, which are not significantly different. The 1416p system detects slightly higher levels but has the disadvantage of higher CVs.

Trends are evident when analysing the proviral DNA burden and the infectious viral burden. The cats infected with viruses from the asymptomatic stage of disease had significantly higher proviral DNA burdens in the PBMCs and significantly higher infectious virus burdens as measured by QVI. The viral RNA load measurements are less clear and although the cats infected with symptomatic isolates appear to have higher viral RNA loads in the PBMCs, this difference is not significant and the levels are still low by the two systems utilised.

The QVI results reflected the proviral burdens of the PBMCs, suggesting that cats inoculated with asymptomatic stage isolates developed infections with higher rates of viral replication and infectivity compared to cats inoculated with isolates from the terminal disease stage which developed significantly lower proviral burdens in the PBMCs and lower infectious virus titres by QVI.

Few studies have been conducted to study viral dynamics after i.m. inoculation of FIV, although one study determined that even when 10 to 100 fold greater amounts of live virus were inoculated by the i.m. route, viral loads in peripheral blood were lower than when virus was inoculated by the i.p. route (Rigby et al. 1997). In addition, seroconversion was found to occur sooner after i.m. compared to i.p. inoculation (Rigby et al. 1997). Another study of two well-characterised isolates demonstrated that the route of inoculation affected levels of $CD4^+$ cytopenia, and both virus type and route of infection influenced plasma viraemia as well as tissue and PBMC proviral burdens (Burkhard et al. 2002). The i.m. route of inoculation may have affected the extent of plasma viraemia by stimulating the immune system to a greater degree compared to that by other routes of inoculation, leading

to greater suppression of viral production and release into the circulation. Interestingly, the two cats inoculated with clone FIV-$GL8_{414}$ developed greater viral RNA loads in plasma and it may be that inoculation of a heterogeneous population of virus with potentially greater antigenicity, such as isolates from cats in the field, may stimulate the immune system to a greater degree compared to a homogeneous population in the clone inoculum. It would be necessary to study the humoral and cell mediated immune responses of each of the cats to ascertain whether virus type can affect immune response after i.m. inoculation.

In conclusion, this study has revealed that cats inoculated with asymptomatic isolates developed higher peak proviral loads in PBMCs and by 15 weeks p.i. 3/4 developed proviral loads higher than 3%. In contrast, only 1/4 symptomatic isolates developed a final proviral load greater than 3%. The differences in PBMC proviral load between cats A709 and A710 suggested that host factors also play a role in the pathogenesis of the disease. QVIs supported the findings in the PBMC proviral burdens. However, further study of the immune system status of these animals may reveal possible explanations for the low and sometimes undetectable viral RNA loads throughout the study. The relationship between viral RNA load, proviral DNA load and infectious viral burden remains unclear. There are inherent problems with the measurement of field isolates by Taqman methods due to differences in sequence at the primer and probe binding sites. Furthermore, the real-time PCR measurement of virus in this fashion does not measure replication-competent virus. Further work is required to investigate the relationship between each of these measurements.

Chapter Five

THE ROLE OF THE *env* GENE IN THE PATHOGENESIS OF FIV

5.1. Introduction

The envelope glycoprotein of FIV contains the principal determinants of virus/cell interactions, tropism and fusogenicity (Pancino et al. 1995) as well as the principal immunodominant domain (Lombardi et al. 1993; de Ronde et al. 1994). Laboratory-adapted isolates of FIV that have proven susceptible to vaccine protection have expanded host cell tropism *in vitro*, which has been shown to correlate with an increased positive charge in the V3 loop (Verschoor et al. 1995; Siebelink et al. 1995b) similar to findings in HIV (de Jong et al. 1992). This expanded tropism has also been correlated with the sole usage of the chemokine receptor CXCR4 for viral entry into cells. *In vivo* these laboratory-adapted isolates produce lower viral and proviral loads, less disruption of the lymphocyte populations and no inversion of the CD4:CD8 ratio. Interestingly, studies from field isolates documented in this thesis have revealed that isolates from cats with terminal disease more readily use CXCR4 for viral entry than isolates from cats with asymptomatic disease. These results were paralleled with the findings of lower proviral loads and less perturbation of the lymphocyte subsets and no inversion of the CD4:CD8 T lymphocyte ratio in naïve cats that were infected with isolates from terminal cases. Taken together, these findings led to the hypothesis that Env may be a determinant of pathogenicity *in vivo* and that tropism and receptor usage *in vitro* may predict the pathogenicity of an isolate *in vivo*.

This study examines the *in vitro* tropism of five isolates: the original prototype viruses derived from molecular clones, FIV-GL8$_{MYA}$ (G8$_{MYA}$) and FIV-PET$_{F14}$ (PET$_{F14}$), and three chimaeras consisting of an G8$_{MYA}$ backbone containing the *env* genes of the two field isolates F0425H$_{as}$, F0827H$_{s}$ as well as PET$_{F14}$. The G8$_{MYA}$ backbone was isolated from the molecular clone CP3 in the low copy number plasmid pBR328 following the excision of PET *env*. The chimaeras were designated G8M(425), G8M(827) and G8M(F14.7). By subjecting the clones and chimaeras to a panel of assays *in vitro*, their ability to use CXCR4 was characterised. Their behaviour *in vivo* was then examined, to determine the relationship between receptor usage and tropism *in vitro* to pathogenicity *in vivo*. Thus the

insertion of various *env* genes from well-characterised prototype clones and field isolates into the same viral backbone to create a range of similar clones, differing only in the *env* gene region, enabled the elucidation of the role of *env* in determining the pathogenicity of an isolate.

5.2. Materials and methods

5.2.1. Production of chimaeras

5.2.1.1. Preparation of DNA

DNA was prepared from cell pellets of infected Mya-1 cells (described in Section 2.7.) using a blood kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and was stored at -20°C until required.

5.2.1.2. Amplification of the *env* gene

The full length of the envelope glycoprotein (*env)* gene was amplified using the polymerase chain reaction (PCR). DNA samples were titrated in two-fold dilutions from 400ng to 50ng and then aliquoted. HiFidelity (Roche) PCR master mix was used in the PCR reaction and the primers corresponded to the 5' cleavage site of the L-SU (5'-TAG ACG CGT AAG ATT TTT AAG GTA TTC-3') and the *Nde* I site 3' of the Rev responsive element (3'-CCC TTT GAG GAA GAT GTG TCA TAT GAA TCC ATT-5'), a segment which incorporates the *Mlu*I and *Nde*I restriction sites. The amplified products were separated by agarose gel electrophoresis, excised and purified using Qiaquick gel extraction kit, following the manufacturer's instructions. The products were then digested with *Mlu*I and *Nde*I, re-purified to remove the restriction enzymes and then ligated into the pre-digested $GL8_{MYA}$ vector.

5.2.1.3. Transformation of competent cells

The ligations were then transformed into the *E.coli* competent cells ONE SHOT® Invα'F (Invitrogen, U.K.). Vials of competent cells were defrosted on ice before adding 1μl of the ligation reaction. The mixture was incubated on ice for a further 30 minutes, before the competent cells were heat shocked at 42°C for 30 seconds and then placed on ice for 2 minutes before 450μl of SOC medium (see Appendix A.3.) was added. The mixture was

incubated for 90 minutes at 30°C in a water bath before plating on L-agar plates containing ampicillin (50μg/ml), which were then incubated for 24 hours in a 30°C incubator.

5.2.1.4. Amplification and purification of plasmid DNA

Colonies were selected from the agar plates (see Appendix A.3.) and cultured in 3ml of L-broth (see Appendix A.3.) supplemented with ampicillin (50μg/ml) overnight at 30°C in an orbital incubator. Plasmid DNA was collected using a Qiagen MiniPrep Plasmid DNA kit (Qiagen) (using the principle first described by Vogelstein (Vogelstein and Gillespie 1979)) following the manufacturer's instructions. Confirmation of successful insertion of the *env* gene was achieved by digestion of the product with *Mlu* 1 and *Nde* 1 restriction enzymes for 60 minutes and examining the product on a 1% agarose gel containing ethidium bromide. Inserts of 2.5Kb represented the full-length *env* product.

5.2.1.5. Identification of the *env* insert

To confirm that the *env* detected following Mlu/Nde digestion represented the novel *env*, and not a residual *env* from the parent molecular clone, each plasmid was digested with the restriction enzyme *Kpn* I for 60 minutes at 37°C. The products were separated on a 1% agarose gel and could be seen at 1659bp and 10967bp. Alternatively, if the FIV-PET *env* from the parent vector CP3 had been reinserted, then only one product band would have been detectable at 12626bp since FIV-PET has a single *Kpn* 1 site. A further digest with the restriction enzyme *Afl* II was conducted to confirm that the gene was distinct from FIV-$GL8_{MYA}$ *env*. Digestion of the field isolate *env* was carried out in parallel with that of FIV-$GL8_{MYA}$ and FIV-PET_{F14}. FIV-PET_{F14} has eight *Afl* II restriction sites whereas FIV-$GL8_{414}$ has seven (see Table 5.a.).

5.2.1.6. Amplification of positive clones

Cultures of clones with the *env* gene successfully inserted were expanded overnight in 200ml of L-broth supplemented with ampicillin (50μg/ml) at 30°C in an orbital incubator. Plasmid DNA was then purified using a Qiagen MaxiPrep Endofree Plasmid DNA kit (Qiagen), following the manufacturer's instructions. This is a procedure based on the alkaline lysis of bacterial cells (Birnboim and Doly 1979).

Table 5.a. Restriction sites and fragment sizes within the FIV genomes FIV-PET_{F14} and FIV-$GL8_{Mya}$ with *Afl*II.

FIV-PET_{F14}								
restriction site (nucleotide position)	1621	3440	3558	4466	5018	7972	8257	9474
fragment length (bp)	1621	1819	118	908	552	2954	285	1217
FIV-$GL8_{Mya}$								
restriction site (nucleotide position)	1620	3439	4465	6618	7968	8253	9467	
fragment length (bp)	1620	1819	1026	2153	1350	285	1214	

Digestion of primary isolate *env* alongside PET_{F14} and $G8_{Mya}$ confirmed successful insertion of the primary isolate *env*.

5.2.1.7. Transfection of clones in 293T cells

Poly-L-lysine plates were set up overnight with 1.5 x 10^5 293T cells per well in 3 ml of 10% DMEM and incubated in a humid incubator at 37°C. The following morning 2.5µg of each plasmid was diluted with DMEM to 100µl and 12.5µl of Superfect (3mg/ml) (Qiagen), mixing by pipetting up and down five times. The mixture was incubated at room temperature to allow complex formation. The medium was removed from the plates which were then washed once with serum free DMEM. 600µl of complete medium was added to the Superfect mixture and then transferred gently to the plates. After three hours, incubation at 37°C the medium was aspirated and the cells were washed twice in serum-free DMEM. Three ml of complete medium were added and the plates were incubated at 37°C in a CO_2 incubator. Three days after transfection the supernatant was harvested and passed through a 0.45µm filter. 200µl was tested by p24 antigen ELISA. 1ml of supernatant from positive wells was stored at -70°C and 1.5ml was added to 10^6 Mya-1 cells in a T25 culture flask and made up to 5ml with complete RPMI containing IL-2. The cultures were tested by p24 antigen ELISA every 3 days until positive when culture fluids were harvested, filtered through a 0.45µm filter, aliquoted and stored at -70°C.

5.2.2. *In vitro* tropism of the clones

The clones were subjected to a panel of *in vitro* assays. Tropism studies on (HO6T1)CrFK cells and AH927 FX4E cells were carried out as described in Chapter 3.

5.2.3. Infection of kittens

5.2.3.1. Virus inoculum

The cloned viruses were titrated as described in Chapter 2. Inocula were prepared in RPMI medium containing 1% BSA. Five groups of three kittens received 250 $TCID_{50}$ of virus (see Table 5.b.) by i.m. inoculation.

5.2.3.2. Collection of samples

Blood samples were collected in EDTA on 0, 3, 6, 12, and 15 weeks p.i. After the final sampling, post-mortem examinations were carried out. Proviral DNA loads of the PBMCs and plasma viral RNA loads were measured at each time point (method described in Chapter 4) and FACS analysis was carried out at 0, 3, 6, 12 and 15 weeks p.i. (see Chapter

6). At post-mortem examination, peripheral and mesenteric lymph nodes were harvested and processed. Virus isolation was carried out on PBMCs from the samples taken at each time point except at 15 weeks p.i. when QVIs were carried out (all methods described in Chapter 2).

5.2.3.3. Tissue samples

Mesenteric and peripheral lymph nodes were collected at post-mortem examination and processed for measurement of proviral DNA load. QVIs were carried out on each of the mesenteric lymph node samples in cats receiving either clone $G8_{Mya}$ or PET_{F14}, as described in Chapter 2.

5.2.4. Viral RNA and proviral DNA

Viral RNA and proviral DNA measurements were conducted as described in Chapter 4.

5.2.5. Virus isolation and quantitative virus isolation

The methodology for virus isolation and quantitative virus isolation is described in sections 2.5.3. and 2.5.4.

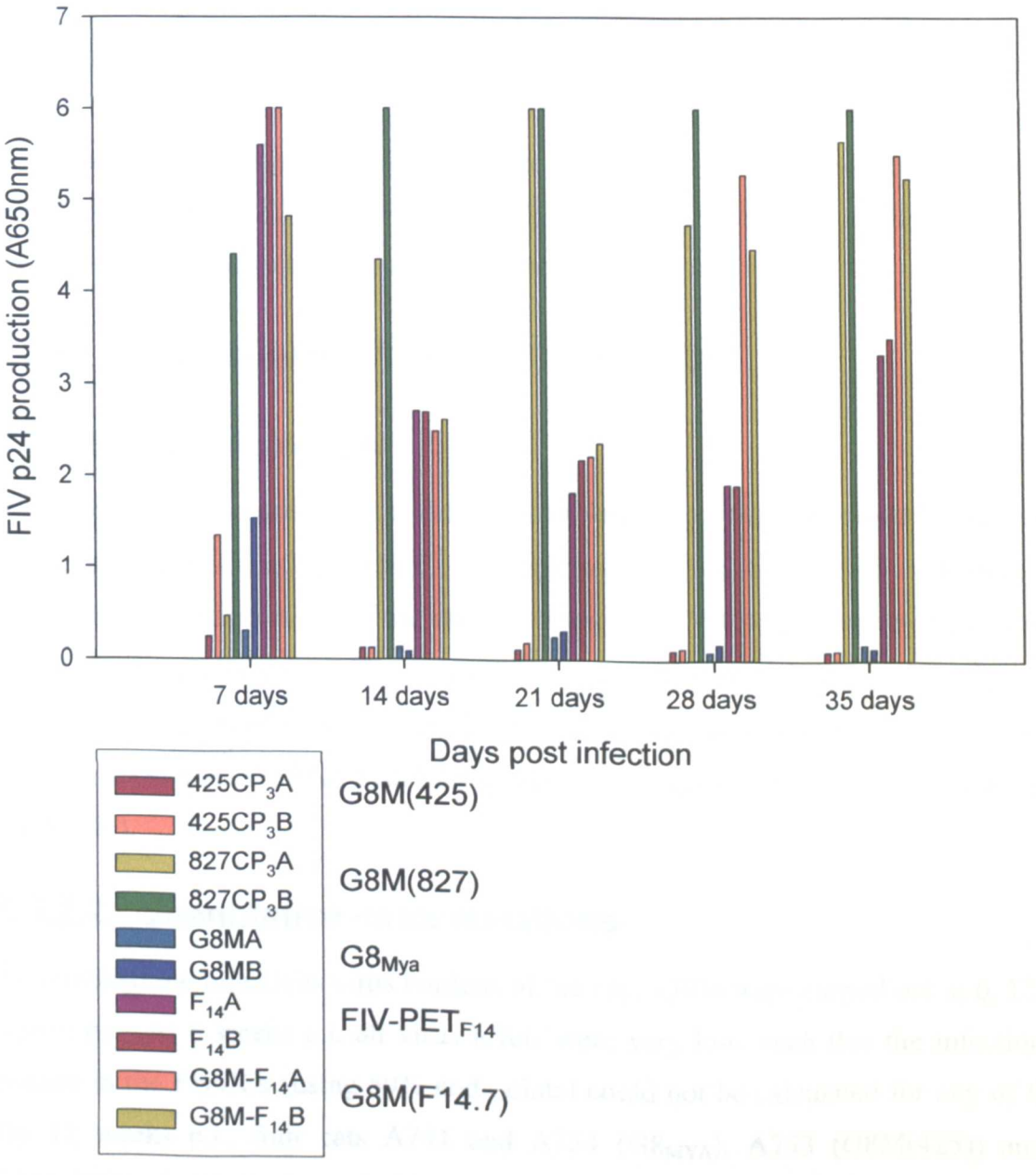
5.3. Results

5.3.1. *In vitro* tropism

5.3.1.1. Tropism on CrFK(HO6T1) cells

The cloned virus containing the *env* gene from the symptomatic cat $F0827H_s$, namely G8M(827), successfully infected CrFK(HO6T1) cells by both cell-free infection and cocultivation. In contrast, the clone containing *env* from the asymptomatic isolate $F0425H_{as}$, G8M(425), only achieved a short period of infection of CrFK(HO6T1) cells following cocultivation until 7 days p.i. The clone G8M(F14.7) and the parental clone PET_{F14} both achieved productive and persistent infection of the cells by cell-free infection and cocultivation. Each clone produced a transient cytopathic effect by both methods of infection (cell-free infection or cocultivation) at 21 days and 28 days p.i. respectively. As a result, these cultures were not subcultured at these time points, however fresh medium was added to each flask and the remaining cells continued to grow. The parental FIV-$G8_{MYA}$ clone produced only a transient productive infection on cocultivation with infected Mya-1 cells and by 14 days p.i. p24 could no longer be detected (see Figure 5.a.).

Figure 5.a. Tropism on CrFK(HO6T1) cells.



A: cultures - cell-free infection, B: cultures - cocultivation with infected Mya-1 cells.

Virus was incubated overnight in cultures of CrFK (HO6T1) cells. The cells were washed the next day and fresh medium added with subculturing twice per week. p24 production was measured by FIVp24 antigen ELISA (IDEXX) at 7 day intervals.

5.3.1.2. Tropism on AH927 FX4E cells

Only the FIV-PET_{F14} clone induced any cytopathic effect in AH927 FX4E cells. RT activity was highest in the laboratory-adapted FIV-PET_{F14} but both chimaeras containing field isolate *env* genes did infect the cells. Interestingly, greater p24 readings were obtained from the culture with the asymptomatic *env* clone G8M(425) when compared to the culture with the symptomatic *env* clone G8M(827). The $G8_{MYA}$ clone, as predicted, was unable to infect these cells whereas the PET_{F14} conferred the ability to use CXCR4 when inserted into the $G8_{MYA}$ backbone (see Figure 5.b.).

5.3.2. *In vivo* studies of the *env* chimaeras

5.3.2.1. Virus isolation

Virus isolation was carried out at each time of sampling. No virus could be isolated from cats receiving the chimaera G8M(F14.7) consistently throughout the 15 week study period. Virus was isolated from 1/3 of the cats receiving the FIV-$G8_{MYA}$ clone by three weeks p.i. and all three cats by six weeks p.i. Cultures of PBMC established from all other cats had detectable levels of p24 by three weeks p.i. Virus was undetectable in two cats receiving PET_{F14} (A750 and A755) at 9 and 15 weeks p.i. as well as cat A747 at 15 weeks p.i. (see Table 5.b.).

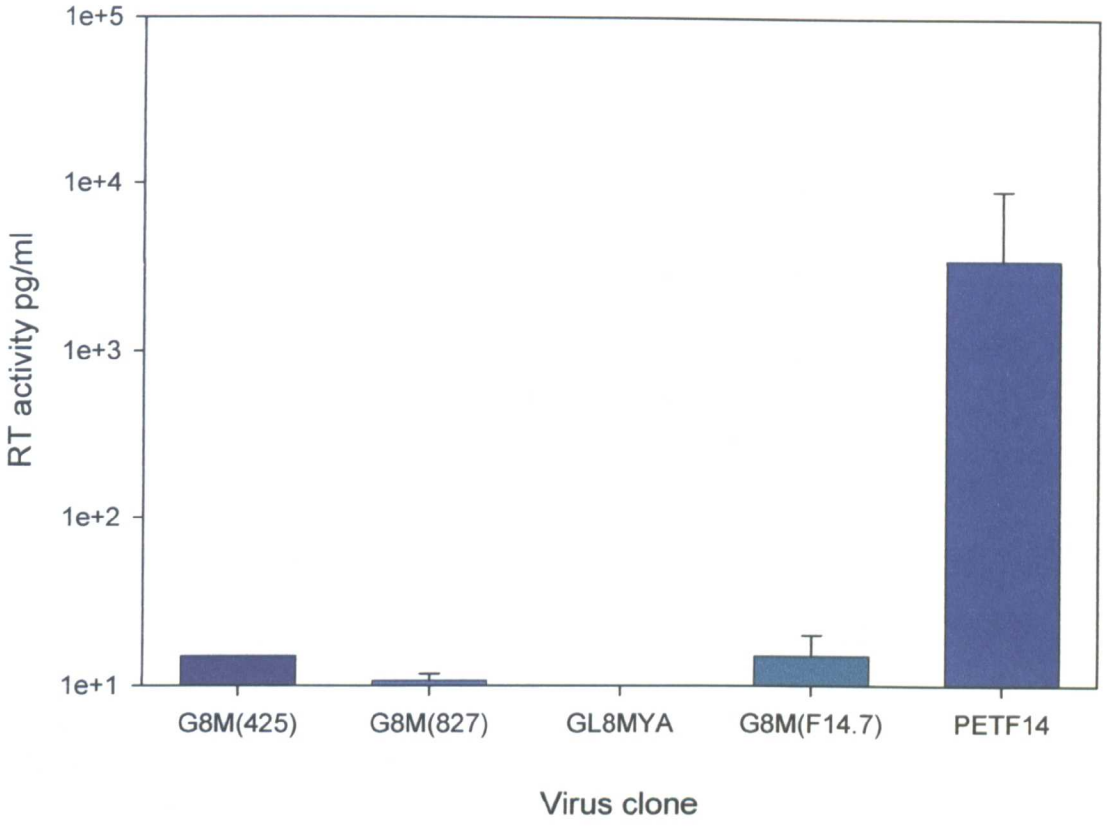
5.3.2.2. Quantitative virus isolations

To establish the infectious virus burdens of the cats, QVIs were carried out at 6, 12 and 15 weeks p.i. At 6 weeks p.i. all virus levels were very low, such that the infectious virus burden in the PBMCs (using 50% end points) could not be calculated for any of the cats. By 12 weeks p.i., four cats A741 and A754 ($G8_{MYA}$), A753 (G8M(425)) and A746 (G8M(827)) had infectious viral burdens that were sufficiently high to calculate 50% end points. By 15 weeks p.i. this number had increased to six cats (A741 and A754 ($G8_{MYA}$), A743, A744 and A753 (G8M(425)) and A746 (G8M(827)). Three cats had undetectable virus, consistent with the virus isolation assays (A747, A750 and A755) (see Tables 5.c.).

5.3.2.3. Detection of viral RNA loads by RT-PCR

The 1416p system was used to detect viral RNA. The highest viral RNA loads recorded in the plasma were from the group receiving $G8_{MYA}$ at 9 weeks p.i. but there was not a significant difference between the groups at this time point. However, G8M(425) had

Figure 5.b. Infection of the cell line AH927 transfected with feline CXCR4.



Virus stocks were incubated with AH927 FX4E cells for 1 hour at 37°C. Cultures were washed and fresh medium added and then incubated for 10 days when virus replication was measured using RT activity assays (Cavidi Tech).

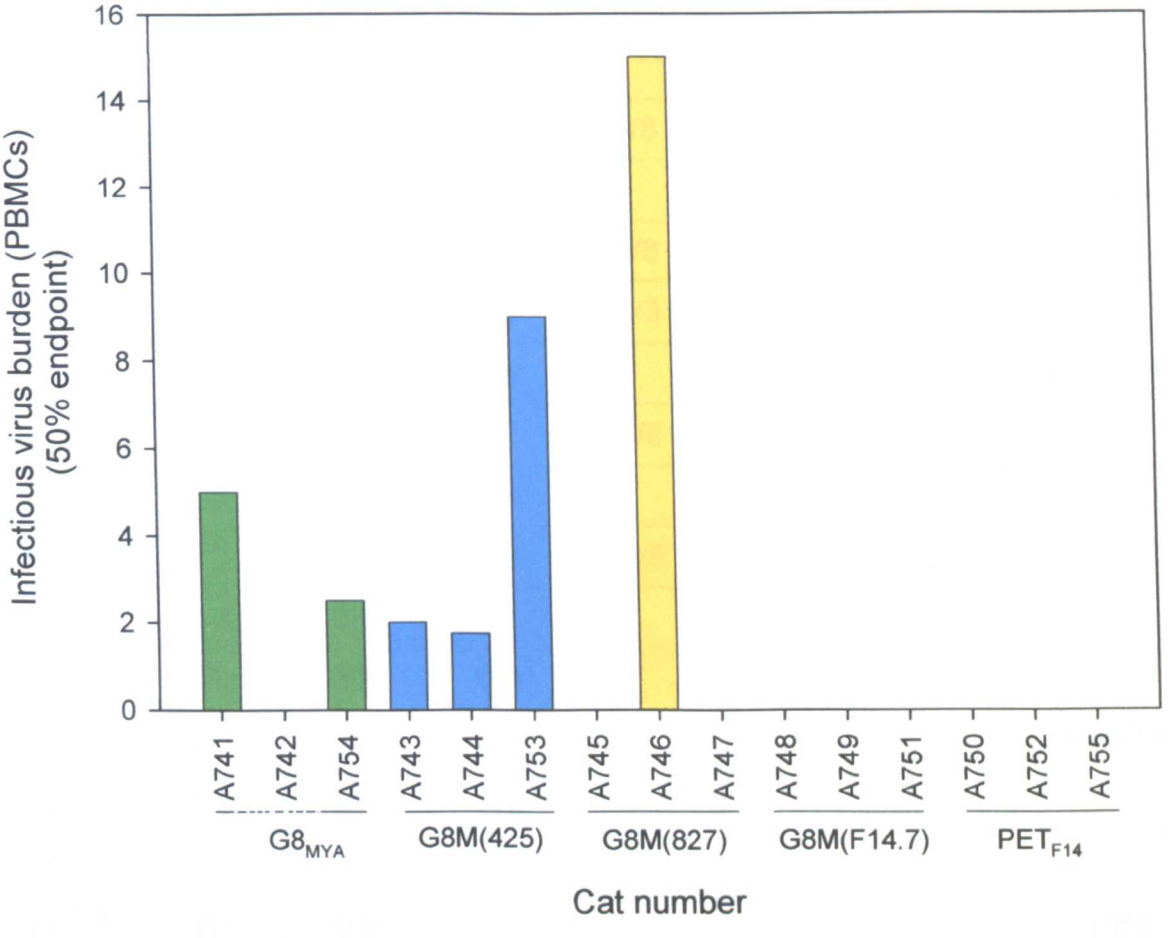
Table 5.b. Virus isolation in Mya-1 cells.

Cat	Virus	Week 3	Week 6	Week 9	Week 12	Week 15
A741	G8Mya	-	+	+	+	+
A742		-	+	+	+	+
A754		+	+	+	+	+
A744	G8M(425)	+	+	+	+	+
A743		+	+	+	+	+
A753		+	+	+	+	+
A745	G8M(827)	+	+	+	+	+
A746		+	+	+	+	+
A747		+	+	+	+	-
A748	G8M(F14.7)	-	-	-	-	-
A749		-	-	-	-	-
A751		-	-	-	-	-
A750	PET	+	+	-	+	-
A752		+	+	+	+	+
A755		+	+	-	+	-

10^6 PBMCs were cultured with 2 x 10^6 Mya-1 cells in 5ml RPMI and IL-2.

Cultures were tested for p24 production (FIV p24 ELISA, IDEXX).

Figure 5.c. The infectious viral burden within the PBMCs 15 weeks p.i.



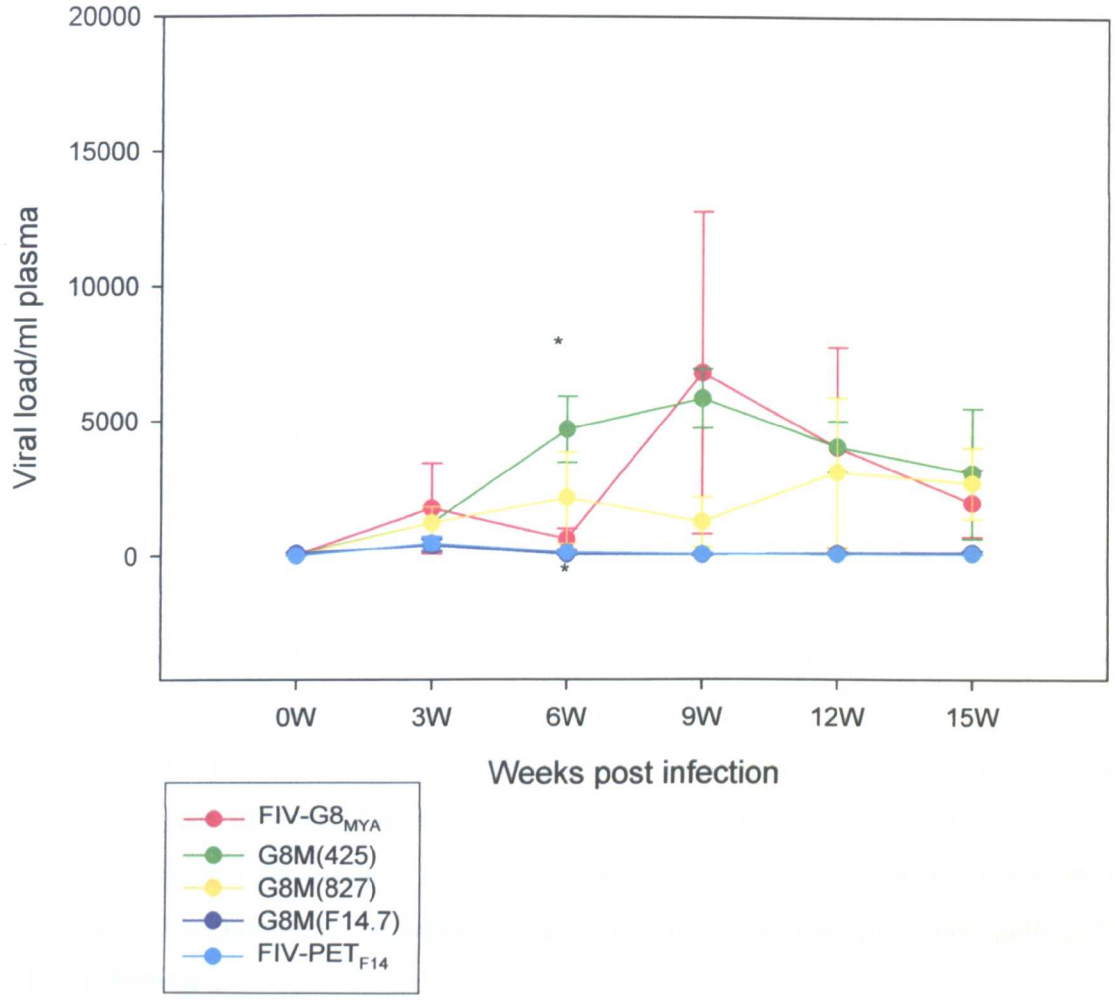
Positive cultures were detected using p24 FIV antigen ELISA. Infectious viral burdens were calculated using the 50% end point method as described by Reed and Muench, 1937.

Table 5.c. 15 week QVI assays (PBMCs).

	Proportion of cells infected at:						
	10^4	3x 10^3	1x 10^3	3x 10^2	30	10	Virus inoculum
A741	8/8	2/8	4/8	0/8	0/8		G8Mya
A742	2/8	0/8	0/8				
A754	7/8	3/8	1/8	0/8	0/8		
A743	6/8	2/8	0/8	0/8			G8M(425)
A744	7/8	0/8	0/8				
A753	8/8	7/8	4/8	1/8	0/8	0/8	
A745	2/8	0/8	0/8				G8M(827)
A746	8/8	8/8	5/8	2/8	0/8	0/8	
A747	0/8	0/8					
A748	1/8	0/8	0/8				G8M(F14.7)
A749	2/8	0/8	0/8				
A751	1/8	0/8	0/8				
A750	0/8	0/8					PET
A752	1/8	0/8					
A755	0/8	0/8					

Each dilution of PBMCs was tested in groups of eight. An FIV p24 antigen ELISA was used to detect positive cultures 7 days p.i. The Reed and Meunch method (Reed and Meunch 1937) was used to calculate the infectious viral burden within the cells.

Figure 5.d. Mean viral RNA load measurements in the plasma of each group at 0-15 weeks p.i.



RNA viral loads in plasma were measured by real-time RT-PCR using the 1416p system (Klein, 1999).

*= $p<0.039$, FIV-PET_{F14} and $p<0.042$, G8M(F14.7).

The cats are grouped by inoculate received. The group mean and SE shown.

significantly greater loads at 6 weeks p.i. (see Figure 5.d.) when compared to the clone PET_{F14} and the chimaera G8M(F14.7) ($p<0.039$ and $p<0.042$ respectively, Tukey test). The greatest viral RNA load was recorded in the group receiving $G8_{MYA}$ (6794 virus/ml plasma (SE ± 5988)) with the viral load in cat A741 reaching a level of 18 700 virus/ml plasma. However, there was a large variation within this group, reflected in the large standard error. The cats infected with clone PET_{F14} and the chimaera G8M(F14.7) developed only low viral RNA loads within the plasma with a slight peak at 3 weeks p.i. (440 ± 250 SE and 380 ± 231 SE). Generally, the chimaera with the *env* gene from terminal isolate F0827H induced viral loads intermediate from $G8_{MYA}$, G8M(425) and PET_{F14} and G8M(F14.7).

5.3.2.4. Proviral DNA loads from PBMCs and tissues

There was evidence of contamination in the proviral load assays and the results were considered unreliable.

5.4. Discussion

From the studies described in Chapter 3 of this thesis it was found that isolates from cats in the asymptomatic stage of the disease were less able to utilise the chemokine receptor CXCR4 *in vitro* and induced greater proviral DNA loads in infected cats, whereas isolates from cats in the terminal stages of the disease had a greater affinity for CXCR4 and induced lower proviral loads *in vivo*. By comparing the *env* genes of these viruses in a common viral backbone, it was hoped to clarify the role of the *env* gene in the pathogenesis of FIV infection.

Interestingly, the ability to utilise CXCR4 by a clone or chimaera was not consistent throughout all the *in vitro* studies. The chimaera G8M(827) and G8M(F14.7) were able to infect the CrFK(HO6T1) cells efficiently by cell-free infection and cocultivation, demonstrating the ability to utilise CXCR4 alone. However, the same viruses were much less efficient at infecting the AH927 FX4E cells (transduced so as to express the CXCR4 receptor), an assay that is also designed to identify viruses with the ability to use CXCR4 alone. The reason for this difference is unknown. Possibly the presentation of CXCR4 in each cell varies, as CXCR4 may have different conformations and molecular weights depending on the cell line (Baribaud et al. 2001; Lapham et al. 2002) or perhaps an additional molecule is involved in FIV entry. The ability of laboratory-adapted isolates

such as PET_{F14} to infect many CXCR4-expressing cell lines with great efficiency may be due to an expansion of CXCR4-tropism or some determinant outwith the *env*.

The virus with the *env* gene from the asymptomatic cat $F0425H_{as}$ and the pathogenic clone $G8_{MYA}$ both induced greater plasma viral RNA loads, although a significant difference was seen only at 6 weeks p.i. (see Figure 5.d.). The ability to induce high viral loads and high infectious viral burdens appears to be inversely correlated with the ability to utilise CXCR4 *in vitro*. Both G8M(425) and $G8_{MYA}$ have a reduced ability to utilise CXCR4 *in vitro* but produce higher viral loads in the plasma. Interestingly, the chimaera containing the *env* gene from the terminal field isolate produced viral RNA loads which, in general, were intermediate. This isolate demonstrated a greater ability to utilise CXCR4 than the chimaera containing the *env* gene from an asymptomatic isolate. The inverse correlation between CXCR4-tropism and viral RNA loads suggests that viruses which readily utilise CXCR4 *in vitro* may display decreased pathogenicity *in vivo*.

Unfortunately, the proviral load data from the PBMCs and the tissues collected post-mortem was unreliable. There was evidence of contamination within the assay and the proportion of the coefficients of variation >30% undermined the validity of the data; therefore, these data could not be included. However, the infectious viral burdens achieved in this study by the chimaeras G8M(425), G8M(827) and the clone $G8_{MYA}$ were lower than those recorded in the earlier study described in Chapter 4. The dose of inoculum and route of inoculation was identical to the previous study so this phenomenon may be due to the nature of cloned chimaeras. Inserting a novel *env* within the $G8_{MYA}$ viral backbone may result in suboptimal functioning of the virus, as proteins derived from the novel gene may be less compatible with the parental viral structural proteins e.g. MA. Nevertheless, the isolation of the *env* genes from different isolates and the construction of chimaeras with the same viral backbone permitted a comparison of the biological behaviour attributable to each *env*. The results presented in this chapter are consistent with the *env* gene having a role in determining the pathogenicity of an isolate as well as its cell tropism *in vitro*.

Chapter Six

ANALYSIS OF LYMPHOCYTE POPULATIONS

6.1. Introduction

The principal target for FIV, like HIV, is the $CD4^+$ T lymphocyte (Dalgleish et al. 1984), although CD4 is not used by FIV as a receptor or coreceptor (Hosie et al. 1993; Norimine et al. 1993; Willett and Hosie 1999) and infection with both viruses leads to depletion of the $CD4^+$ subpopulation (Ackley et al. 1990; Novotney et al. 1990; Hoffman-Fezer et al. 1992). The degree of immune impairment that is characteristic of FIV infection is associated with the decline in $CD4^+$ T lymphocyte numbers (Torten et al. 1991). $CD8^+$ T lymphocytes are also targets for FIV infection (Brown et al. 1991) but their numbers tend to increase (Willett et al. 1993) resulting in an inversion of the CD4:CD8 T lymphocyte ratio. This expanded cell population was found to express lower levels of CD8 ($CD8^{low}$) (Lehmann et al. 1992; Willett et al. 1993) and increased levels of major histocompatibility complex II (MHC II) (Willett et al. 1993). The $CD8^+$ T lymphocyte population has been further defined in that the CD8 marker on the cell surface exists as either a homodimer molecule CD8αα or the heterodimer CD8αβ (Shimojima et al. 1998a; Shimojima et al. 1998b). In FIV infection an expansion of the CD8αβ population has been demonstrated but the β-chain has been shown to have much lower expression, therefore, the subpopulations have been designated $CD8\alpha^+\beta^{low}$ or $CD8\alpha^+\beta^-$. Studies in humans receiving highly active antiretroviral therapy (HAART) have been shown to express increased levels of the CD8 lymphocyte heterodimer CD8αβ, which is correlated with increased expression of molecules for lymphocyte activation, adhesion and cytotoxic T cell activity, leading to the possibility that this subpopulation of cells may be used to analyse the immune status of HIV infected individuals (Schmitz et al. 1998). FIV-infected cats have an increased $CD8\alpha^+\beta^{low}$ population and these cells have been shown to have anti-FIV activity and also to lack the L-selectin marker ($CD62L^-$) (Bucci et al. 1998b; Gebhard et al. 1999). Studies of the pathogenicity of two FIV isolates revealed that the more pathogenic isolate, FIV-GL8, caused rapid expansion of the $CD8\alpha^+\beta^{low}$ subpopulation in the early stages of infection (Hosie et al. 2002), accompanied by lower $CD4^+$ T lymphocyte numbers and higher proviral burdens in the PBMCs (Hosie et al. 2002). In contrast, the clone FIV-PET produced lower proviral burdens in the PBMCs and had no effect on CD4 or CD8 T

lymphocyte populations. Therefore, it was suggested that more pathogenic isolates may induce greater perturbation of the lymphocyte populations.

Using the above criteria, we studied the dynamics of the lymphocyte subpopulations of five groups of two SPF cats described in Chapter 4 that were infected with either field isolates or the infectious molecular clone FIV-$GL8_{414}$. Viral isolates from cats in the asymptomatic stages of infection induced greater $CD8^+$ T lymphocyte expansion compared to isolates from cats in the terminal stages of disease. In Chapter 5, CXCR4 usage *in vitro* was shown to correlate with low infectious viral burden, demonstrated by QVI and low proviral loads in the PBMCs. Figure 6.a. summarises the viral origins of the isolates used in the two studies. In this chapter we examine the correlation of disease stage and lymphocyte activation. Furthermore, we examine the role of Env in this process by isolating *env* genes from viruses collected from cats at different stages of infection and then inserting these *env* genes into a $GL8_{MYA}$ viral backbone and comparing the dynamics of lymphocyte activation with that of the prototype clones PET_{F14} and $GL8_{MYA}$.

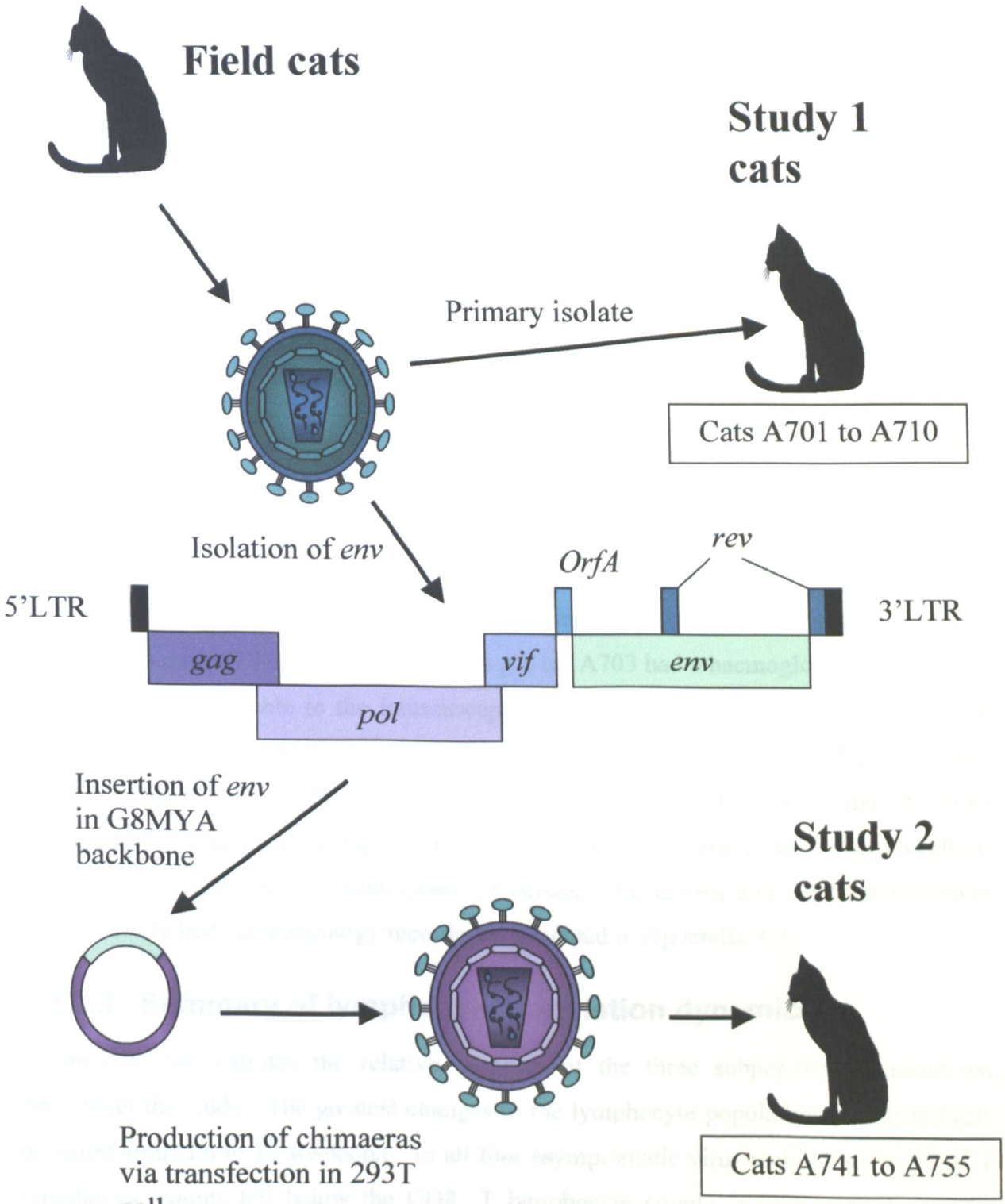
6.2. Materials and methods

6.2.1. Antibodies

Antibodies were used either unconjugated or conjugated to phyco-erythrin (PE) or fluorescein isothiocyanate (FITC). Anti-feline CD4-FITC (vpg34) and CD8αβ-PE (vpg9) originated in the Retrovirus Research Laboratory and were produced by Serotec Ltd., Oxford, United Kingdom; anti-feline CD8α (12A3) was obtained from Y. Nishimura, University of Tokyo, Tokyo, Japan; and anti-feline CD8β-FITC and -PE (FT2) was obtained from Southern Biotechnology Ltd., Birmingham, Alabama. FITC-conjugated anti-feline CD8α (12A3) was prepared using FITC coupling reagent (Pierce Chemical company Rockford, Illinois) according to the manufacturer's instructions. Unconjugated primary antibodies were detected using a FITC- or PE-coupled $F(ab')_2$ fragment of sheep anti-mouse immunoglobulin G whole molecule (Sigma).

PBMCs were isolated from blood samples collected into EDTA following whole blood lysis in 0.88% ammonium chloride/0.01M Tris-HCl pH7.4 and resuspended in 200µl of phosphate buffered saline supplemented with 0.1% sodium azide (PBA) and 1% bovine serum albumin. A volume of 30µl of cells from each sample was then incubated for 30

Figure 6.a. Schematic representation of primary isolates used in the study and the provenance of chimaeras used in study 2.



minutes on ice with primary antibodies recognising feline CD4, feline CD8, feline CD8α and feline CD8β. The cells were then washed twice with PBA by centrifugation before the addition of PE-conjugated f(ab')$_2$-fragment of sheep anti-mouse IgG, incubated on ice for 30 minutes and washed. Following the final incubation with FITC-conjugated secondary antibody, the cells were washed twice with PBA before analysis on an EPICS Elite flow cytometer using the EXPO analysis software package. Lymphocytes were live-gated on the basis of size and granularity with 10 000 events being collected for each sample. In addition, whole blood collected in EDTA was used for routine haematological analysis.

6.3. Results

6.3.1. The field isolates

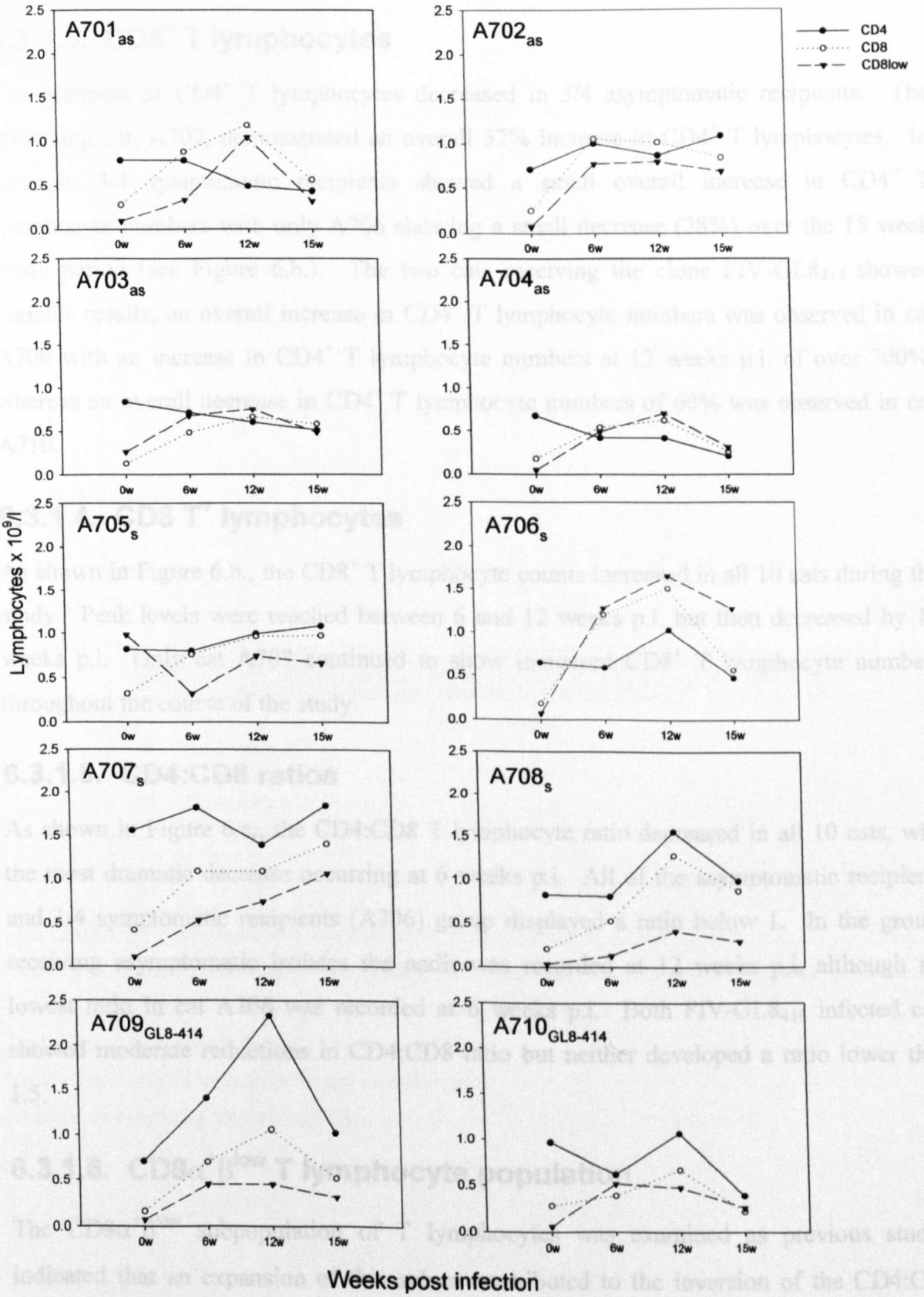
6.3.1.1. Routine haematology of cats A701 to A710

A study of healthy experimental juvenile cats showed marked variation in haematological parameters (Anderson et al. 1971). In this 15-week study all cats had mildly lowered haematocrits at least once, but only cats A701 and A703 had markedly lowered levels at 15 and 6 weeks p.i. respectively, however haemoglobin levels were generally nearer or within the reference range (10-15 g/dl). At 6 weeks p.i. cat A703 had a haemoglobin level of 7.07 g/dl, possibly attributable to the intussusception and corrective surgery; cat A701 had a level of 7.85 g/dl at 15 weeks p.i. Neutropenia was recorded in six cats A702, A705 and A706 at 15 weeks p.i., A709 at 15 weeks p.i. and A704 and A710 at 6, 12 and 15 weeks p.i. Cat A703 had toxic changes at 6 weeks p.i. presumed due to the intussusception. There was no correlation or trend observed between the groups and the haematological parameters studied. Haematology records are tabulated in Appendix A.1.

6.3.1.2. Summary of lymphocyte population dynamics

Figure 6.b. demonstrates the relative numbers of the three subpopulations examined throughout the study. The greatest changes in the lymphocyte population of infected cats occurred around 6 to 12 weeks p.i. In all four asymptomatic virus recipients, the $CD4^+$ T lymphocyte counts fell below the $CD8^+$ T lymphocyte counts at least once during the study. In three of four cats the $CD8\alpha^+\beta^{low}$ cell population was also greater than the $CD4^+$ population at least once during the study. Of the symptomatic isolate recipients, only

Figure 6.b. Lymphocyte dynamics throughout the course of the study.



A701-A704 were asymptomatic virus recipients, A705-A708 were symptomatic virus recipients. A709-A710 received the clone FIV-GL8414. A decline in CD4 lymphocyte numbers can be seen in A701, A703, A704, A706 and A710.

A706 showed a similar trend but a more marked expansion of the $CD8^+$ T lymphocyte population was observed in this cat.

6.3.1.3. $CD4^+$ T lymphocytes

The numbers of $CD4^+$ T lymphocytes decreased in 3/4 asymptomatic recipients. The remaining cat, A702, demonstrated an overall 52% increase in $CD4^+$ T lymphocytes. In contrast, 3/4 symptomatic recipients showed a small overall increase in $CD4^+$ T lymphocyte numbers with only A706 showing a small decrease (28%) over the 15 week study period (see Figure 6.b.). The two cats receiving the clone FIV-$GL8_{414}$ showed variable results; an overall increase in $CD4^+$ T lymphocyte numbers was observed in cat A709 with an increase in $CD4^+$ T lymphocyte numbers at 12 weeks p.i. of over 300%, whereas an overall decrease in $CD4^+$ T lymphocyte numbers of 60% was observed in cat A710.

6.3.1.4. CD8 T^+ lymphocytes

As shown in Figure 6.b., the $CD8^+$ T lymphocyte counts increased in all 10 cats during the study. Peak levels were reached between 6 and 12 weeks p.i. but then decreased by 15 weeks p.i. Only cat A707 continued to show increased $CD8^+$ T lymphocyte numbers throughout the course of the study.

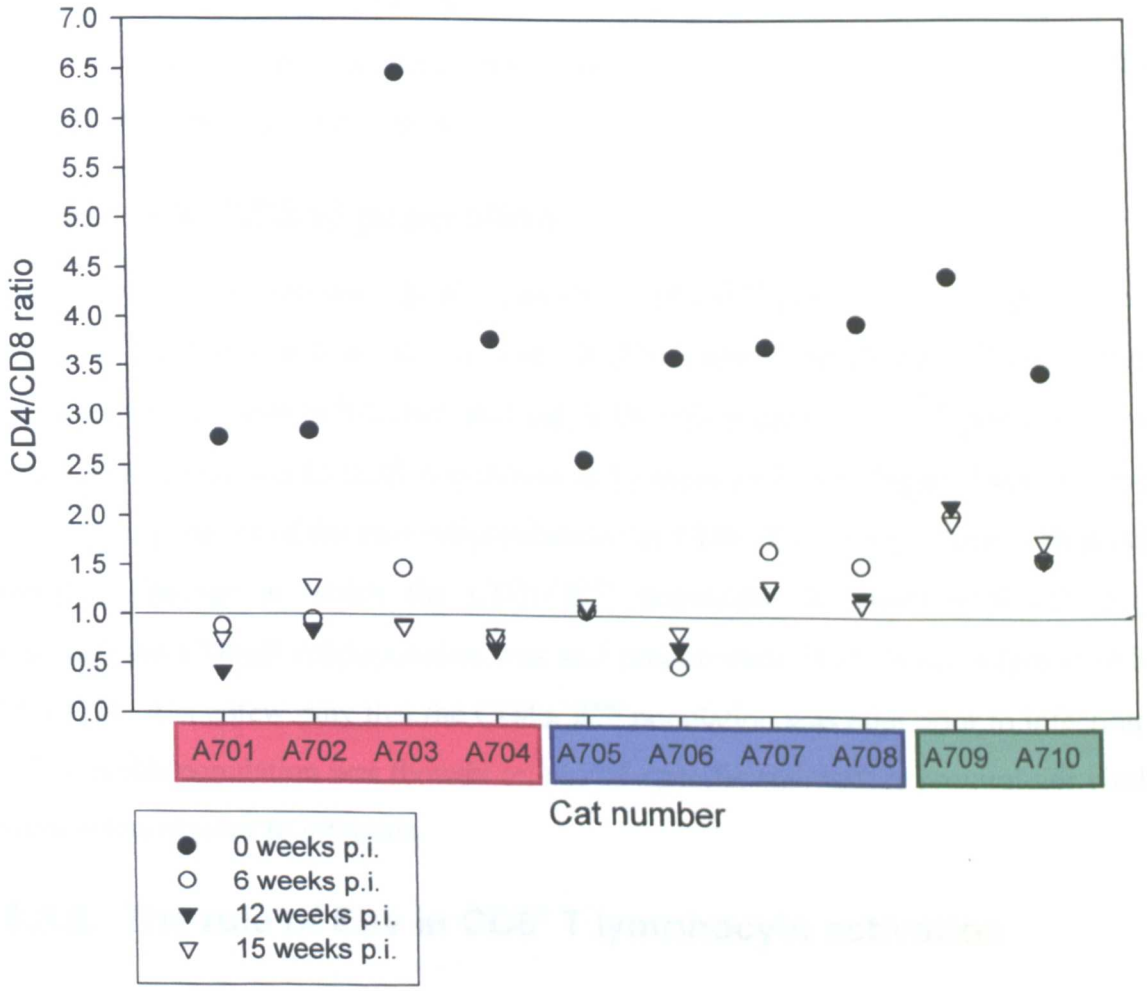
6.3.1.5. CD4:CD8 ratios

As shown in Figure 6.c., the CD4:CD8 T lymphocyte ratio decreased in all 10 cats, with the most dramatic decrease occurring at 6 weeks p.i. All of the asymptomatic recipients and 1/4 symptomatic recipients (A706) group displayed a ratio below 1. In the groups receiving asymptomatic isolates the nadir was recorded at 12 weeks p.i. although the lowest ratio in cat A706 was recorded at 6 weeks p.i. Both FIV-$GL8_{414}$ infected cats showed moderate reductions in CD4:CD8 ratio but neither developed a ratio lower than 1.5.

6.3.1.6. $CD8\alpha^+\beta^{low}$ T lymphocyte population

The $CD8\alpha^+\beta^{low}$ subpopulation of T lymphocytes was examined as previous studies indicated that an expansion of this subset contributed to the inversion of the CD4:CD8 ratio and may characterise more pathogenic viruses (Hosie et al. 2002). In general, the cats

Figure 6.c. $CD4^+$:$CD8^+$ lymphocyte ratios in PBMCs throughout the study.



The CD4:CD8 T lymphocyte ratio was calculated for each cat at 0, 6, 12 and 15 weeks p.i.

The black bar indicates the ratio 1. All asymptomatic recipients have at least one time point where the ratio is below 1 and only A706 of the symptomatic recipients is below 1.

Red = cats receiving virus from the asymptomatic phase

Blue = cats receiving virus from the symptomatic phase

Green = cats receiving virus clone $GL8_{414}$

receiving the asymptomatic isolates had a higher percentage of their $CD8^+$ lymphocyte population represented by $CD8\alpha^+\beta^{low}$ lymphocytes when compared to the cats receiving isolates from terminal cases. Although these findings were not statistically significant, a trend was clearly evident (see Figure 6.d.). Cat A706 displayed a dramatic change in lymphocyte numbers and the CD8αβ lymphocyte count of this individual was the highest of all 10 cats at 6 and 12 weeks p.i., consistent with a total increase of the $CD8^+$ T lymphocyte population (see Figure 6.e.).

6.3.1.7. The CD8αβ population

The CD8αβ population was greater than the $CD8\alpha^+\beta^{low}$ population throughout the study except in cat A703 at 6 weeks p.i. and cat A705 where the $CD8\alpha^+\beta^{low}$ population was markedly higher prior to infection and cat A706 where the $CD8\alpha^+\beta^{low}$ population was 2.5 times greater than the CD8αβ population at 15 weeks p.i. (see Figure 6.e.). Overall, the relative proportions of the two subpopulations of $CD8^+$ T cells were similar to previously reported findings in which the $CD8\alpha^+\beta^{low}$ population increased with FIV infection although the CD8αβ subpopulation was still predominant in the early stages (Gebhard et al. 1999). It is noteworthy that the $CD8\alpha^+\beta^{low}$ population was high prior to infection in cat A705 as this population was thought to be FIV-specific and neither proviral nor viral loads were detected prior to infection.

6.3.2. The role of Env in $CD8^+$ T lymphocyte activation

6.3.2.1. Routine haematological analysis of cats A741 to A755

Of the cats inoculated with chimaeric viruses containing *env* from field isolates in the G8M backbone, three cats (A741, A748, A750) had low haematocrits accompanied by low haemoglobin levels at the time of infection. However, these increased to near the reference range by 3 weeks p.i. Six cats were neutropenic at some time during the study, four (A742, A743, A745, and A750) at 15 weeks p.i. A754 at 0 and 15 weeks p.i. and A755 at 12 weeks p.i. Moderate neutrophilia was recorded in two A746 and A751 at 3 and 6 weeks p.i. Full haematological results were unavailable for the following cats at week 0 – A744, A745, A746, A747, A753 and A755.

6.3.2.2. The CD4:CD8 T lymphocyte ratio

In general, among the cats, there was a mild decline in the CD4:CD8 T lymphocyte ratio following infection with only cat A748 showing an increased ratio during the time course of the study. The CD4:CD8 T lymphocyte ratio did not fall below 1 in any of the cats at any time during the 15 week study period (see Figure 6.f.).

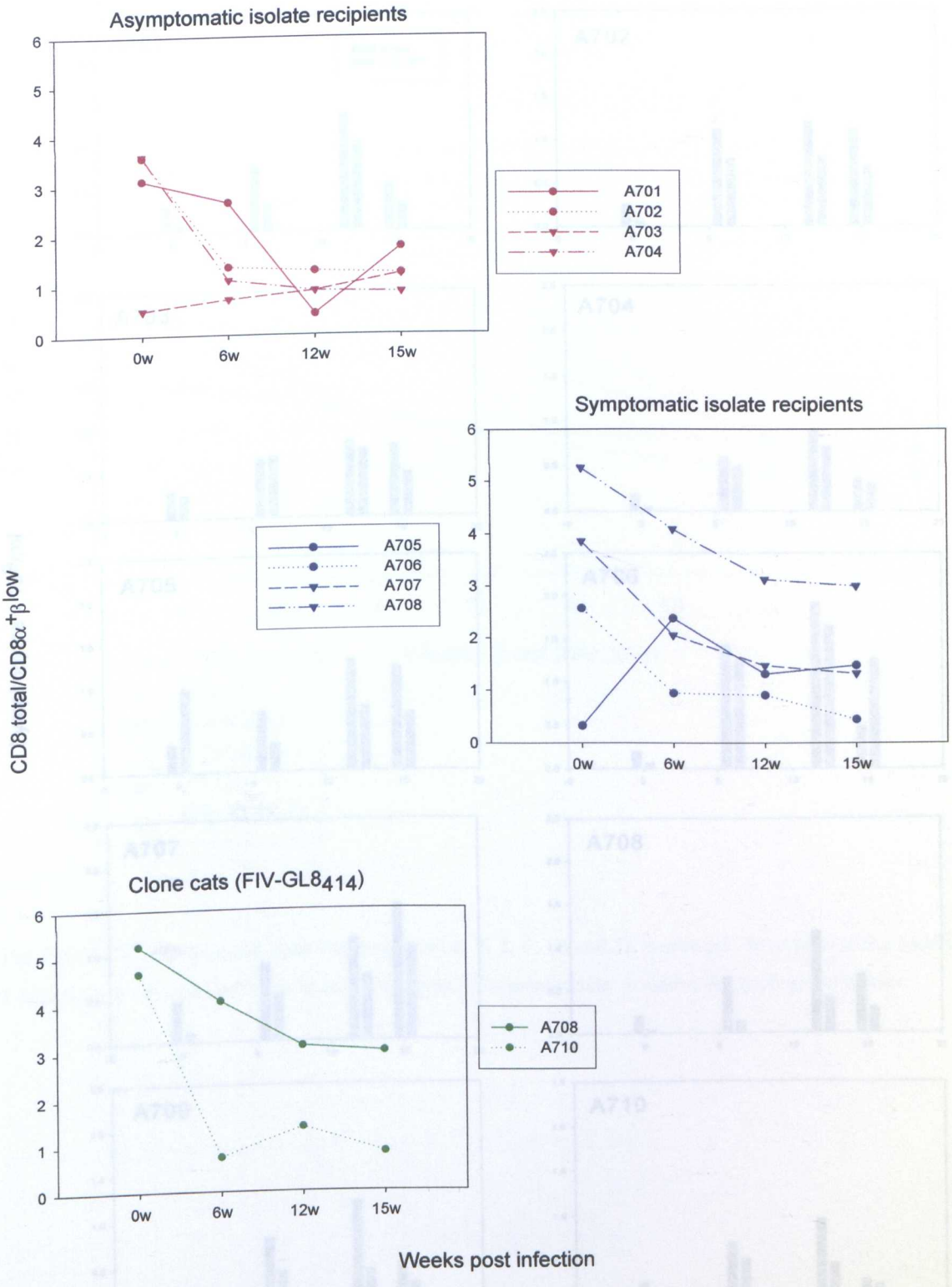
6.3.2.3. The activated T lymphocyte subset: $CD8\alpha^{+}\beta^{low}$ T lymphocytes

The chimaera G8M(425) and the clone FIV-$G8_{MYA}$ induced significant expansion of the percentage of $CD8\alpha^{+}\beta^{low}$ subpopulation. The G8M(425) chimaera induced a rapid expansion from a mean of 6.53% (± 0.61 SE) at 3 weeks p.i. to 9.9% (± 0.35 SE) at 15 weeks p.i. (see Figure 6.g.). $G8_{MYA}$ induced a gradual expansion of the $CD8\alpha^{+}\beta^{low}$ population within the infected cats. The mean maximum population was 7.53% (± 0.5 SE) at 15 weeks p.i. There was a statistically significant greater expansion of the $CD8\alpha^{+}\beta^{low}$ lymphocyte subset following infection with FIV-G8M(425) compared to G8M(F14.7) from 3 weeks p.i. until post-mortem examination at 15 weeks p.i. ($p<0.05$ at 3-12 weeks p.i. and $p<0.01$ by 15 weeks p.i., Tukey test). Similarly, G8M(425) induced a significantly greater expansion of this lymphocyte subset compared to PET_{F14} and G8M(827) at 15 weeks p.i. ($p<0.001$ and $p<0.007$, respectively). The prototype clone $G8_{MYA}$ also induced a statistically significant increase of the $CD8\alpha^{+}\beta^{low}$ subset compared to G8M(F14.7) and PET_{F14} ($p<0.01$ and $p<0.02$, respectively) at 15 weeks p.i.

6.4. Discussion

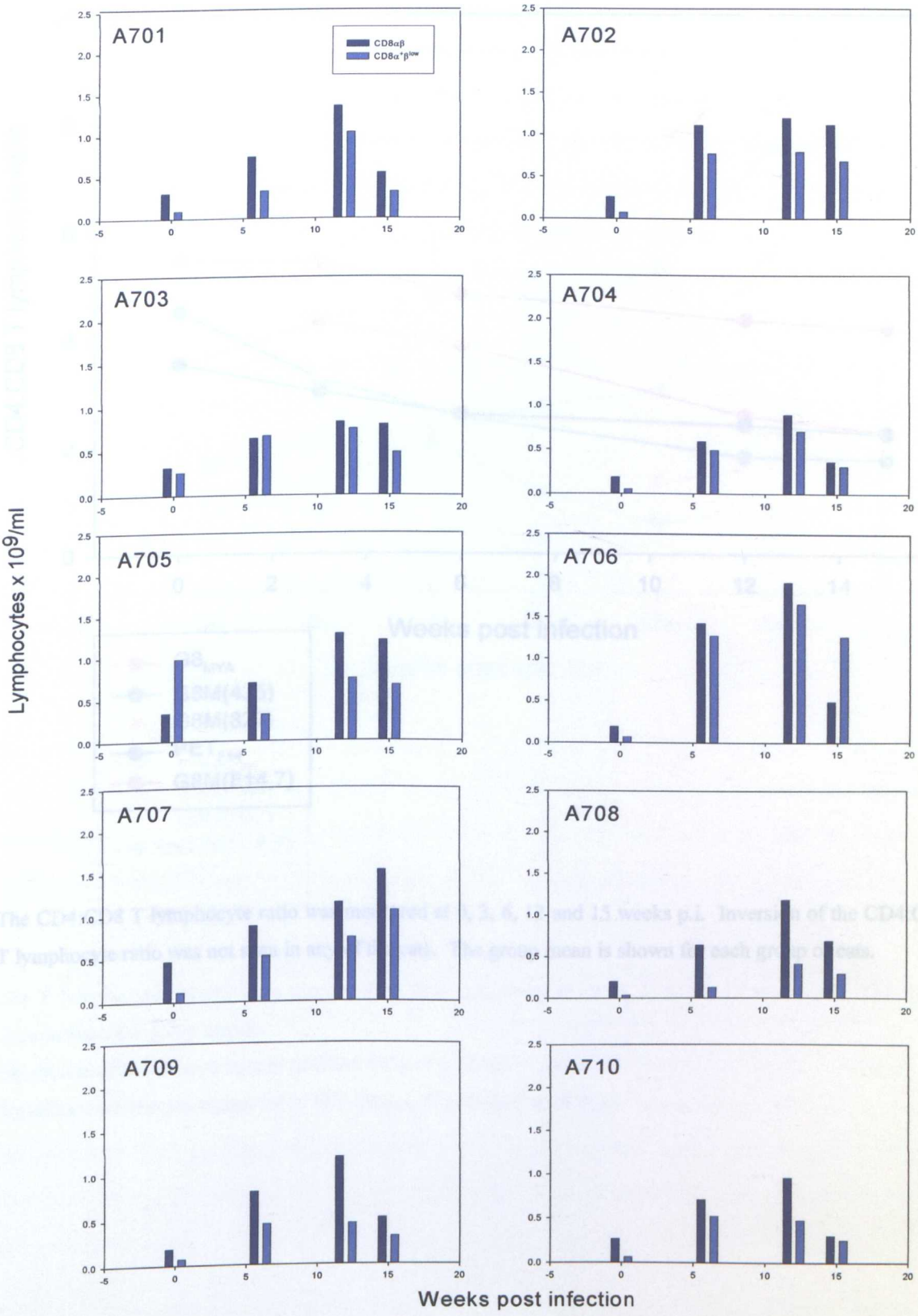
When the lymphocyte subpopulations were examined following infection with four field isolates, the greatest changes were observed in cats infected with the asymptomatic isolates $F0425H_{as}$ and $F0556H_{as}$, with the CD4:CD8 ratios of all four recipients falling to less than 1 during the 15 week study. In contrast, cats inoculated with the symptomatic isolates maintained higher CD4:CD8 ratios with a decrease below 1.0 being observed only in a single cat. Interestingly, the cats inoculated with the virus derived from the FIV-$GL8_{414}$ molecular clone did not develop ratios below 1.5. FIV-GL8 is a well-described isolate known to be pathogenic and resistant to vaccine induced protection (Hosie et al. 1995; Hosie and Flynn 1996b; Hosie et al. 1998b; Hosie et al. 2000) and inducing high proviral loads post infection (Hosie et al. 2002). Therefore the maintenance of CD4:CD8

Figure 6.d. CD8 population ratios.



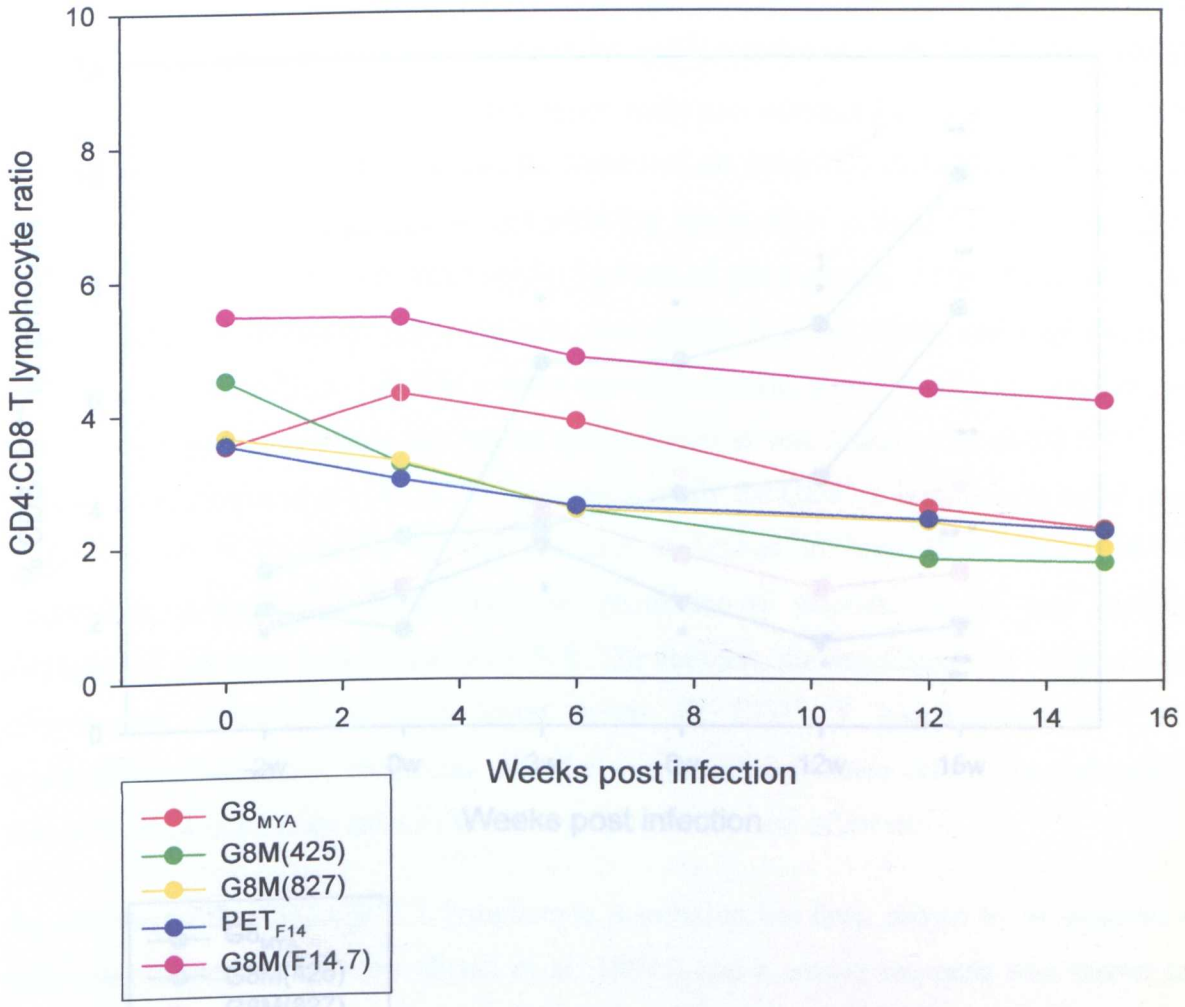
CD8total/CD$\alpha^{+}\beta^{low}$ ratios were plotted for each cat throughout the 15 week study. The cats were grouped as asymptomatic, symptomatic and clone recipients.

Figure 6.e. CD8αβ and CD8α^{+}β^{low} absolute numbers.



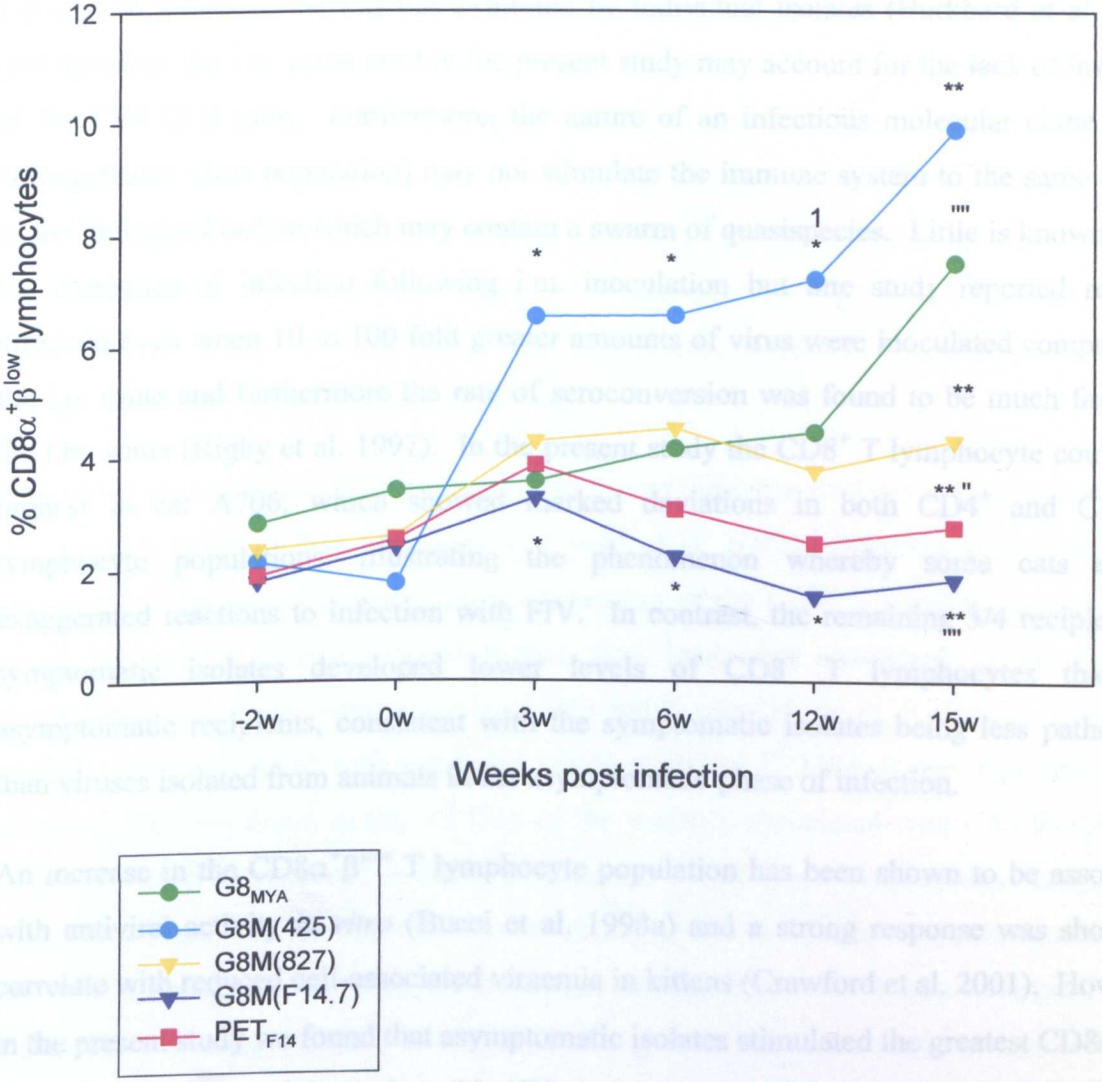
The CD8αβ and CD8α^{+}β^{low} T lymphocyte subsets were measured at 0, 6, 12 and 15 weeks p.i. Each cat is represented by an individual histogram.

Figure 6.f. CD4:CD8 T lymphocyte ratio.



The CD4:CD8 T lymphocyte ratio was measured at 0, 3, 6, 12 and 15 weeks p.i. Inversion of the CD4:CD8 T lymphocyte ratio was not seen in any of the cats. The group mean is shown for each group of cats.

Figure 6.g. CD8αβ T lymphocyte populations.



The T lymphocyte subsets were measured by flow cytometry at –2, 0, 3, 6, 12, 15 weeks p.i. The figure demonstrates the group means.

Significant differences compared to G8M(425). (* $p<0.05$, ** $p<0.01$).

Significant differences compared to FIV-$G8_{MYA}$. (" $p<0.05$, "" $p<0.01$).

ratios in the FIV-GL8$_{414}$ infected cats in the present study was unexpected. However, in previous studies where inversion of the CD4:CD8 ratio was observed, cats were inoculated with FIV-GL8 by the i.p. route, (Hosie et al. 2000). The route of infection has been reported to influence the kinetics exhibited by individual isolates (Burkhard et al. 2002) and therefore the i.m. route used in the present study may account for the lack of inversion of the CD4:CD8 ratio. Furthermore, the nature of an infectious molecular clone (i.e. a homogeneous virus population) may not stimulate the immune system to the same degree as the biological isolate which may contain a swarm of quasispecies. Little is known about the dynamics of infection following i.m. inoculation but one study reported reduced viraemia even when 10 to 100 fold greater amounts of virus were inoculated compared to the i.p. route and furthermore the rate of seroconversion was found to be much faster by the i.m. route (Rigby et al. 1997). In the present study the $CD8^+$ T lymphocyte count was highest in cat A706, which showed marked deviations in both $CD4^+$ and $CD8^+$ T lymphocyte populations, illustrating the phenomenon whereby some cats exhibit exaggerated reactions to infection with FIV. In contrast, the remaining 3/4 recipients of symptomatic isolates developed lower levels of $CD8^+$ T lymphocytes than the asymptomatic recipients, consistent with the symptomatic isolates being less pathogenic than viruses isolated from animals in the asymptomatic phase of infection.

An increase in the $CD8\alpha^+\beta^{low}$ T lymphocyte population has been shown to be associated with antiviral activity *in vitro* (Bucci et al. 1998a) and a strong response was shown to correlate with reduced cell-associated viraemia in kittens (Crawford et al. 2001). However, in the present study we found that asymptomatic isolates stimulated the greatest $CD8\alpha^+\beta^{low}$ expansion in the recipients but this did not correlate with decreased levels of cell-associated virus. Indeed, the proviral loads of the asymptomatic isolate recipients were stable or continued to increase to the end of the 15 week study (see Figure 4.c.). Interestingly, the cats inoculated with FIV-GL8$_{414}$ developed lower $CD8\alpha^+\beta^{low}$ lymphocyte levels than the asymptomatic recipients, in contrast to previous studies. However, it may be postulated that a cloned population of a single virus rather than a swarm may stimulate the immune system to a lesser degree or that the route of infection altered the outcome as discussed above.

In HIV-1 infection, patients in the terminal stages of disease tend to be infected with SI, T-lymphotropic viruses that have been shown to have greater sequence heterogeneity than

(NSI) monocytotropic variants, which are generally found in the asymptomatic stage of the disease (Chesebro et al. 1992), during which time the virus replicates most rapidly (Connor and Ho 1994). We postulate that asymptomatic FIV variants may stimulate the $CD8\alpha^{+}\beta^{low}$ population if a similarly high rate of replication and concurrent production of variant viruses occurs. However the present study was too short to test whether increased $CD8\alpha^{+}\beta^{low}$ numbers correlated with decreased cell-associated virus. Also the sample size was very small so caution must be exercised in interpreting the results. However, the results of this study suggest that further investigations are merited, to examine more viruses isolated from the different stages of infection in larger groups of cats, which may highlight differences in replication rate and immunogenicity between isolates from asymptomatic and terminal stages of disease.

Recently it was reported that viruses with increased virulence evolved in cats infected with an FIV-PET strain of low pathogenicity (Hosie et al. 2002). Infection with FIV-PET is characterised by low viral loads and no expansion of the $CD8^{+}$ T lymphocyte population while the variant viruses induced higher viral loads and expansion of the $CD8^{+}$ T lymphocyte population when inoculated into naïve cats (Hosie et al. 2002). Since this evolution was correlated with a reduced ability to utilise CXCR4, it was suggested that a mutation that was found in the V3 loop of the variants, associated with CXCR4-tropism, led to reversion to virulence *in vivo*. In Chapters 3 and 4, we demonstrated that isolates from early stages of infection had a lesser ability to utilise CXCR4 and subsequently that this phenotype correlated with the induction of higher proviral loads, inversion of the CD4:CD8 T lymphocyte ratio and significantly greater infectious viral burdens following *in vivo* infection of cats. Although the degree of lymphocyte activation in cats inoculated with asymptomatic isolates was not statistically significantly greater in cats inoculated with isolates from the terminal stages of disease, a trend was identified which prompted us to examine the degree of lymphocyte activation induced by the Env proteins from the field isolates already examined. The proportion of the $CD8^{+}$ T lymphocyte population *in vivo* represented by the activated phenotype $CD8\alpha^{+}\beta^{low}$ was significantly higher in cats following infection with the chimaera containing the asymptomatic *env* (G8M(425)) and the prototype clone FIV-$G8_{MYA}$ compared to the symptomatic isolate (G8M(827)) and the FIV-PET_{F14} clone and chimaera (G8M(F14.7)). These results suggest that isolates which use CXCR4 more readily *in vitro* induce less activation of the $CD8^{+}$ T lymphocyte

population *in vivo* and support the hypothesis of Hosie et al. (Hosie et al. 2002) indicating that CXCR4-tropism may correlate with the degree of $CD8^+$ T lymphocyte activation.

Cats inoculated with the clones FIV-$G8_{MYA}$, FIV-PET_{F14} or the chimaeras G8M(425), G8M(827) and G8M(F14.7) did not display inversion of the CD4:CD8 T lymphocyte ratio even though they received matched doses of virus compared to the biological field isolate trial. This may be attributable to lower infectious viral burdens, as demonstrated by the QVIs (see Figures 4.f. and 5.c.), suggesting that the rate of replication of these cloned viruses may be slower than the biological isolates. Furthermore, the degree of lymphocyte activation demonstrated by the cloned and chimaeric viruses was markedly less than that of the biological isolates suggesting that, as previously discussed, a homogeneous population of virus does not stimulate the expansion of the $CD8^+$ T lymphocyte subpopulation to the same degree as a mixed population of viral quasispecies.

In conclusion, the results presented in this chapter demonstrate that Env is a major determinant influencing activation of the lymphocyte populations and that the Env proteins from the asymptomatic isolate and the clone FIV-$G8_{MYA}$ induce significantly greater activation of the $CD8\alpha^+\beta^{low}$ cell population compared with the FIV-PET or symptomatic virus Env proteins. It appears that the Env proteins mediate their effect on virulence/immune activation via the interaction with CXCR4 and therefore further work will be required to clarify the role of CXCR4-tropism in lymphocyte activation.

Chapter Seven

THE ENVELOPE GLYCOPROTEIN OF FIV IN ISOLATES FROM ASYMPTOMATIC AND SYMPTOMATIC CATS

7.1. Introduction

Primary isolates of HIV-1 have distinct biological characteristics *in vitro* and can be distinguished by replication rate, cell tropism and syncytium inducing capacity (Cheng-Mayer et al. 1988; Schuitemaker et al. 1992; Connor and Ho 1994). Isolates may be classified as syncytium-inducing (SI) or nonsyncytium-inducing (NSI) based on their ability to form syncytia upon infection of the T lymphocyte cell line MT-2 (Koot et al. 1992). Monocytotropic, NSI isolates are thought to be transmitted and furthermore have found to be the phenotype that is isolated most readily in early infection (Schuitemaker et al. 1992; Zhu et al. 1993; Connor et al. 1993a). In contrast, SI isolates appear later in infection in about 50% of patients (Tersmette et al. 1989b; Koot et al. 1993) and this has been shown to correlate with disease progression, coinciding with a marked reduction in $CD4^+$ T lymphocyte count *in vivo* and expanded cell tropism *in vitro* (Connor et al. 1997).

HIV-1 has been shown to employ cofactors, G-protein coupled receptors, for the infection of cells. The α-chemokine receptor CXCR4 is required for entry of T cell-adapted, SI strains (Broder and Berger 1995; Bleul et al. 1996; Feng et al. 1996; Oberlin et al. 1996), and monocytotropic, NSI strains have been shown to use the β-chemokine receptor CCR5 (Alkhatib et al. 1996; Deng et al. 1996; Dragic 1996). However, some viruses are capable of using more than one molecule as a coreceptor, for example, use of the chemokine receptors CCR3 and CCR2b has been documented (Choe et al. 1996; Doranz et al. 1996).

The major determinant of the cell tropism of HIV is the envelope glycoprotein with receptor usage being closely associated with cell tropism and biological phenotype (Björndal et al. 1997). Conflicting reports have been published regarding the determinants of cell tropism within Env, highlighting the adaptability of lentiviruses to overcome blocks to cell infection. The determinants of macrophage-tropism lie between the V1 and V3 regions of the Env and have ranged from a single amino acid mutation to a combination of mutations spanning the region (Cordonnier et al. 1989; O'Brien et al. 1990; Hwang et al.

1991; Shioda et al. 1991; Shioda et al. 1992; Westervelt et al. 1992; Koito et al. 1994; Koito et al. 1995).

T cell-tropism and SI phenotype determinants also lie within the V2 and V3 loops where an increase in length of V2 has been associated with the time of conversion from NSI to SI (Fouchier et al. 1995). Further, an increase in positive charge of the V3 loop has been reported to coincide with the SI phenotype (de Jong et al. 1992; Fouchier et al. 1992) and this phenomenon was further characterised by the 11/25K rule whereby a basic residue at either of these positions within the V3 loop had a high prediction rate for the SI phenotype (Hoffman et al. 2002). A hypervariable locus on the V2 loop has been reported to be predictive for NSI to SI phenotype conversion (Groenik et al. 1993).

The envelope glycoprotein of FIV has a similar structure to that of the primate lentiviruses. It consists of two subunits, the surface glycoprotein (SU or gp120), and the transmembrane (TM or gp41) protein. Within the SU and TM are a number of variable regions. Selection pressure enforced upon the virus by the host immune response leads to the acquisition of mutations within these regions. The error-prone nature of the viral reverse transcriptase results in the accumulation of mutations in the *env* sequence while the host immune response applies a selective pressure resulting in resistant viruses. The variation in the amino acid sequence is clustered in the nine hypervariable regions of FIV - six of which occur in the SU with the remaining three in the TM (Pancino et al. 1993b). The changes in the amino acid sequence of Env are thought to occur at a ten-fold greater rate of change than that in the *gag* or *pol* genes (Greene et al. 1993), suggesting that they may be driven by the host immune response.

The envelope glycoprotein of FIV, like HIV, has several important regions. The principal neutralising domain is located in the V3 region (Lombardi et al. 1993), as are determinants of cell fusogenicity (Pancino et al. 1995) and cell tropism (Verschoor et al. 1995; Siebelink et al. 1995b). The V3-V4 region of the SU and TM contain determinants of macrophage-tropism (Vahlenkamp et al. 1997; Vahlenkamp et al. 1999) and also four amino acids between the fusion protein and the membrane spanning region of TM have been shown to inhibit CrFK-tropism (Lombardi et al. 1996).

The first aim of this study was to compare the *env* gene sequences of four field isolates, two asymptomatic ($F0425H_{as}$ and $F0556H_{as}$) and two symptomatic isolates, ($F0795H_{s}$ and

F0827H_s), to identify amino acid sequences which might correlate with receptor utilisation, cell tropism, the ability to adapt to *in vitro* culture and virulence *in vivo*. The second part of the study evaluated *env* sequence changes which led to infection of CrFK(HO6T1) cells with two symptomatic isolates (F0795H_s and F0827H_s), by either (a) cell-free infection or by (b) cocultivation.

7.2. Materials and Methods

7.2.1. Preparation of DNA

The envelope gene was sequenced from plasmid DNA prepared as described in Chapter 5.

7.2.2. Sequencing the *env* genes

The *env* gene was sequenced using a panel of primers which spans the entire length of the *env* gene (see Figure 7.a.), FIVenv6190f (5'-GGC AGT TGC AAT CTA CAT TA-3'), FIVenv6353f (5'-ATG AAA AAG GGC CAC TAA ATC-3'), FIVenv6490f (5'-GAAGAAGGAAATGCAGGTAAG-3'), FIVenv7223f (5'-GTA CAG ACC CAT TAC AAA TCC-3'), FIVenv8274f (5'-GCA TCA AGT ACT AGT AAT AGG-3'), FIVenv8294r (5'-CCT ATT ACT AGT ACT TGA TGC TC-3'), FIVenv8461r (5'CCC CCA AAG TTA TAT TTC C-3') and sequencing was carried out on an ABI 3100 capillary sequencer using Big Dye terminator 2 by the methods described by Rosenblum (Rosenblum et al. 1997) and Sanger (Sanger et al. 1977). The numbering of the primers corresponds to their location in the FIV-$GL8_{MYA}$ molecular clone. Statistical analysis of results was carried out using the Wisconsin sequence analysis package (Devereux et al. 1984) (Genetics Computer Group, Inc., Wisconsin, Madison). Sequence data was analysed using Seqed and BESTFIT (Smith and Waterman 1981) and sequences were compared with published sequence data using BLAST (Lipman and Pearson 1988) (National Centre for Biotechnology Information).

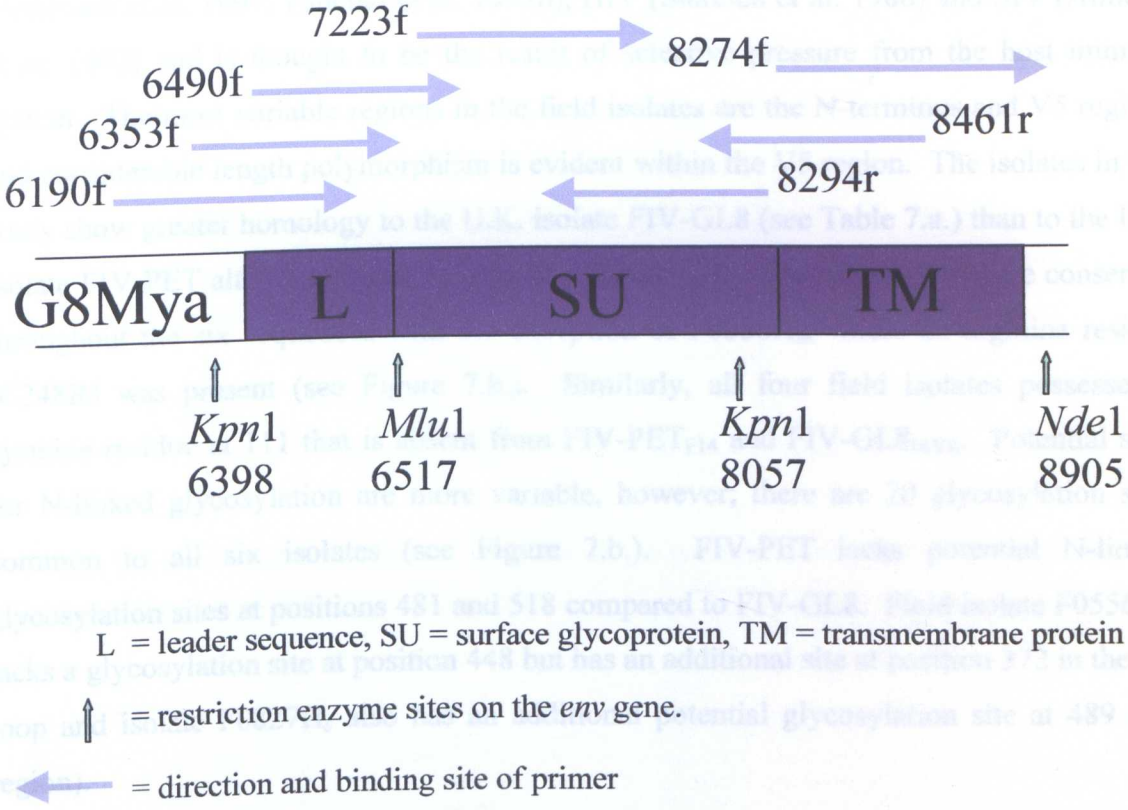
7.3. Field isolate sequences

7.3.1. Results

7.3.1.1. Comparison of *env* sequences

The full-length *env* genes of the four field isolates used in the *in vivo* study described in Chapter 4 were sequenced in order to identify sequence motifs that might be associated

Figure 7.a. Primer map on the envelope gene of FIV.



L = leader sequence, SU = surface glycoprotein, TM = transmembrane protein

= restriction enzyme sites on the *env* gene.

= direction and binding site of primer

with virulence and cell tropism. Variations in the *env* sequence occur throughout the gene but localise largely to the variable regions that form the external loops of the Env protein (see Figure 7.b.). This phenomenon is well documented for FIV (Phillips et al. 1990; Morikawa et al. 1991; Pancino et al. 1993b), HIV (Starcich et al. 1986) and SIV (Almond et al. 1992) and is thought to be the result of selective pressure from the host immune system. The most variable regions in the field isolates are the N-terminus and V5 regions and considerable length polymorphism is evident within the V5 region. The isolates in this study show greater homology to the U.K. isolate FIV-GL8 (see Table 7.a.) than to the U.S. isolate FIV-PET although all are subtype A viruses. All cysteine residues were conserved throughout the six sequences with the exception of F0556H_{as} where an arginine residue (C248R) was present (see Figure 7.b.). Similarly, all four field isolates possessed a cysteine residue at 111 that is absent from FIV-PET_{F14} and FIV-$GL8_{MYA}$. Potential sites for N-linked glycosylation are more variable, however; there are 20 glycosylation sites common to all six isolates (see Figure 7.b.). FIV-PET lacks potential N-linked glycosylation sites at positions 481 and 518 compared to FIV-GL8. Field isolate F0556H_{as} lacks a glycosylation site at position 448 but has an additional site at position 372 in the V3 loop and isolate F0827H_s also has an additional potential glycosylation site at 489 (V4 region).

7.3.1.2. Changes in the N-terminus of Env

The V1 and V2 regions occur in the putative leader sequence of *env* but do not occur on the mature Env protein and therefore are not included in Figure 7.b. (Morikawa et al. 1991; Verschoor et al. 1993; Pancino et al. 1993b). However, notable differences occur in the sequences of the N-termini (see Figure 7.b. and Table 7.b.). At amino acid 111 all four primary isolates possess a cysteine residue, while this is absent from FIV-PET_{F14} and FIV-$GL8_{MYA}$. Notable variation clusters occur where acidic residues have been gained by some of the primary isolates, F0425H_{as} at $S_{134}D$, F0556H_{as} at $S_{135}E$ and F0827H_s at $S_{134}D$. At amino acid 138 all four field isolates have a positive residue lysine, resulting in a cluster of highly charged residues in this region among the field isolates. A further cluster of changes is present at sites 255 and 256 (see Figure 7.b.) where F0556H_{as} has gained a positive charge ($R_{256}K$), F0795Hs has lost +1 ($K_{255}R$) and F0827H_s has a shift in positive charges ($K_{255}R$ and $R_{256}K$). Similarly, three of the isolates possess negative charges or a polar charge at 313 ($Y_{313}D/Q$, see Figure 7.b.).

Table 7.a. Field isolate nucleotide sequence comparison against the prototype viruses FIV-GL8 and FIV-PET.

Isolate	FIV-GL8 (%)	FIV-PET (%)
F0425H	93.69	92.33
F0556H	93.41	91.33
F0795H	95.44	92.16
F0827H	93.93	92.69

The field isolate *env* nucleotide sequence compared to the prototype viruses FIV-PETF14 and FIV-GL8 using SeqEd (Smith et al. 1981).

Figure 7.b. The amino acid sequence of the envelope glycoproteins.

```
          1
GL8414    MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EEGPLNPGIN PFRVPGITET
PETF14    MAEGFAANRQ WIGLEEAEEL LDFDIATQMS EEGPLNPGVN PFRVPGITEK
F0425H    MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK
F0556H    MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK
F0795H    MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK
F0827H    MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK

          51                                  ↓SU
          EKQDYCNMLQ PKLQALRNEI QEVKLEEGNA GKFRRARFLR YSDETILSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRARFLR YSDESVLSLV
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDETILSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDETVLSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDETILSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDEHVLSLI

          101
          HLFIGYCTYL LNRKELGSLR HDIDIEAPQE ECYSSREQGI TDNIKYGKRC
          HAFIGYCIYL GNRNKLGSLR HDIDIEAPQE ECYNNREKGT TDNIKYGRRC
          NLFVGYCTYL CRKNELGTLR HDIDIEVPQE ECYDNREKGI TDNIKYGRRC
          HLFIGYCTYL CNRNNLGSLR HDIDIEAPQE ECYNEKEKGI TDNIKYGRRC
          YLFIGYCTYA CNRNRLGSLR HDIDIEAPQE ECYNNREKGT TENIKYGGRC
          HLFIGYCTYL CNSRKLGSLR HDIDIEAPQE ECYDNKEKGT TANIKYGRRC

          151
          FIGTAGLYLL LFIGVGIYLG TAKAQVVWRL PPLVVPVEES EIIFWDCWAP
          CLGTVTLYLI LFIGIIIYSQ TTNAQVVWRL PPLVVPVEES EIIFWDCWAP
          IIGTATLYLL LFIGIIIYTK TTSAQVVWRL PPLVVPVEES EIIFWDCWAP
          FIGTAVLYLI LFIGIIVHLQ TTSAQVVWRL PPLVVPVEES EIIFWDCWAP
          FIGTAGLYLL LFIGVGIYLG TTKAQVVWRL PPLVVPVEES EIIFWDCWAP
          IIGTAALYLI LFTGIIIYTQ TANAQVVWRL PPLVVPVDES EIIFWDCWAP

          201                 •
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAKEIWGTL FKKATRQCRR
          EEPACQDFLG AMIHLKAKTN ISIREGPTLG NWAREIWATL FKKATRQCRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAREIWGTL FKKATRQCRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAREIWGTL FKKATRQRRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAKEIWGTL FKKATRQCRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAREIWGTL FKKATRQCRR

          251    •            •     •                          •
          GRIWKRWNET ITGPLGCANN TCYNISVIVP DYQCYLDRVD TWLQGKVNVS
          GRIWKRWNET ITGPSGCANN TCYNVSVIVP DYQCYLDRVD TWLQGKINIS
          GRIWKRWNET ITGPVGCANN TCYNISVIVP DYQCYLDRVD TWLQGKVNIS
          GRIWKKWNET ITGPLGCANN TCYNISVIIP DYQCYLDRVD TWLQGKVNIS
          GRIWRRWNET ITGPLGCANN TCYNISVIVP DYQCYLDRVD TWLQGKVNIS
          GRIWRKWNET ITGPLGCANN TCYNISVVIP DYQCYLDRVD TWLQGKVNIS

          301                                •     •     •
          LCLTGGKMLY NKYTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE
          LCLTGGKMLY NKVTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE
          LCLTGGKMLY NKDTKQLSYC TDPLQIPLIN YTFGPNLTCM WNTSQIQDPE
          LCLTGGKMLY NKYTKQLSYC TDPLQIPLIN YTFGPNQTCK WNTSQIQGPE
          LCLTGGKMLY NKDTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE
          LCLTGGKMLY NKQTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE

          351       V3          •
          IPKCGWWNQI AYYNSCRWES TDVKFHCQRT QSQPGLWLRA ISSWKQRNRW
          IPKCGWWNQM AYYNSCKWEE AKVKFHCQRT QSQPGSWFRA ISSWKQRNRW
          IPKCGWWNQK AYYNSCRWEK TDVKFHCQRT QSQPGSWIRA ISSWRQRNRW
          IPKCGWWNQG AYYNSCRWES TNVTFHCQRK QSQPGSWRRA ISSWRQRNRW
          IPKCGWWNQK AYYNSCRWES TDVKFHCQRK QSQPGSWSRA ISSWKQRNRW
          IPKCGWWNQK AYYNSCRWEQ TDVKFQCQRT QSQPGSWHRT ISSWKQSNRW

          401                 •   •                          •
          EWRPDFESEK VKVSLQCNST KNLTFAMRSS GDYGEVTGAW IEFGCHRNKS
          EWRPDFESKK VKISLQCNST KNLTFAMRSS GDYGEVTGAW IEFGCHRNKS
          EWRPDFESEK VKVSLQCNST KNLTFAMRSS GDYGEVTGAW IEFGCHRNKS
          EWRPDFESEK VKVSLQCNST KNLTFVMRSS GDYGEVVGAW IEFGCHRKKS
          EWRPDFESEK VKISLQCNST KNLTFAMRSS SDYGEVMGAW IEFGCHRNKS
          EWRPDFESEK VKISLQCNST KNLTFAMRSS GDYGDITGAW IEFGCHRNKS
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        451 V4                •                 •         •           •
GL8414  KLHTEARFRI RCRWNVGDNT SLIDTCGETQ NVSGANPVDC TMYANRMYNC
PETF14  KLHAEARFRI RCRWNVGSNT SLIDTCGNTQ KVSGANPVDC TMYSNKMYNC
F0425H  RRHSEARFRI RCRWNIGDNT SLIDTCGNTQ NVSGANPVDC TMYENKMYNC
F0556H  KLHDEARFRI RCRWNEGDNA SLIDTCGKTQ NISGANPVDC TMYANRMYNC
F0795H  RLHTEARFRI RCRWNVGDNT SLIDTCGKTQ NVSGANPVDC TMYTNRMYNC
F0827H  KLHTEARFRI RCRWNIGDNT SLIDTCGKTQ NVTGANPVNC TMYANSMYNC

        501                   •                 •       V5          •
        SLQNGFTMKV DDLIMHFNMT KAVEMYNIAG NWSCTSDLPP TWGYMNCNCT
        SLQNGFTMKV DDLIMHFNMK KAVEMYNIAG NWSCTSDLPS SWGYMNCNCT
        SLQNGFTMKV DDLIMHFNMT KAVGMYNIAG NWSCTSDLPP TWGYMNCNCT
        SLQSGFTMKV DDLIMHFNMT KAVELYNIAG NWSCTSDLPR DWGYMNCNCT
        SLQNGFTMKI DDLIMHFNMT KAVEMYNIAG NWSCTSDLPP TWGYMNCNCT
        SLQNGFTMKV DDLIMHFNMT KAVEMYNIAG NWSCKSDLPP TWGYMNCNCT

        •551                                                V6
        NSSST..N.S VKMACPKNQG ILRNWYNPVA GLRQSLEKYQ VVKQPDYLVV
        NSSSS..YSG TKMACPSNRG ILRNWYNPVA GLRQSLEQYQ VVKQPDYLVV
        NSSDN..SND KKMECPGKQG ILRNWYNPVA GLRQSLEKYQ VVKQPDYLVV
        NGSDT....T TKMACPGKQG ILRNWYNPVA GLRQSLERYQ VVKQPDYLVV
        NSTTSGGT.S NKMACPNNQG ILRNWYNPVA GLRQSLEKYQ VVKQPDYLVV
        NSTSSVSTSS VQMACPSHQG ILRNWYNPVA GLRQSLEKYQ VVKQPDYLVV

        601         SU↓TM
        PGEVMEYKPR RKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYHQVLAT
        PEEVMEYKPR RKRAAIHVML ALAAVLSIAG AGTGATAIGM VTQYHQVLAT
        PEEVMEYKPR KKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYQQVLAT
        PEEVMEYKPR RKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYHQVLAT
        PGEVMEYKPR RKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYHQVLAT
        PGEVMEYKPR RKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYHQVLAT

        651
        HQETIEKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG
        HQEAVEKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG
        HREAIDKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG
        HQEAIDKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG
        HQEAIEKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL STAFAMQELG
        HQEAIEKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG

        701                 •   •           •       •
        CNQNQFFCKV PPELWKRYNM TINQTIWNHG NITLGEWYNQ TKELQQKFYE
        CNQNQFFCKI PPELWTRYNM TINQTIWNHG NITLGEWYNQ TKDLQQKFYE
        CNQNQFFCKV PLELWKRYNM TINQTIWNHG NITLGEWYNQ TKGLQQKFYE
        CNQNQFFCKV PPELWQRYNM TINQTIWNHG NITLGEWYNQ TKKLQKNFYE
        CNQNQFFCKI PLELWEGYNM TINQTIWNHG NITLGEWYNQ TKDLQQKFYE
        CNQNQFFCKV PLILWQRYNM TINQTVWNHG NITLGEWYNQ TKDLQQKFYE

        751
        IIMNIEQNNV QGKKGLQQLQ EWEDWVGWIG NIPQYLKGLL GGILGIGLGI
        IIMDIEQNNV QGKKGIQQLQ KWEDWVGWIG NIPQYLKGLL GGILGIGLGV
        IIMDIEQNNV QGKQGLQQLQ KWEDWVGWIG NIPKYLKGLL GGILGIGLGV
        IIMDIEQNNV QGKKGLQQLQ EWESWVGWIR NIPQYLKGLM GGILGIGLGV
        IIMDIERNNV QGKKGLQQLQ EWEDWVGWIG NIPQYFRGLL GGILGIGLGV
        IIMDIEQNNV QGKKGLQQLQ EWEDWVGWIG NIPQYLKGLL GGILGIGLGV

        801                                                     851
        LLLILCLPTL VDCIRNCISK VLGYTVIAMP EIDDEEETVQ MELRKNGRQC GMS EKEEE
        LLLILCLPTL VDCIRNCIHK ILGYTVIAMP EVEGEEIQPQ MELRRNGRQC GMS EKEEE
        LLLILCLPTL VDCIRNCIHK ILGYTVIAMP EVEEEEIQPQ MELRRNGRQC GMS EKEEE
        LLLILCLPTL VDCIRNCIHK ILGYTVIAMP EVDREEFVQPQ MELRRNGRQC DMS EKEEE
        LLLILCLPTL VDCIRNCFHK ILGYTVIAMP EVEEEEIQPQ MELRRNGRQR GIS EEEEE
        LLLILCLPTL VDCIRNCIHK ILGYSVIAMP EIEGEEIQSQ MELRRNGRQC GIS EKEEE
```

Comparison of the primary isolates with FIV-GL8 and FIV-PET. Unique sequences are highlighted in blue and residues different from FIV-GL8 but the same as FIV-PET are highlighted in green. Variable regions are highlighted in grey. • highlights glycosylation sites and isolates involved are underlined. ↓ represents the start of the SU and TM subunits

Table 7.b. Amino acid differences in the variable loops of the *env* gene.

Isolate	V1								
F0425H	H101N	I104V		L111C	N112R	R113K	K114N		
F0556H				L111C			K114N	E115N	
F0795H	H101Y		L110A	L111C			K114N	E115R	
F0556H				L111C		R113S	K114R	E115K	

Isolate																		
F0425H	A127V	S134D	S135N		Q138K		K148R	F151I	G156T		V165I	G166I			L169T	G170K	A172T	K173S
F0556H		S134N	S135E	R136K	Q138K	D142E	K148R		G156V	L160I	V165I	G166I	I167V	Y168H		G170Q	A172T	K173S
F0795H		S134N	S135N		Q138K	D142A	K148G										A172T	
F0827H		S134D	S135N	R136K	Q138K		K148R	F151I	G156A	L160I	V165I	G166I			L169T	G170Q		K173N

Isolate	V3												
F0425H	I360K	S370K					L386S	L388I		K395R			
F0556H	I360G		D372N	K374T		T380K	L386S	L388R		K395R			
F0795H	I360K					T380K	L386S	L388S				V413I	
F0827H	I360K	S370Q			H376Q		L386S	L388H	A390T		R397S	V413I	

Isolate	V4								
F0425H	K450R	L452R	T454S	V466I		E478N			
F0556H			T454D	V466E	T470A	E478K	V482I		
F0795H	K450R					E478K			
F0827H				V466I		E478K		S483T	D489N

Isolate	V5															
F0425H					S554D	T555N			N558S	*559N	S560D	V561K		A564E	K567G	N568K
F0556H	P540R	T541D	S552G		S554D				N558*		S560T	V561T			K567G	N568K
F0795H				S553T	S554T	T555S	*556G	*557G	N558T			V561N			K567N	
F0556H				S553T		T555S	*556V	*557S	N558T	*559S			K562Q		K567S	N568H

Sequences are compared against FIV-GL8$_{414}$ with FIV-GL8$_{414}$ amino acid notation on the left and the field isolate notation on the right. Field isolate residues which match that of FIV-PET are highlighted in green.

7.3.1.3. The V3 region

At amino acids 360 and 388 all four primary isolates display unique residues and at residues 386 all four isolates possess a serine, similar to FIV-PET (see Figure 7.b. and Table 7.b.). Compared to FIV-GL8, F0425H_{as} possesses a positively charged residue at positions 360 and 370 ($I_{360}K$ and $S_{360}K$), isolate F0556H_{as} possesses a positive charge at 380 and 388 ($T_{380}K$ and $L_{388}R$), and also possesses a potential glycosylation site at residue 372 ($D_{372}N$). Both of these isolates have a decreased positive charge at residue 395 ($K_{395}R$, a loss of +1). Isolate F0795H_s possesses a positive charge at residues 360 and 380 ($I_{360}K$ and $T_{380}K$) and isolate F0827H_s has a positive charge at residue 360 ($I_{360}K$), and at residue 388 ($L_{388}H$), but possesses the polar residues glutamine at 370 and 376 ($S_{370}Q$ and $H_{376}Q$) and lastly has less positive charge at residue 397 ($R_{397}S$) by possessing a serine residue.

7.3.1.4. The V4 region

Many unique changes have occurred in the V4 region (see Figure 7.b.). F0425H_{as} possesses differences in charge compared to FIV-GL8 at residues 451 and 452 ($K_{451}R$ and $L_{452}R$). F0556H_{as} has negative charges at residues 454 and 466 ($T_{454}D$ and $V_{466}E$) and possesses a positive charge at residue 478 ($E_{478}K$) as do F0795H_s and F0827H_s (see Table 7.b.). Only the isolate F0827H_s possesses a potential glycosylation site at residue 489 ($D_{489}N$). However, all the isolates except FIV-PET_{F14} possess a potential glycosylation site at residue 481.

7.3.1.5. The V5 region

Length polymorphism is the main feature of the V5 region (see Figure 7.b.). In comparison to FIV-GL8, F0425H_{as} has one insertion, F0556H_{as} has one deletion, whereas F0795H_s has two insertions and F0827H_s has three insertions. The two asymptomatic isolates, F0425H_{as} and F0556H_{as}, have more charged residues in this region than the symptomatic isolates (see Figure 7.b.).

7.3.2. Discussion

The biological behaviour and molecular properties of field isolates of FIV are unknown, hence this study examined four isolates, two from asymptomatic cats and two from terminally ill cats, which had displayed quite different properties during *in vitro* tropism studies. The *env* sequences were analysed as this gene has previously been shown to

contain determinants of host cell tropism and fusogenicity (Phillips et al. 1990; Pancino et al. 1995) as well as the principal immunodominant domain (Lombardi et al. 1993). The four field isolates show greatest homology to the FIV-GL8 prototype virus which is the more virulent of the two prototypic viruses used in this study, but there was no correlation with stage of disease and *env* sequence homology to either the more virulent FIV-GL8 or the laboratory-adapted FIV-PET. The greatest differences amongst the six viruses compared were located in the N-terminal and V5 regions as may be predicted since the hypervariable regions are thought to arise due to selective pressure from the host immune response (Siebelink et al. 1993; Pancino et al. 1993a). Surprisingly, however, the V3 loop, the site of specific amino acid mutations which are associated with cell tropism and the ability to form syncytia for both FIV and HIV (Chesebro et al. 1992; Shioda et al. 1992; Shioda et al. 1994), is not the region of greatest divergence. Virus cultured *in vitro* would not experience immunological pressure, which would explain the lack of changes within the V3 neutralising domain. In HIV, the V1/V2 region has been shown to be a determinant of macrophage-tropism (Westervelt et al. 1992; Koito et al. 1994) and the V2 region was shown to modulate the conformation of the required V3 region (Koito et al. 1995). However, no correlation to clinical staging was evident among V1/V2 sequences in HIV-1 from asymptomatic and symptomatic patients (Hughes et al. 1997). The relevance of the large degree of variation in the V1 and V2 region of FIV is unclear as the differences occur in the putative leader sequence and are not present in the mature Env protein (Verschoor et al. 1993) although the first coding region for the *rev* gene is found in this site (Phillips et al. 1992). A further area of interest was the cluster of charge changes occurring in the C2 region. In HIV-1 the binding sites for CD4, CCR5 and CXCR4 occur within the N-terminal region of SU, more specifically the V1/V2 stem and bridging sheet, V3 and conserved regions of the constant region C4 (Rizzuto et al. 1998; Kwong et al. 1998; Basmaciogullari et al. 2002). V1 or V2 variable loops have not been assigned for FIV SU, however, the region N-terminal to the FIV V3 loop requires investigation for similar determinants of cell tropism which may constitute the receptor binding domain of FIV SU.

As previously reported (Sodora et al. 1994) V5 was the only region where length polymorphisms occurred. Mutations in this region were shown to play a role in evading the immune response (Siebelink et al. 1993) and broad neutralisation resistance in the laboratory-adapted strain FIV-PET in long-term viral revertants was attributed to an S557N

mutation in V5 (Bendinelli et al. 2001). These findings suggest that the immune response exerts selective pressure on the V5 region.

The viruses in this study came from cats infected in the field and will thus contain a range of virus types, or quasispecies within each population. The sequences shown here may represent the most common or average viruses in the population, or may represent virus types that are more conducive to PCR amplification. Incomplete primer extension and template switching during PCR has been documented and so these sequences may even represent a hybrid sequence of viruses within the population (Odelberg et al. 1995). Biological cloning as described by Connor (Connor et al. 1993b) would allow further investigation of the quasispecies contained within each isolate and the identification of viruses with different tropism properties which may offer greater information about sequence variation within an infected individual. Such information may clarify the correlation of sequence variation and biological behaviour *in vivo* and the relationship with tropism and chemokine receptor usage *in vitro*.

Finally, linear sequence analysis may render little information about gene motifs and possible correlations of receptor usage and virulence whereas 3-dimensional analysis (Yamaguchi-Kabata and Gojobori 2000) may reveal more information about sites which are structurally related but distant in linear sequence.

7.4. Sequences changes that permit growth in CrFK(HO6T1) cells

7.4.1. Results

7.4.1.1. Analysis of sequence changes

Two isolates from cats in the terminal stage of disease, F0795H_s and F0827H_s achieved limited and persistent infection of CrFK(HO6T1) cells by cell-free infection, respectively and both achieved persistent infection following cocultivation with infected Mya-1 cells (see Chapter 3). The *env* genes from the virus cultures were sequenced to identify any common nucleic acid sequence which correlated with CrFK(HO6T1)-tropism.

F0795H_s achieved only transient infection of CrFK(HO6T1) cells by cell-free infection at 17 days post infection. Comparison between the A (cell-free infection) and B (cocultivation) cultures 795A and 795B respectively, revealed that identical mutations occurred within the variable loops at residues 396, 413 and 451 ($Q_{396}H$, $I_{413}V$ and $R_{451}K$)

(see Figure 7.c. and Table 7.c.). Within the variable loops charge changes occurred in 3/4 of the variable regions in both cultures. The constant regions of each F0795H_s culture also revealed matching mutations, $S_{432}G$, $M_{438}K$ and $R_{496}K$, notably the latter two mutations resulted in acquisition of further positive charge within this region (see Figure 7.c.). Further mutations common to both isolates occurred within the transmembrane protein, $A_{600}E$, $T_{605}A$, $S_{642}Y$, $E_{717}K$ and $F_{787}L$ but only two of the mutations in the transmembrane protein (at residues 600 and 717) result in a charge change (see Figure 7.c.). All cysteine residues were conserved. The virus sequenced from the A culture lost a potential glycosylation site at site 448. However, all other potential glycosylation sites were conserved (see Figure 7.c. and Table 7.c.).

F0827H_s achieved persistent infection of CrFK(HO6T1) cells from 31 days post infection. Six matching mutations occurred throughout the gene, $A_{172}T$ and $L_{265}S$ occurring in the N-terminus, $K_{360}N$, $K_{488}E$ within the variable loops and a further two occurring within the TM protein, $L_{712}P$ and $V_{726}I$ (see Figure 7.d. and Table 7.c.). Charge changes occurred at only two of these residues, 360 and 488, however, the mutation at residue 712 resulted in an additional proline residue and might therefore induce significant structural changes. Virus from culture B displayed minor changes at residues 265 and 313 ($K_{265}R$ and $Q_{313}H$). Further, changes occurred in 2/4 of the variable loops in culture A and 3/4 of the variable loops in culture B. In the V5 region, sequence 827B lost five amino acid residues and several mutations occurred ($V_{561}D$, $Q_{562}K$, $M_{563}L$, and $H_{568}R$) resulting in a gain of positive charge (+4) in that region of Env. The virus from culture B lost a potential glycosylation site at 481, similar to FIV-PET. All cysteine residues were conserved.

7.4.1.2. Common motif changes between cocultivated and cell-free infection of HO6T1 cells.

There were no universal motif changes within all four isolates, however, two residues were identified where a mutation was common to several isolates. K360N occurred in 795B, 827A and 827B while 795A displayed a $K_{360}I$ mutation (see Table 7.d.). Residue 496 in the V4 variable region gained a positive charge in all isolates except 827A, where a small nonpolar amino acid was acquired.

Figure 7.c. The envelope glycoprotein sequences of the parental virus F0795H$_s$ and viruses from cell-free infection and cocultivation of CrFK(HO6T1) cells respectively.

```
          1
F0795H    MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK
F0795H_A  MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK
F0795H_B  MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK

          51                                  L↓SU                V1
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDETILSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDESILSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDETILSLI

          101                      V2
          YLFIGYCTYA CNRNRLGSLR HDIDIEAPQE ECYNNREKGT TENIKYGGRC
          HLFIGYCTYV CNRNRLGSLR HDIDIEAPQE ECYNNREKGT TENIKYGKRC
          YLFIGYCTYA CNRNRLGSLR HNIDIEAPQE ECYNNREKGT TENIKYGGRC

          151
          FIGTAGLYLL LFIGVGIYLG TTKAQVVWRL PPLVVPVEES EIIFWDCWAP
          FIGTAGLYLL LFIGVGIYLG TTKAQVVWRL PPLVVPVEES EIIFWDCWAP
          FIGTAGLYLL LFIGVGICLG TTKAQVVWRL PPLVVPVEES EIIFWDCWAP

          201                •
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAKEIWGTL FKKATRQCRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG IWAKEIWGTL FKKATRQCRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAKEIWGTL FKKATRQCRR

          251
          GRIWRRWNET ITGPLGCANN TCYNISVIVP DYQCYLDRVD TWLQGKVNIS
          GRIWRRWNET ITGPLGCANN TCYNISVIVP DYQCYLDRVD TWLQGKVNIS
          GRIWRRWNET ITGPLGCANN TCYNISVIVP DYQCYLDRVD TWLQGKVNIS

          301                          •     •     •
          LCLTGGKMLY NKDTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE
          LCLTGGKMLY NKDTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE
          LCLTGGKMLY NKDTKQLSYC TDPLQTPLIN YTFGPNQTCM WNTSQIQDPE

          351        V3
          IPKCGWWNQK AYYNSCRWES TDVKFHCQRK QSQPGSWSRA ISSWKQRNRW
          IPKCGWWNQI AYYNSCRWES TDVKFHCQRK QSQPGSWRRA ISSWKHRNRW
          IPKCGWWNQN AYYNSCRWER TDVKFHCQKT QSQPGSWSKT ISSWRHRNRW

          401                •     •                       •
          EWRPDFESEK VKISLQCNST KNLTFAMRS SSDYGEVMGAW IEFGCHRNKS
          EWRPDFESEK VKVSLQCNST KNLTFAMRS SGDYGEVKGAW IEFGCHRKKS
          QWRPDFESEK VKVSLQCNST RNLTFAMRS SGDYGEVKGAW IEFGCHRNKS

          451   V4             •                   •                 •
          RLHTEARFRI RCRWNVGDNT SLIDTCGKTQ NVSGANPVDC TMYTNRMYNC
          KLHSEARFRI RCRWNVGDNT SLIDTCGKTQ NVSGANPVDC TMYTNKMYNC
          KLHTEARFRI RCRWNVGDNT SLIDTCGKTQ NVSGANPVDC TMYANKMYNC
```

```
          501                •                •        V5        •
F0795H    SLQNGFTMKI DDLIMHFNMT KAVEMYNIAG NWSCTSDLPP TWGYMNCNCT
F0795H_A  SLQNGFTMKI DDLIMHFNMT KAVEMYNIAG NWSCTSDLPP TWGYMNCNCT
F0795H_B  SLQNGFTMKI DDLIMHFNMT KAVEMYNIAG NWSCTSDLPP TWGYMNCNCT

          551                                              V6
          NSTTSG..GT SNKMACPNNQ GILRNWYNPV AGLRQSLEKY QVVKQPDYLV
          NRTSEGTRGT SNKMACPNNQ GILRNWYNPV AGLRQSLEKY QVVKQPDYLV
          NDTTS..RGN GNKMACPNNQ GILRNWYNPV AGLRQSLEKY QVVKQPDYLV

          601           ↓
          VPGEVMEYKP RRKRAAIHVM LALATVLSMA GAGTGATAIG MVTQYHQVLA
          VPGEVMEYKP RRKRAAIHVM LALATVLSIA GAGTGATAIG MVTQYHQVLE
          VPGEVMEYKP RRKRAAIHVM LALATVLSMA GAGTGATAIG MVTQYHQVLE

          651
          THQETIEKVT EALKINNLRL VTLEHQVLVI GLKVEAMEKF LSTAFAMQEL
          THQEAIEKVL EALKINNLRL VTLEHQVLVI GLKVEAMEKF LYTAFAMQEL
          THQEAIEKVT QALKINNLRL VTLEHQVLVI GLKVEAMEKF LYTAFAMQEL

          701                •    •                   •
          GCNQNQFFCK IPLELWEGYN MTINQTIWNH GNITLGEWYN QTKDLQQKFY
          GCNQNQFFCK IPLDLWKGYN MTINQTIWNH GNITLGEWYN QTKDLQRKFY
          GCNQNQFFCK IPLELWKRYN MTINQTIWNH GNITLGEWYN QTKDLQQKFY

          751
          EIIMDIERNN VQGKKGLQQL QEWEDWVGWI GNIPQYFRGL LGGILGIGLG
          EIIMDIERNN VQGKKGLQQL QEWEDWVGWI GNIPQYLRGL LGGILGIGLG
          EIIMDIERNN VQGKKGLQQL QEWEDWVGWI GNIPQYLRGL LGGILGIGLG

          801
          VLLLILCLPT LVDCIRNCFH KILGYTVIAM PEVEEEEIQP QMELRRNGRQ
          VLLLILCLPT LVDCIRNCIH KILGYTVIAM PEVEEEEIQP QMELRRNGRQ
          VLLLILCLPT LVDCIRNCFH KILGYTVIAM PEVEEEEITV QMELRRNGRQ

          851
          RGISEEEEE*
          RGISEKEEE*
          RGISEKEEE*
```

The variable loops are highlighted in grey, potential glycosylation sites are marked by • and affected sequences underlined for clarity, the start of the SU and the TM are marked by ↓. Unique mutations are highlighted in blue and residues identical to FIV-PET are highlighted in green.

Table 7.c. Mutations recorded in the env gene sequence allowing infection of (CrFK)HO6T1 cells.

Isolate									
795A	Y101H	A110V			G148K				
795B				D122N			Y168C		
827A									A172T
827B			L119V			I160L		T169I	A172T

V1/V2 loop

Isolate																
795A	K360I								S388R				Q396H			I413V
795B	K360N			S370R		R379K	K380T			R389K	A390T	K395R	Q396H		E401Q	I413V
827A	K360N	N364S	E369K		K378E											
827B	K360N							S382N						S397R		

V3 loop

Isolate				
795A	R451K	T454S		
795B	R451K			T494A
827A			K478E	
827B			K478E	

V4 loop

Isolate												
795A	S552R		T554S	S555E		*557T	*558R					
795B	S552D				G556*		*558R	T560N	S561G			
827A												
827B	S552*	T553*	S554*	S555*	V556*				V561D	Q562K	M563L	H568R

V5 loop

* = no amino acid at this site. Numbers represent the residue site within the gene. The original amino acid notation is recorded on the left and the novel motif is recorded on the right of the site number. Common mutations are highlighted.

Figure 7.d. The envelope glycoprotein sequences of F0827H_s and viruses from cell-free infection and cocultivation with CrFK(HO6T1) cells respectively.

```
          1
F0827H    MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK
F0827H_A  MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK
F0827H_B  MAEGFAANRQ WIGPEEAEEL LDFDIATQMN EKGPLNPGIN PFRVPGITEK

          51                                  L↓SU
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDEHVLSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDEHVLSLI
          EKQNYCNILQ PKLQDLRNEI QEVKLEEGNA GKFRRVRFLR YSDENILSLI

          101 V1                    V2
          HLFIGYCTYL CNSRKLGSLR HDIDIEAPQE ECYDNKEKGT TANIKYGRRC
          HLFIGYCTYL CNSRKLGSLR HDIDIEAPQE ECYDNKEKGT TANIKYGRRC
          HLFIGYCTYL CNSRKLGSVR HDIDIEAPQE ECYDNKEKGT TANIKYGRRC

          151
          IIGTAALYLI LFTGIIIYTQ TANAQVVWRL PPLVVPVDES EIIFWDCWAP
          IIGTAALYLI LFTGIIIYTQ TTNAQVVWRL PPLVVPIDES EIIFWDCWAP
          IIGTAALYLL LFTGIIIYIQ TTNAQVVWRL PPLVVPVDES EIIFWDCWAP

          201          •
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAREIWGTL FKKATRQCRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAREIWGTL FKKATRQCRR
          EEPACQDFLG AMIHLKASTN ISIQEGPTLG NWAREIWGTL FKKATRQCRR

          251  •        •     •                          •
          GRIWRKWNET ITGPLGCANN TCYNISVVIP DYQCYLDRVD TWLQGKVNIS
          GRIWRKWNET ITGPSGCANN TCYNISVVIP DYQCYLDRVD TWLQGKVNIS
          GRIWRRWNET ITGPSGCANN TCYNISVVIP DYQCYLDRVD TWLQGKVNIS

          301                          •     •     •
          LCLTGGKMLY NKQTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE
          LCLTGGKMLY NKQTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE
          LCLTGGKMLY NKHTKQLSYC TDPLQIPLIN YTFGPNQTCM WNTSQIQDPE

          351  V3
          IPKCGWWNQK AYYNSCRWEQ TDVKFQCQRT QSQPGSWHRT ISSWKQSNRW
          IPKCGWWNQN AYYSSCRWKQ TDVKFQCQRT QSQPGSWHRT ISSWKQSNRW
          IPKCGWWNQN AYYNSCRWEQ TDVKFQCQRT QNQPGSWHRT ISSWKQRNRW

          401                •     •                          •
          EWRPDFESEK VKISLQCNST KNLTFAMRSS GDYGDITGAW IEFGCHRNKS
          EWRPDFESEK VKISLQCNST KNLTFAMRSS GDYGDITGAW IEFGCHRNKS
          EWRPDFESEK VKISLQCNST KNLTFAMRSS GDYGDVTRAW IEFGCHRNKS

          451 V4                     •           •     •          •
          KLHTEARFRI RCRWNIGDNT SLIDTCGKTQ NVTGANPVNC TMYANSMYNC
          KLHTEARFRI RCRWNIGDNT SLIDTCGETQ NVTGANPVNC TMYANTMYNC
          KLHTEARFRI RCRWNIGDNT SLIDTCGETQ NVAGANPVNC TMYANRMYNC
```

```
                501                •                   •        V5          •
F0827H     SLQNGFTMKV DDLIMHFNMT KAVEMYNIAG NWSCKSDLPP TWGYMNCNCT
F0827H_A   SLQNGFTMKV DDLIMHFNMT KAVEMYNIAG NWSCKSDLPP TWGYMNCNCT
F0827H_B   SLQNGFTMKV DDLIMHFNMT KAVEMYNIAG NWSCKSDLPP TWGYMNCNCT

               •551                                              V6
           NSTSSVSTSS VQMACPSHQG ILRNWYNPVA GLRQSLEKYQ VVKQPDYLVV
           NSTSSVSTSS VQMACPSHQG ILRNWYNPVA GLRQSLEKYQ VVKQPDYLVV
           N.....STSS DKLACPSRQG ILRNWYTPVA GLRQSLEKYQ VVKQPDYLVV

           601           ↓TM
           PGEVMEYKPR RKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYHQVLAT
           PGEVMEYKPR RKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYHQVLAT
           PGEVMEYKPR RKRAAIHVML ALATVLSMAG AGTGATAIGM VTQYHQVLAT

           651
           HQEAIEKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG
           HQEAIEKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG
           HQEAIEKVTE ALKINNLRLV TLEHQVLVIG LKVEAMEKFL YTAFAMQELG

           701                •    •         •         •
           CNQNQFFCKV PLILWQRYNM TINQTVWNHG NITLGEWYNQ TKDLQQKFYE
           CNQNQFFCKV PPILWQRYNM TINQTIWNHG NITLGEWYNQ TKDLQQKFYE
           CNQNQFFCKV PPILWQRYNM TINQTIWNHG NITLGEWYNQ TKDLQQKFYE

           751
           IIMDIEQNNV QGKKGLQQLQ EWEDWVGWIG NIPQYLKGLL GGILGIGLGV
           IIMDIEQNNV QGKKGLQQLQ EWEDWVGWIG NIPQYLKGLL GGILGIGLGV
           IIMDIEQNNV QGKKGLQQLQ EWEDWVGWIG NIPQYLKGLL GGILGIGLGV

           801
           LLLILCLPTL VDCIRNCIHK ILGYSVIAMP EIEGEEIQSQ MELRRNGRQC
           LLLILCLPTL VDCIRNCIHK ILGYSVIAMP EIEGEEIQSQ MELRRNGRQC
           LLLILCLPTL VDCIRNCIHK ILGYSVIAMP EIEGEEIQSQ MELRRNGRQC

           851
           GISEKEEE*
           GISEKEEE*
           GISEKEEE*
```

Variable loops are highlighted in grey, potential glycosylation sites are marked by • and the affected sequences underlined. ↓ represents the start of the SU and the TM subunits. Unique mutations are highlighted in blue and mutations identical to FIV-PET are highlighted in green.

7.4.2. Discussion

The ability of some FIV isolates to infect CrFK(HO6T1) cells is CXCR4-dependent (Hosie et al. 1998a; Egberink et al. 1999) and correlates with an increase in charge of the third variable region (Verschoor et al. 1995; Siebelink et al. 1995b) brought about by an E to K mutation at either position 407 or 409. In this part of the study, we aimed to compare sequences of two isolates from terminally ill cats which infected CrFK(HO6T1) cells by both cell-free infection and cocultivation in order to explore sequence changes which may correlate with this expansion in tropism.

An E to K mutation did not occur in any of the four virus sequences examined and, in contrast to previous studies, no increase in positive charge in the V3 loop was found. Interestingly, all viruses which achieved persistent infection of CrFK(HO6T1) cells (795B, 827A and 827B) had a $K_{360}N$ mutation in the V3 loop, resulting in a decrease in charge. The significance of mutations outside the variable loops, common to both A and B isolates, is unknown but nonvariable regions have been found to influence phenotype in HIV (Hoffman et al. 2002). Greater numbers of mutations were observed in viruses sequenced from cultures infected by cocultivation compared to those obtained following cell-free infection. It is conceivable that further mutations may have been introduced during the further passage of CRFK(HO6T1) supernatants in Mya-1 cells prior to amplification of the *env* gene by PCR, however, previous studies have indicated that Mya-1 cells do not apply a selective pressure (data not shown). The PCR process itself has the inherent potential to introduce errors, although proofreading enzymes were used in these studies to minimise such errors.

A lysine to asparagine or glutamine mutation at site 481 in the V4 loop has previously been reported in cats inoculated with laboratory-adapted isolates which reverted to more pathogenic, neutralisation-resistant isolates (Bendinelli et al. 2001). It is interesting that the B culture of isolate $F0827H_s$ lost a glycosylation site, also at position 481, suggesting that this residue may be a determinant of broad neutralisation resistance, consistent with the results of Siebelink et al. (Siebelink et al. 1995a).

In order to characterise further the role of each mutation in CrFK(HO6T1)-tropism, it will be necessary to examine each mutation within the context of the original *env* as was done previously for HIV-1 (Boyd et al. 1993). This would require isolating each motif change

and inserting this into the parent envelope by site-directed mutagenesis. By subsequently cloning the mutated *env* into the $GL8_{MYA}$ viral backbone, the function of each mutation that was identified in this chapter could be investigated.

Chapter Eight

GENERAL DISCUSSION

8.1. Introduction

The first aim of this study was to investigate the *in vitro* tropism of a range of primary isolates from cats at different stages of the disease and compare their phenotypes with the well-characterised prototype viruses, FIV-GL8 and FIV-PET, both of which have been the subject of many vaccine trials. The second aim of the study was to investigate the biological behaviour of primary isolates from different stages of the disease *in vivo*, in order to examine the relationship between pathogenicity *in vivo* and tropism *in vitro*. The third aim of the study was to examine the role of the env gene in the pathogenicity of FIV.

8.2. Isolates from the terminal stage of disease are more CXCR4-tropic

FIV-GL8 and FIV-PET are two isolates that have been well-characterised *in vitro* and *in vivo*, as discussed in Chapter 3. The characteristics displayed by these two isolates led to the hypothesis that the pathogenicity of an isolate may be characterised by *in vitro* tropism, particularly the ability to utilise CXCR4. *In vitro* assays were employed to examine a panel of field isolates and it was found that isolates from cats in the terminal, symptomatic stages of the disease had a greater ability to utilise CXCR4. Interestingly, this ability was not consistent throughout all of the assays used. The assays based on AH927 cells demonstrated that many isolates from different stages of infection may use CXCR4 for entry, however a greater proportion of symptomatic isolates infected these cells compared to CXCR4-expressing CrFK cells.

CrFK-tropism was less common with only 2 isolates from terminally ill cats (F0795H_s and F0827H_s) achieving infection using cell-free virus. Viral stocks from these infected CrFK cultures were subsequently unable to infect the cell line AH927 FX4E. Furthermore, the FIV-G8M chimaeras containing the CrFK-tropic *env* genes, G8M(827) and G8M(F14.7), (Chapter 5) infected CrFK cells efficiently, but only achieved limited infection of AH927 FX4E cells. Although no blocking assays were conducted in this study to confirm the use of CXCR4 in the cell lines, previous reports document CXCR4-dependent infection of these cell lines (Willett et al. 1997c; Hosie et al. 1998a). The chimaeras G8M(827) and

G8M(F14.7) demonstrated CrFK-tropism but infected AH927 FX4E cells less efficiently. This suggested that some viral component other than Env may be required by FIV-PET and F0827H_s for efficient replication within these cells. The variation in tropism shown by the chimaeras may be due to (a) variation in CXCR4 conformation or presentation within the cell membrane environment or (b) some regions of genomes besides *env* imparts the ability to grow in AH927 FX4E cells. In general, the *in vitro* tropism studies suggest that isolates from more advanced disease stages have a greater affinity for CXCR4.

Different conformations have been described for the human homologue of CXCR4 (Baribaud et al. 2001; Lapham et al. 2002), a phenomenon that may exist with feline CXCR4 but has not yet been investigated. However, de Parseval and Elder have suggested that cofactors such as HSPGs may be involved in virus binding and have also identified a 40kD protein from primary feline T cells by coprecipitation with a gp95-Fc immunoadhesin molecule which is thought to participate in virus binding (de Parseval and Elder 2001). The ability to use different cofactors depended on the cell line and the viral isolate. The recent identification of the 40kD molecule CD134 as a primary receptor for cell entry has clarified receptor usage and tropism. CD134 has been shown to be the principal receptor for FIV cell entry, productive infection and syncytium formation. However, CD134-dependent entry requires the coreceptor CXCR4, shown by the dose-dependent inhibition of HeLa cells expressing feline CD134 by the CXCR4 antagonist AMD3100 (Shimojima et al. 2004).

8.3. CXCR4-tropism correlated with reduced virulence *in vivo*

The emergence of T-tropic, SI forming isolates in HIV-1 coincides with disease progression and marked decreases in $CD4^+$ T lymphocyte numbers, which has led to the hypothesis that these isolates are more pathogenic (Tersmette et al. 1989a; Schellekens et al. 1992; Koot et al. 1993). Similar findings have been reported in FIV where the progression to feline AIDS coincides with reduction in $CD4^+$ T lymphocytes (Ackley et al. 1990; Torten et al. 1991; Hoffman-Fezer et al. 1992). Contrary to this popular hypothesis, infection of SPF cats with isolates from asymptomatic cats induced higher proviral loads, higher infectious viral burdens, as measured by QVI, and inversion of the CD4:CD8 T lymphocyte ratio when compared to infections of cats inoculated with isolates derived from cats in the AIDS-like phase of the disease. The asymptomatic isolates exhibited FIV-GL8 like characteristics *in vivo* as well as *in vitro*.

8.4. The use of proviral DNA loads as a measurement of pathogenicity

As in HIV-1 infection, the predictive value of proviral DNA load in disease progression of FIV is still unknown. From the numerous studies in HIV-1 infection that have compared the two parameters, proviral load would appear to be most useful in the measurement of successful HAART, where non-responders have consistently high proviral DNA loads (Russell et al. 2001). The use of proviral load as a prognostic indicator in HIV-1 infection of infants was championed by Di Rossi and colleagues as rapid increases in proviral DNA loads within the PBMCs to very high levels correlated with early onset AIDS (de Rossi et al. 1996). However, other workers reported no correlation between proviral DNA load and disease progression (Cone et al. 1998). Reimann et al. reported higher proviral DNA loads in macaques infected with pathogenic SHIVs, which produced a marked decrease in the CD4+ T cell population, but the same clones also achieved higher viral loads than the less pathogenic SHIVs. Therefore, the use of proviral DNA in SIV infection as a prognostic indicator also remains unclear (Reimann et al. 1999).

The measurement of proviral load has received some scrutiny over recent years and the expression of proviral load as a fraction of 10^6 cells has been criticised because cell populations are not constant throughout the disease, as some cell populations have a tendency to decrease with disease progression. Therefore, the proposition that proviral load should be measured against blood volume (per ml of blood) has been made (Cone et al. 1998; Aleman et al. 1999) as this is a constant denominator throughout the disease. However, in the studies described in this thesis, proviral loads are expressed as a fraction of 10^6 cells.

Although retrovirus proviral DNA exists in many forms, only full-length integrated proviral DNA is replication-competent. In HIV-1 the majority of proviral DNA is found as the unintegrated form within the cytoplasm (Chun et al. 1997) or as defective genomes with deletions in the provirus (Sanchez et al. 1997). Therefore, the method of measurement may affect the levels detected. In the present study samples were not analysed for deletions within the provirus or the amount of unintegrated proviral DNA. However, QVI assays measuring the infectious viral burden in the PBMCs demonstrated a similar trend to that demonstrated by the proviral measurements from PCR, indicating that the proviral loads represent replication-competent proviral DNA rather than unintegrated

or defective DNA. An additional consideration is that virus type and route of infection have been shown to influence the dynamics of provirus in the PBMCs during experimental infection (Burkhard et al. 2002). The proportion of proviral load data with coefficients of variation >30%, particularly using the 1416p system, warrants caution when interpreting the results in isolation. As this system did not give an indication of the levels of replication-competent virus *in vivo,* the *in vitro* measurement of infectious viral load by means of QVI was a useful adjunct when assessing viral titres calculated by molecular methods.

8.5. Could route of infection affect viral RNA load in plasma?

The reasons for the low plasma viral RNA loads in this study are unclear. Some real-time PCR primers and probe mismatches were identified and the effect of different mismatches is unknown but several factors are known to influence the overall efficiency (see Chapter 4). However, the proviral measurements demonstrated that the same primers and probes were adequate for measurement of the field viruses, and therefore the cause of the low viral RNA loads remains unclear. If the route of infection influences viral dynamics then this is significant when testing vaccines and it will be important to establish challenge systems that mimic closely the natural route of transmission.

Detailed studies of the immune responses induced following infection with the field isolates may clarify the reasons behind this low virus release into plasma. Detailed examination of both CTL and noncytolytic responses (Flynn et al. 1995; Bucci et al. 1998b; Flynn et al. 2002), as well as the humoral immune response (Hosie and Jarrett 1990), would be important to investigate the possibility that immune responses are suppressing viral production.

8.6. Expansion of the CD8$\alpha^{+}\beta^{low}$ population by asymptomatic isolates

An expansion of the CD8$\alpha^{+}\beta^{low}$ T lymphocyte subset coinciding with the *in vivo* evolution of an FIV-PET isolate to a more virulent state has been documented. This expansion was accompanied by increased plasma viral RNA and PBMC proviral DNA burden *in vivo* (Hosie et al. 2002).

In this study, FACS analysis revealed that isolates from cats in the asymptomatic stage of disease induce greater activation of the $CD8\alpha^{+}\beta^{low}$ subpopulation in naïve SPF cats compared to symptomatic isolates. The phenomenon of noncytolytic $CD8^{+}$ cell anti-FIV activity awaits further clarification. This population of cells was found to expand during acute FIV infection (Lehmann et al. 1992; Willett et al. 1993) and was later shown to suppress cell-associated and plasma viral expression (Jeng et al. 1996). Indeed total clearance, by noncytolytic mechanisms, of virus from intravaginally infected kittens was attributed to these cells (Bucci et al. 1998a). However, other reports demonstrate that the $CD8\alpha^{+}\beta^{low}$ phenotype is not solely responsible for viral suppression (Crawford et al. 2001) and indeed the $CD8\alpha^{+}\beta^{hi}$ population has been shown to exhibit similar antiviral activity (Flynn et al. 2002). The ability to induce a suppressor response in FIV-infected cats is variable. Some cats may demonstrate no inducible anti-FIV suppressor activity (Bucci et al. 1998a; Bucci et al. 1998b; Choi et al. 2000). Furthermore, the properties exhibited by the suppressor cells vary greatly: there was neither any correlation between the percentage of $CD8\alpha^{+}\beta^{low}$ cells in the population and the extent of antiviral activity exhibited (Bucci et al. 1998b), nor between the extent of $CD8\alpha^{+}\beta^{low}$ expansion and clinical disease (Jeng et al. 1996).

In this study, the asymptomatic stage isolates induced greater expansion of the $CD8\alpha^{+}\beta^{low}$ T lymphocyte population. The findings that these isolates induced greater proviral DNA burdens, greater infectious viral burdens and greater inversion of the CD4:CD8 T lymphocyte ratio *in vivo*, led to the conclusion that these asymptomatic stage isolates are more pathogenic. Furthermore, the results depicted in Figure 6.g. indicate that *env* is a major determinant involved in the expansion of this lymphocyte population. Further work is required to further define the function of this cell population.

Major limitations of this project were a) the small number of viruses investigated and b) the small number of animals in each group, with the result that any statistical analyses must be interpreted cautiously. The results have, nevertheless, demonstrated trends that will prompt further investigation of field isolates using similar methods. By selecting two viruses from terminally ill cats, which were both CrFK-tropic, it is possible that more FIV-PET like viruses were selected, thus influencing the polarity of the results. The symptomatic viruses showed a range of tropism properties, from being able to infect CrFK cells and AH927 cells to infecting only IL-2 dependent cells Mya-1. Hence, it would be

interesting to examine more isolates in detail to determine the relationship between pathogenicity *in vivo* and cell tropism *in vitro*.

The viral populations within these primary isolates are poorly defined. Analysis of the quasispecies isolated from each individual by heteroduplex mobility assay (Delwart et al. 1993; Bachmann et al. 1997) and biological cloning (Connor et al. 1993b) may reveal further useful information regarding phenotypes. The replication rates, tropism and receptor usage of viruses within the quasispecies may reveal correlations with stage of infection. By further categorising the quasispecies within each isolate, it may be possible to identify common amino acid motifs responsible for each phenotype. In addition, analysis of the viral populations in each of the cats infected with primary isolates (A701-A708) throughout the 15 week study period would be worthwhile in order to examine the dynamics of the quasispecies *in vivo*.

8.7. Are terminal isolates the cause or the consequence of disease?

In HIV-1 infection, progression to disease coincides with the emergence of SI CXCR4 T-tropic isolates in about 50% of infected individuals. However it has been debated whether these isolates arise as a cause or a consequence of $CD4^+$ T cell depletion (Cheng-Mayer et al. 1988; Fenyö et al. 1989; Tersmette et al. 1989a; Von Gegerfelt et al. 1991; Koot et al. 1992). SI isolates are typically found to occur in patients with moderately reduced $CD4^+$ T cell counts, suggesting that they replicate following the onset of immune system dysfunction (Miedema et al. 1990). However, once SI isolates appear, a threefold decrease in $CD4^+$ T cell numbers occurs. This outcome contrasts to that observed in people infected with only NSI viruses where, in general, a more prolonged, gradual decrease in $CD4^+$ T cell numbers is seen. Therefore, the hypothesis that SI variants are more pathogenic and the cause of $CD4^+$ T cell depletion has become popular. Reports of rapidly progressing disease in individuals infected from people with only SI variants support this hypothesis (Cheng-Mayer et al. 1988; Fenyö et al. 1989; Tersmette et al. 1989a; Koot et al. 1992; Schuitemaker et al. 1992; Roos et al. 1992; Groenik et al. 1993). Evidence from infection of pig-tailed macaques with late stage SI T-tropic SIV isolates that develop early onset simian AIDS suggests that SI viruses do indeed drive the progression to AIDS (Kimata et al. 1999).

However, if SI variants were more pathogenic, one would expect these viruses to predominate in individuals infected with a mixed population of viruses, which is not the case. The "division rate" theory suggests that SI CXCR4-tropic isolates are restricted due to their ability to infect only naïve T cells, which divide at a very low rate in contrast to memory cells, which are targets for NSI CCR5-tropic isolates and replicate at a much higher rate. The replication of the SI infected naïve T cells is only stimulated when the memory T cell count decreases (Davenport et al. 2002).

The pathogenesis of HIV/AIDS remains to be clearly defined. However, the trends evident from this study suggest that isolates from the asymptomatic stage of FIV infection produce higher proviral loads, greater perturbation of the lymphocyte populations and higher infectious viral burdens, and may therefore be classified as more pathogenic than the viruses isolated from the terminal stage of disease. While further work with additional primary isolates is required to confirm the generality of these findings, the trends reported here indicate the need to direct future FIV vaccine studies against primary isolates, particularly those from the asymptomatic stage of the disease, as these viruses are the most likely to be transmitted in nature.

Appendices

Appendix A.1. Haematology results for cats A701 to A704 at 0, 6, 12 and 15 weeks p.i.

	A701				A702				A703				A704			
Test	0w	6w	12w	15 w	0 w	6w	12w	15w	0w	6w	12w	15w	0 w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	7.70	7.08	7.97	ND	7.44	6.85	6.92	6.84	7.62	4.99	7.2	5.99	6.36	6.24	7.06	5.83
Hb g/dl (10-15)	10.5	9.8	10.9	7.85	12.0	10.9	11.2	10.8	11.0	7.07	10.3	8.26	9.9	9.4	10.7	8.79
HCT % (30-45)	29.0	26.3	28.8	20	32.0	29.2	28.8	28.4	29.2	19.4	27.3	22.3	26.8	25.9	29.0	22.9
MCV fl (39-55)	37.7	37.2	36.1	ND	43.1	42.7	41.7	41.5	38.2	38.9	37.9	37.2	42.2	41.5	41.2	39.3
MCH pg (12.5-17.5)	13.7	13.9	13.7	ND	16.2	15.9	16.2	15.7	14.4	14.2	14.3	13.8	15.5	15.1	15.2	15.1
MCHC g/dl (30-36)	36.2	37.2	38	ND	37.5	37.2	38.8	37.9	37.7	36.4	37.8	37.1	36.8	36.3	36.9	38.3
WBC $x10^9$/l (5.5-15.5)	18.3	12.1	8.74	5.83	11.8	9.12	9.64	7.77	17.0	6.38	11.7	8.62	11.1	3.87	4.23	2.53
Band neutrophils $x10^9$/l	1.098	0	0	0	0.12	0	0	0	0.51	0	0	0	0.22	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	13.36	8.11	4.89	3.15	8.38	3.37	3.86	2.25	11.22	2.74 *	6.44	3.77	6.88	1.28	1.1	0.99
Lymphocytes $x10^9$/l (1.5-7)	3.29	3.63	3.67	2.51	2.36	5.20	5.30	5.28	4.42	3.06	5.15	4.40	2.89	2.17	2.83	1.32
Monocytes $x10^9$/l (0-0.85)	0.37	0.12	0.18	0.12	0.35	0.18	0.19	0.23	0.17	0.26	0.12	0.34	0.56	0.23	0.17	0.20
Eosinophils $x10^9$/l (0-1.5)	0.18	0.24	0	0.06	0.59	0.36	0.29	0	0.68	0.19	0	0.17	0.56	0.16	0.13	0.02
Basophils $x10^9$/l	0	0	0	0	0	0	0	0	0	0.06	0	0	0	0.04	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0.06	0	0	0	0	0	0

* = toxic change

Appendix A.1. (contd) Haematology results for cats A705 to A708 at 0, 6, 12 and 15 weeks p.i.

	A705				A706				A707				A708			
Test	0w	6w	12w	15w	0 w	6w	12w	15w	0w	6w	12w	15w	0 w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	6.02	6.73	7.21	5.71	7.6	6.34	7.52	6.23	6.72	6.87	7.91	7.28	6.46	5.97	6.36	5.41
Hb g/dl (10-15)	9.2	10.3	11.4	9.02	10.9	9.34	11.1	9.32	10.0	10.4	12.3	10.9	9.1	9.27	10.2	8.81
HCT % (30-45)	25.2	27.6	29.4	24.0	29.2	24.7	29.0	23.6	26.8	27.3	31.3	28.6	24.6	23.6	26.1	21.8
MCV fl (39-55)	41.9	41.0	40.8	42.1	38.3	38.9	38.6	37.9	39.8	39.8	39.6	39.3	38.0	39.5	41.	40.2
MCH pg (12.5-17.5)	15.4	15.3	15.8	15.8	14.3	14.7	14.8	15.0	14.8	15.1	15.6	15.0	14.1	15.5	16	16.3
MCHC g/dl (30-36)	36.6	37.4	38.7	37.8	37.3	37.8	38.3	39.5	37.2	37.9	39.4	38.3	37.1	39.2	38.9	40.5
WBC $x10^9$/l (5.5-15.5)	15.64	8.74	10.2	6.35	19.4	8.49	10.1	5.73	14.8	14.0	12.0	13.8	16.9	15.5	16.7	12.8
Band neutrophils $x10^9$/l	0	0	0	0	0.39	0	0	0	0.15	0	0	0	0.17	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	11.89	3.93	4.79	1.14	15.5	3.31	3.13	2.06	7.55	4.06	4.32	3.45	10.31	8.06	6.35	6.4
Lymphocytes $x10^9$/l (1.5-7)	2.82	3.32	4.08	4.64	1.9	4.42	6.56	3.38	6.22	9.24	6.96	9.94	3.89	5.27	8.85	4.86
Monocytes $x10^9$/l (0-0.85)	0.16	0.35	0.20	0.19	0.58	0.42	0.20	0.29	0.44	0.42	0.12	0.28	0.51	0.31	0	0.13
Eosinophils $x10^9$/l (0-1.5)	0.78	1.05	0.92	0.25	0.78	0.26	0.20	0	0.44	0.28	0.48	0.14	2.03	1.86	1.50	1.15
Basophils $x10^9$/l	0	0.09	0.20	0.13	0.19	0.08	0	0	0	0	0.12	0	0	0	0	0.26
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A.1. (contd) Haematology results for cats A709 and A710 at 0, 6, 12 and 15 weeks p.i.

	A709				A710			
Test	0w	6w	12w	15w	0w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	8.4	6.68	7.27	6.18	7.32	6.97	7.68	6.98
Hb g/dl (10-15)	12.6	9.90	11.0	9.41	11.5	11.0	11.5	10.1
HCT % (30-45)	34.4	26.9	29.5	24.5	30.6	29.3	30.2	26.8
MCV fl (39-55)	41.1	40.3	40.6	39.6	41.8	42.0	39.4	38.4
MCH pg (12.5-17.5)	15.0	14.8	15.2	15.2	15.8	15.7	15.0	14.5
MCHC g/dl (30-36)	36.6	36.8	37.3	38.4	37.7	37.5	38.0	37.7
WBC $x10^9$/l (5.5-15.5)	12.2	8.07	9.12	4.69	15.0	3.63	5.48	2.51
Band neutrophils $x10^9$/l	0.12	0	0	0	0.6	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	8.66	2.66	1.92	1.45	9.15	0.11	1.04	0.25
Lymphocytes $x10^9$/l (1.5-7)	2.44	4.84	6.93	3.14	3.75	3.09	4.22	2.16
Monocytes $x10^9$/l (0-0.85)	0.37	0	0.09	0.05	0.75	0.22	0.16	0.02
Eosinophils $x10^9$/l (0-1.5)	0.61	0.56	0.18	0.05	0.75	0.22	0	0.05
Basophils $x10^9$/l	0	0	0	0	0	0	0.06	0.02
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0

Appendix A.2. Haematology results for cats A741, A742 and A754 at 0, 3, 6, 12 and 15 weeks p.i.

	A741					A742					A754				
Test	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	5.4	6.7	6.93	7.05	6.08	6.76	6.54	7.3	7.55	7.1	6.58	7.15	7.07	7.78	6.49
Hb g/dl (10-15)	7.48	8.93	8.98	9.79	8.22	9.38	9.4	10.2	11.2	10.5	8.67	9.19	8.76	10.3	8.6
HCT % (30-45)	23	26	25.5	26.4	22.3	27.9	26.5	29.1	30.3	29.1	25	26.3	25.2	27.9	24.1
MCV fl (39-55)	42.5	38.8	36.8	37.3	36.7	41.3	40.5	39.9	40.2	41	38	36.8	35.7	35.9	37.1
MCH pg (12.5-17.5)	13.9	13.3	13	13.8	13.5	13.9	14.4	14	14.8	14.7	13.2	12.9	12.4	13.3	13.3
MCHC g/dl (30-36)	32.6	34.3	35.2	37.1	36.8	33.6	35.4	35	36.8	36	34.7	35	34.8	36.9	35.7
WBC $x10^9$/l (5.5-15.5)	12.9	10.1	13.1	12.7	7.44	5.9	8.05	7.58	11.4	2.62	3.82	8.4	8.05	7.23	5.2
Band neutrophils $x10^9$/l	0.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	9.55	5.66	7.99	4.06	3.27	2.83	3.62	3.11	5.24	0.31	1.87	4.87	3.86	2.53	2.13
Lymphocytes $x10^9$/l (1.5-7)	2.45	3.33	3.93	6.22	3.57	2.36	3.86	3.49	5.13	2.07	1.45	2.52	3.54	4.41	2.86
Monocytes $x10^9$/l (0-0.85)	0.26	0.20	0.66	0.51	0.30	0.12	0.08	0.15	0	0.03	0.04	0.08	0.40	0.14	0
Eosinophils $x10^9$/l (0-1.5)	0.26	0.81	0.52	1.90	0.30	0.53	0.48	0.76	1.03	0.18	0.42	0.92	0.24	0.14	0.10
Basophils $x10^9$/l	0.26	0.10	0	0	0	0.06	0	0.08	0	0.03	0.04	0	0	0	0.10
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A.2. (contd) Haematology results for cats A743, A744 and A753 at 0, 3, 6, 12 and 15 weeks p.i.

	A743					A744					A753				
Test	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	6.08	7.14	7.25	7.2	10.3	nd	6.84	7.38	7.77	7.02	nd	8.15	6.67	7.48	6.61
Hb g/dl (10-15)	8.79	9.74	9.87	10.4	14.3	nd	9.79	10.3	11.5	10.3	nd	11.8	9.97	11.6	9.6
HCT % (30-45)	25.6	28.5	28.4	27.9	40.6	nd	27.5	28.6	30.6	28.1	nd	33.7	27.1	30.2	27.1
MCV fl (39-55)	42.2	39.9	39.1	38.7	39.5	nd	40.2	38.8	39.3	40.1	nd	41.4	40.6	40.3	41
MCH pg (12.5-17.5)	14.5	13.6	13.6	14.5	13.9	nd	14.3	14	14.8	14.7	nd	14.5	14.9	15.5	14.5
MCHC g/dl (30-36)	34.3	34.2	34.8	37.3	35.2	nd	35.6	36.1	37.7	36.8	nd	35.1	36.8	38.3	35.4
WBC $x10^9$/l (5.5-15.5)	13.1	17.4	9.54	7.41	4.77	nd	8.35	13.5	11.4	9.03	nd	9.68	11.8	11.5	8.65
Band neutrophils $x10^9$/l	0	0	0	0	0	nd	0	0	0	0	nd	0	0	0	0.09
Neutrophils $x10^9$/l (2.5-12.5)	7.99	12.88	4.96	4.08	1.91	nd	4.59	6.75	6.73	3.34	nd	4.36	6.02	5.18	4.24
Lymphocytes $x10^9$/l (1.5-7)	3.41	2.96	3.53	2.74	2.72	nd	2.76	4.59	3.99	4.42	nd	4.26	4.25	5.75	3.81
Monocytes $x10^9$/l (0-0.85)	0.39	0.52	0.19	0.22	0.05	nd	0.33	1.22	0.11	0.54	nd	0.29	0.59	0.12	0.26
Eosinophils $x10^9$/l (0-1.5)	1.31	1.04	0.67	0.30	0.10	nd	0.50	0.94	0.57	0.54	nd	0.77	0.83	0.46	0.17
Basophils $x10^9$/l	0	0	0.19	0.07	0	nd	0.17	0	0	0.18	nd	0	0.12	0	0.09
Normoblasts $x10^9$/l	0	0	0	0	0	nd	0	0	0	0	nd	0	0	0	0

nd = not done.

Appendix A.2. (contd) Haematology results for cats A745, A746 and A747 at 0, 3, 6, 12 and 15 weeks p.i.

	A745					A746					A747				
Test	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	nd	6.83	6.81	6.62	5.83	nd	7.21	6.47	6.81	6.51	nd	7.29	8.01	8.31	6.21
Hb g/dl (10-15)	nd	9.43	9.29	9.28	8.22	nd	10.3	9.32	10.1	9.44	nd	10	10.7	11.7	8.61
HCT % (30-45)	nd	27.7	27.4	25.9	22.6	nd	29.6	26.3	26.9	26.2	nd	28.7	30.6	31.3	24.2
MCV fl (39-55)	nd	40.5	40.3	39.1	38.9	nd	41.1	40.6	39.6	40.2	nd	39.4	38.2	37.7	39
MCH pg (12.5-17.5)	nd	13.8	13.6	14	14.1	nd	14.3	14.4	14.8	14.5	nd	13.7	13.3	14.1	13.9
MCHC g/dl (30-36)	nd	34	33.9	35.9	36.3	nd	34.8	35.5	37.5	36.1	nd	34.8	34.6	37.3	35.6
WBC $x10^9$/l (5.5-15.5)	11.2	16.7	10.6	12.6	7.22	16.6	25.5	15.8	13.2	14.9	nd	17.4	11.7	15.7	7.08
Band neutrophils $x10^9$/l	0	0	0	0	0	0	0	0	0	0	nd	0	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	7.06	9.35	4.45	5.54	2.46	11.45	19.12	9.48	6.6	9.54	nd	10.79	8.31	9.26	4.74
Lymphocytes $x10^9$/l (1.5-7)	3.47	6.180	5.19	6.05	4.26	4.48	3.57	3.16	5.15	4.77	nd	5.39	2.57	5.02	1.77
Monocytes $x10^9$/l (0-0.85)	0.45	0.50	0.32	0.13	0.14	0.17	0.51	0.47	0.40	0.30	nd	0.52	0.23	0.47	0.14
Eosinophils $x10^9$/l (0-1.5)	0.22	0.67	0.53	0.76	0.29	0.50	2.04	2.69	1.06	0.30	nd	0.70	0.58	0.94	0.42
Basophils $x10^9$/l	0	0	0.11	0.13	0.07	0	0.26	0	0	0	nd	0	0	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0	nd	0	0	0	0

nd = not done.

Appendix A.2. (contd) Haematology results for cats A748, A749 and A751 at 0, 3, 6, 12 and 15 weeks p.i.

	A748					A749					A751				
Test	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	4	6.98	8.2	7.82	6.99	6.98	8.07	9.81	8.04	6.92	5.5	7.48	7.22	7.53	7.25
Hb g/dl (10-15)	5.92	9.66	10.5	10.5	9.42	9.94	10.8	13.8	11.4	9.95	8.12	10.6	10.1	11.1	10.4
HCT % (30-45)	16	26.6	30.8	28.7	26	28.8	31.2	36.8	31	27.5	23.8	30.4	28.3	29.5	29.8
MCV fl (39-55)	39.9	38.1	37.6	36.6	37.2	41.2	38.6	37.6	38.6	39.8	43.2	40.6	39.2	39.1	41.1
MCH pg (12.5-17.5)	14.8	13.8	12.9	13.5	13.5	14.2	13.4	14.1	14.1	14.4	14.8	14.1	14.1	14.7	14.3
MCHC g/dl (30-36)	37.1	36.3	34.3	36.8	36.3	34.5	34.6	37.4	36.6	36.2	34.1	34.8	35.9	37.5	34.8
WBC $x10^9$/l (5.5-15.5)	4.74	13.7	16.2	4.9	6.54	8.54	13.5	9.34	9.14	17.4	14.5	10.1	23.9	10	8.28
Band neutrophils $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0.239	0	0
Neutrophils $x10^9$/l (2.5-12.5)	2.61	9.45	8.1	2.25	3.53	4.36	7.16	5.23	4.48	9.05	9.57	5.56	17.21	6.2	3.15
Lymphocytes $x10^9$/l (1.5-7)	1.75	3.15	6.97	2.16	2.75	3.33	4.32	2.99	2.56	6.09	4.35	3.74	4.78	2.8	4.88
Monocytes $x10^9$/l (0-0.85)	0.10	0.27	0.32	0.05	0.06	0.08	0.54	0.19	0.18	0.17	0.44	0.30	0.72	0.2	0.08
Eosinophils $x10^9$/l (0-1.5)	0.28	0.82	0.65	0.44	0.20	0.77	1.48	0.84	1.92	2.09	0.14	0.50	0.72	0.8	0.17
Basophils $x10^9$/l	0	0	0	0	0	0	0	0.09	0	0	0	0	0.24	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix A.2. (contd) Haematology results for cats A750, A752 and A755 at 0, 3, 6, 12 and 15 weeks p.i.

	A750					A752					A755				
Test	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w	0w	3w	6w	12w	15w
RBC $x10^{12}$/l (5-10)	5.47	6.05	7.29	7.16	9.48	6	6.9	7.07	8.07	7.75	nd	6.25	7.23	7.32	6.98
Hb g/dl (10-15)	7.65	8.21	9.17	9.83	13	9.11	10.1	10.5	12.5	11.8	nd	8.84	10.2	10.6	10.3
HCT % (30-45)	22.5	23.5	27.3	26.9	35.6	26.2	28.9	28.6	32.9	31.7	nd	26	28.9	28.8	29.5
MCV fl (39-55)	41.2	38.8	37.5	37.6	37.6	43.8	41.9	40.5	40.7	40.9	nd	41.6	40	39.4	42.2
MCH pg (12.5-17.5)	14	13.6	12.6	13.7	13.8	15.2	14.6	14.8	15.4	15.2	nd	14.1	14.1	14.5	14.8
MCHC g/dl (30-36)	33.9	35	33.5	36.5	36.6	34.7	35	36.6	37.9	37.1	nd	34	35.1	36.9	35
WBC $x10^9$/l (5.5-15.5)	7.27	10.2	18.7	9.35	4.66	10.2	18.7	14.9	19.7	12.1	nd	13	15.0	7.27	10.6
Band neutrophils $x10^9$/l	0	0	0.19	0	0	0	0	0	0	0	nd	0	0	0	0
Neutrophils $x10^9$/l (2.5-12.5)	4.29	3.98	8.42	3.09	1.82	6.43	12.90	8.49	14.97	7.02	nd	5.07	5.25	2.40	3.71
Lymphocytes $x10^9$/l (1.5-7)	2.25	4.59	8.04	5.42	2.61	2.86	4.68	5.36	3.74	3.99	nd	7.28	8.55	4.29	6.25
Monocytes $x10^9$/l (0-0.85)	0.14	0.41	0.37	0	0.14	0	0	0.74	0.20	0.24	nd	0.26	0.3	0	0.21
Eosinophils $x10^9$/l (0-1.5)	0.51	1.12	1.50	0.84	0.09	0.82	1.12	0.30	0.79	0.85	nd	0.39	0.9	0.58	0.42
Basophils $x10^9$/l	0.07	0.10	0.19	0	0	0	0	0	0	0	nd	0	0	0	0
Normoblasts $x10^9$/l	0	0	0	0	0	0.10	0	0	0	0	nd	0	0	0	0

nd = not done.

Appendix A.3. Media used in cloning experiments

LB-broth

10g tryptone
5g yeast
10g NaCl

Dissolve in 1 litre of dH_2O and then autoclave. For LB agar, add 1.2-1.5% agarose prior to autoclaving.

SOC medium

20g tryptone
5g yeast
0.5g NaCl

Dissolve in 1 litre of dH_20 and then autoclave. After autoclaving, add 1ml sterile $MgCl_2$, 1ml sterile 250mM KCl and 2.78 ml sterile 2M (36%) glucose/100ml.

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