STUDIES ON THE CELLULAR IMMUNE RESPONSE TO FELINE IMMUNODEFICIENCY VIRUS

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ABBREVIATIONS

aa	Amino acid
ADCC	Antibody-dependent, cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ARC	AIDS-related complex
ß-gal	ß-galactosidase
BHV	Bovine herpesvirus
CA	Capsid
CAEV	Caprine arthritis-encephalitis virus
cpe	Cytopathic effect
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DMSO	Dimethylsulphoxide
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(β-amino ethyl ether)
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ESR	Erythrocyte sedimentation rate
FCS	Foetal calf serum
FDC	Follicular dendritic cells
FeCoV	Feline coronavirus
FeSV	Feline sarcoma virus
FeLV	Feline leukaemia virus
FIP	Feline infectious peritonitis
FIV/GL14	Feline immunodeficiency virus/Glasgow 14 isolate
FIV/GL8	Feline immunodeficiency virus/Glasgow 8 isolate
FIV/PET	Feline immunodeficiency virus/Petaluma isolate
GST	Glutathione S-transferase
HHV-6	Human herpesvirus-6
HIV	Human immunodeficiency virus
IF	Immunofluorescence
Ig	Immunoglobulin
IN	Integrase
IV	Intravenous
K	KiloDaltons
KS	Kaposi's sarcoma
LAK cell	Lymphokine-activated killer cell

LCMV	Lymphocytic choriomeningitis virus
LDA	Limiting dilution analysis
LPS	Lipopolysaccharide
L:T	Lymphocyte:target ratio
MA	Matrix proteins
MAP	Multiple antigenic peptide
m.o.i.	Multiplicity of infection
NC	Nucleocapsid
NFMP	Non-fat milk powder
NK cell	Natural killer cell
O.D.	Optical density
orf	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pCTL	Precursor CTL
pfu	Plaque forming units
PGL	Persistent generalised lymphadenopathy
PHA	Phytohaemagglutinin
PR	Protease
rbc	Red blood cells
rpm	Revolutions per minute
RT	Reverse transcriptase
RPMI	Rosewall Park Memorial Institute medium
RSV	Respiratory syncytial virus
S.I.	Stimulation index
STD	Sexually transmitted disease
SDS	Sodium dodecyl sulphate
SPF	Specific pathogen free
Tris	2-amino-2(hydroxymethyl)-1,3-propandiol
TCR	T-cell receptor
TK	Thymidine kinase
U	Unit
vWT	Wild type vaccinia virus

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DECLARATION

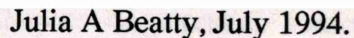
The studies described in this thesis were carried out in the Department of Veterinary Pathology at the University of Glasgow Veterinary School between October, 1990 and March 1994. The author was responsible for all results except where it is stated otherwise.

No part of this thesis has been presented to any university but it has been reproduced in part in the following publication and abstracts:

Beatty JA, Reid G, Rigby M, Willett B, Jarrett O and Browning MJ: Proliferation of peripheral blood lymphocytes from naive and FIV infected cats in response to a recombinant FIV envelope fusion peptide in vitro. In *Proceedings of the First International Conference of Feline Immunodeficiency Virus Researchers,* Abstract 25. University of California, Davis, USA, September 1991.

Beatty JA, Reid G, Rigby MA, Neil JC, Jarrett O and Browning MJ: A recombinant feline immunodeficiency virus envelope fusion protein stimulates peripheral blood lymphocytes from naive cats to proliferate in vitro. Vet Immunol Immunopathol 1992, **35**:143-153.

Beatty J, Flynn N and Jarrett O: Studies on cellular immunity to feline immunodeficiency virus (FIV). In *Proceedings of the International Symposium on Feline Retrovirus Research.* p16. North Carolina State University, USA, 6-9 October, 1993.



Julia A Beatty, July 1994.

SUMMARY

The aim of this study was to investigate cellular immunity to FIV. The main part of the project involved the development of an assay system to detect FIV-specific cytotoxic T lymphocytes (CTL).

In Chapter 1 the general features of the retrovirus family are described. The epidemiology, clinical signs and pathogenesis of human immunodeficiency virus (HIV) infection in man and of feline immunodeficiency virus (FIV) infection in the cat are reviewed in detail. The current knowledge on the host immune response to both of these viruses is summarised.

Chapter 2 covers the materials and methods which have been used routinely throughout these studies.

Chapter 3 describes the development of an autologous, FIV-GAG expressing, ^{51}Cr labelled target cell system for detecting feline cytotoxic effector cells. Peripheral blood mononuclear cells (PBMC), renal fibroblasts, bone marrow fibroblasts and skin fibroblasts were compared on the basis of ease of harvesting, in vitro growth characteristics and target antigen expression. Virus antigen expression in fibroblasts was achieved using a recombinant FIV/vaccinia virus, vFIV/GL14-*gag,* as fibroblasts are not normally infectable by FIV. Skin fibroblasts were selected as the most suitable target cell for the assay. A convenient and effective method of ^{51}Cr labelling of target cells was developed using overnight incubation of target cell monolayers *in situ*.

It was intended to use the prototype cytotoxicity assay to detect secondary cytotoxic responses since these are generally of greater magnitude than primary responses. The conditions necessary for the generation of secondary FIV-specific effector cells from circulating precursor cells were unknown. In Chapter 4, potential sources of endogenously processed viral antigen for the in vitro restimulation of circulating lymphocytes primed by FIV infection in vivo were investigated. Mitogen-activated PBMC from FIV-infected cats, lymphoblasts infected with FIV in vitro followed by fixation and vFIV/GL14-*gag* infected, fixed lymphoblasts were compared for efficiency of preparation and antigen expression. The latter were found to be the most suitable and batches of vFIV/GL14-*gag* infected, fixed lymphoblasts were prepared from the experimental cats and used as stimulator cells in subsequent experiments.

In Chapter 5, the capacity of a prototype assay to measure feline cytotoxic responses was determined. From 3 experimental cats, skin fibroblasts were harvested and

stimulator cells were prepared. Two of these cats (E3 and F23) were then experimentally infected with FIV and the third cat remained uninfected as a control (F22). PBMC were isolated from these cats prior to, and at intervals post-infection or mock-infection. Following a 6-7 day in vitro restimulation period, these bulk cultures were assayed for secondary cytotoxic effector cell activity.

The assay was successful in detecting antigen-specific cytotoxic effector cells in both FIV-infected cats. Cytotoxic cells specific for autologous targets expressing FIV-GAG were detected from 2 weeks post-infection in 1 FIV-infected cat and at 14 weeks post-infection in the other. Killing of heterologous targets cells was also detected from FIV-infected cats although to a lesser extent than autologous targets. No target cell lysis above background was detected using effector cells from the uninfected control cat.

In a separate set of experiments (Chapter 6) the capacity of a recombinant FIV-ENV fusion protein to stimulate PBMC from naive cats to proliferate in vitro was investigated. A region of FIV/GL8 *env*, incorporating the third and fourth variable regions (V3/V4), was cloned, inserted into the pGEX vector and expressed in *Escherichia coli* to yield milligram quantities of the recombinant polypeptide as a fusion protein with glutathione S-transferase (GST). The fusion protein, V3/V4GST, was used in lymphocyte proliferation assays, where it consistently caused PBMC from naive cats to proliferate in a dose-dependent manner. Other FIV fusion proteins produced under identical conditions (V5GST and p24GST) and GST alone did not cause proliferation in this system. The monoclonal antibody vpg15, which has been shown to block infection of susceptible cells in vitro, did not decrease the response to V3/V4GST. Human PBMC did not proliferate in response to V3/V4GST.

In Chapter 7 the results of this thesis are brought together, conclusions drawn and implications discussed. Suggestions for the future direction of this work are included.

CHAPTER 1

GENERAL INTRODUCTION

Feline immunodeficiency virus (FIV) is a pathogenic lentivirus which is endemic in domestic cats throughout the world. FIV infection in the cat is an excellent experimental model of human immunodeficiency virus (HIV) infection in man. The aim of this study was to investigate the cellular immune response to FIV. The principal aim of the project was to develop an assay system capable of detecting FIV-specific CTL. This problem was approached by adapting the ^{51}Cr release cytotoxicity assay, the standard method for detecting CTL, for use in the cat.

In this chapter the general features of the retrovirus family are outlined. Following a description of the structures of HIV and FIV, the epidemiology, pathogenesis and clinical signs of these infections are covered in detail. Current knowledge on the host immune response to HIV and FIV is reviewed. Finally, the general features of CTL responses and their role in antiviral immunity are discussed.

1.1 THE RETROVIRIDAE

1.1.1 Introduction

The study of retroviruses began over 80 years ago when it was shown that a leukaemia (Ellerman and Bang, 1908) and a sarcoma (Rous, 1911) could be transmitted between chickens by the inoculation of tumour extracts. Members of this virus family have since been isolated from many species of animals. Some retroviruses are apathogenic, while others are associated with a wide variety of diseases including malignancies, neurological disorders, wasting diseases and immunodeficiency.

The retroviruses derive their name from their capacity for reverse transcription, a process by which the viral RNA genome is converted into DNA by the viral enzyme reverse transcriptase (RT). The DNA can incorporate stably into the host cell genome as a provirus and, in some cases after a period of latency, direct the cellular machinery to reproduce the virus. It is this integration which underlies the ability of retroviruses to cause persistent infections in animals. A further consequence of chromosomal integration is that some retroviruses recombine with cellular DNA which replaces part of the viral genome. Where this foreign sequence represents a host oncogene, infection of further susceptible cells may result in malignant transformation. Even retroviruses which do not contain oncogenes may still have oncogenic potential since random integration within or adjacent to cellular oncogenes can disrupt their regulation. Conversely, many vertebrate species harbour endogenous proviral sequences in their genomes resulting from historical integration of retroviruses into germ line cells. Aside from the importance of many members as agents of disease, the study of retroviruses is also fuelled by their potential use as vectors for genetic manipulation (Varmus, 1988; Coffin, 1990).

1.1.2 Classification

Retroviruses were originally classified into subfamilies based on their host species and ultrastructural morphology of virus particles. This system was superseded by classification into 3 subfamilies based on their clinical manifestations or effect on host cells in culture. The subfamily Oncovirinae contained the tumour-forming viruses such as mouse mammary tumour virus (MMTV) and feline leukaemia virus (FeLV); Lentivirinae contained the so-called "slow" viruses including maedi-visna virus of sheep, caprine arthritis-encephalitis virus (CAEV) and the human, simian, feline and bovine immunodeficiency viruses (HIV, SIV, FIV, BIV); Spumavirinae contained apparently apathogenic viruses which produced a characteristic vacuolating cytopathic effect (cpe) on susceptible cells, e.g. feline syncytial virus (FeSFV). This classification had drawbacks. For instance, many of the genera of oncogenic retroviruses were no more genetically related to each other than to members of the other subfamilies. Consequently, retroviruses have recently been reclassified into 7 genera (Table 1.1) (Coffin, 1992).

1.1.3 Structure and genomic organisation.

Retroviruses have roughly spherical virions of 80-100nm diameter. The diploid, single-stranded RNA genome is contained within a spherical to rod shaped capsid. There is an outer virus envelope which is derived from the host cell plasma membrane during budding. The virus envelope contains glycoprotein projections or "spikes" which attach to the host cell during adsorption and thus determine the host cell range.

Retroviruses have a diploid, single stranded positive sense RNA (ss+RNA) which is not infectious *per se*. The provirus has 3 major open reading frames (orf); the *gag*, *pol* and *env* genes. Retroviral proteins are often synthesised as polyproteins which undergo cleavage to yield the functional products. The *gag* gene encodes the internal structural proteins of the virus which contain group specific determinants. These are the matrix (MA), capsid (CA) and nucleocapsid (NC) proteins and, in some genera, a fourth protein. The viral protease (PR), RT, and integrase (IN) are encoded by the *pol* gene except in the avian retroviruses where the PR is encoded by *gag*. The *env* gene codes for the surface (SU) and transmembrane (TM) envelope proteins. The envelope proteins are glycosylated with the exception of MuLV-related viruses in which the TM protein remains unglycosylated. The RNA is flanked by non-coding reiterated (R) sequences and adjacent unique 5' (U5) or 3' (U3) sequences which are involved in the initiation and regulation of transcription. More complex mammalian retroviruses also encode non-structural regulatory proteins.

1.1.4 Replication

Retroviruses generally require actively dividing cells for productive infection (Varmus and Swanstrom, 1982). The virus attaches to a specific host cell surface receptor by its envelope glycoproteins. By a process which is not fully understood, the virus envelope fuses with the host cell membrane, either the plasma membrane (HIV) or the endosomal membrane following receptor-mediated endocytosis (most other retroviruses), and the core is internalised. The ss+RNA genome is reverse trancribed into ds±DNA. Retroviral RT lacks an exonucleolytic (proof-reading) activity allowing a high frequency of base misincorporation, thus generating a large number of virus variants which is a characteristic of retroviruses. The ds±DNA intermediate migrates to the nucleus and becomes integrated into the host chromosomal DNA by the viral IN to form a provirus. In the course of reverse transcription there is duplication of the R, U3 and U5 sequences which combine to produce the long terminal repeats (LTR) which flank the proviral DNA. The DNA provirus serves as a template for the transcription of both mRNA, which is translated into viral proteins by the host cell, and genomic RNA. Mature virions assemble either at the plasma membrane or as intracytoplasmic particles and are released from the host cell by budding.

1.1.5 Lentiviruses

Lentiviruses cause lifelong infections characterised by slowly progressive illnesses with prolonged periods of subclinical infection. Host neutralising antibodies fail to clear infection and protective immunity is very difficult to achieve by vaccination. Like other retroviruses, lentiviruses are highly host-specific. In contrast to other retroviruses lentiviruses can productively infect non-dividing cells although activation and/or differentiation is necessary for synthesis of progeny virus. As a result of replication, lentiviruses are often cytopathic. The principal route of transmission of lentiviruses is horizontal, although some (e.g. HIV) are also transmitted vertically (Narayan and Clements, 1989; Narayan and Clements, 1990).

Lentiviral infections of ungulates cause primary diseases. Differences in disease syndromes caused by different lentiviruses are partly due to preferential infection of different target cell types e.g. CAEV is tropic for synovial tissue. In man, macaques and cats, in addition to primary viral disease (lymphadenopathy, pneumonia, neurological signs) secondary diseases are also seen. Secondary diseases are a consequence of lentivirus-induced immunodeficiency.

Table 1.1 Classification of the retroviruses

FAMILY	GENUS	SUB-GENUS	SPECIES
	MAMMALIAN TYPE B ONCOVIRUSES	——	Mouse mammary tumour viruses
	MURINE LEUKAEMIA VIRUS-RELATED VIRUSES	Mammalian type C viruses	e.g. Murine and feline leukaemia viruses
		Reticuloendotheliosis viruses	Avian reticuloendotheliosis virus
		Reptilian type C viruses	Viper virus
	TYPE D RETROVIRUS GROUP	——	e.g. Mason-Pfizer virus
RETROVIRIDAE	AVIAN TYPE C RETROVIRUS GROUP	——	e.g. Avian leukosis virus
	FOAMY VIRUS GROUP	——	e.g. Human foamy syncytium-forming virus
	HTLV-BLV GROUP	——	Human T-cell leukaemia viruses, Bovine leukaemia virus
	LENTIVIRUS GROUP	Primate immunodeficiency viruses	HIV-1, HIV-2, SIV
		Ovine/caprine lentiviruses	Maedi-visna virus, caprine arthritis encephalitis virus
		Equine lentiviruses	Equine infectious anaemia virus
		Feline lentiviruses	Feline immunodeficiency virus
		Bovine lentiviruses	Bovine immunodeficiency virus

Table 1.1

1.2 HUMAN IMMUNODEFICIENCY VIRUS.

1.2.1 Introduction

In 1981 a syndrome characterised by chronic and recurrent opportunistic infections and unusual neoplasms was described in male homosexuals in America (Centers for Disease Control 1981; Gottlieb *et al.*, 1981). The increasing incidence of cases of this acquired immune deficiency syndrome (AIDS) and geographical clustering suggested that an infectious agent was involved. Several groups isolated a novel retrovirus from patients with AIDS and at risk for AIDS (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984). Originally designated human T lymphotropic virus III (HTLV-III) or lymphadenopathy-associated virus (LAV), the virus was eventually classified with the lentiviruses as human immunodeficiency virus (HIV) (Coffin *et al.*, 1986) based on morphological, biological and molecular features shared with equine and ruminant lentiviruses. Sero-epidemiological data and consistent isolation of HIV from patients with AIDS have established HIV as the aetiological agent of AIDS (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Safai *et al.*, 1984; Levy *et al.*, 1984; Schechter *et al.*, 1993; Gallo *et al.*, 1986; Montagnier, 1986). A related lentivirus, antigenically distinct from HIV-1 in surface glycoproteins and designated HIV-2, has been isolated from patients with immunodeficiency in West Africa (Clavel *et al.*, 1986; Albert *et al.*, 1987).

1.2.2 Structure and genomic organisation

The HIV virion contains 2 identical copies of single stranded RNA which, together with viral enzymes, are enclosed in a protein core and surrounded by the envelope which contains viral glycoproteins. There are 3 major orf: the *gag*, *pol* and *env* genes as described above (1.1.3). The GAG polyprotein precursor, p55 is post-translationally cleaved into p24 (CA), p10 (NC) and p17 (MA). The *env* gene encodes the SU and TM envelope proteins which become glycosylated (gp120 and gp41 respectively). In addition to these major genes, HIV-1 also contains 3 genes encoding regulatory proteins; *rev* (regulator of virion protein), *tat* (trans-activator) and *nef* (negative regulatory factor), and 3 encoding proteins involved in virus maturation and release; *vif* (virion infectivity factor), *vpu* (viral protein U) and *vpr* (viral protein R) (Cann and Karn, 1989).

HIV-2 differs from HIV-1 at the genomic level in 2 major respects. HIV-2, in common with the simian immunodeficiency virus (SIV), contains the gene *vpx* not found in HIV-1. The product of *vpx* is required for efficient viral repication and

may be involved in virus entry into the host cell (Kappes *et al.*, 1991; Yu *et al.*, 1993). HIV-2 does not encode a *vpu* protein (Rosenberg and Fauci, 1989).

1.2.3 Host cell range

The principal targets of HIV-1 are T-cells and cells of the monocyte/macrophage lineage, although *in situ* hybridisation has demonstrated mRNA in several other cell types including glial and enterochromaffin cells (Tersmette and Miedema, 1990). The major cellular receptor for HIV-1, the CD4 molecule, was the first retroviral receptor to be identified when 2 groups were able to protect susceptible cells from infection with HIV-1 in vitro using antibodies to CD4 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984). These findings were supported by co-immunoprecipitation of CD4 and HIV ENV using antibodies to either molecule (McDougal *et al.*, 1986) and were confirmed when transfection of the CD4 gene was found to confer susceptibility to HIV-1 infection on previously resistant human cell lines (Maddon *et al.*, 1986). However, it is likely that other molecules are required for virus entry since murine cells transfected with CD4, although able to bind HIV-1, are resistant to infection (Maddon *et al.*, 1986). In addition, some $CD4^-$ human cells can be infected with HIV-1 in vitro (Cao *et al.*, 1990). Cells expressing the Fc receptor may permit entry of HIV-1 in the form of HIV-1-antibody complexes (Takeda *et al.*, 1988; Homsy *et al.*, 1989) although there is controversy as to whether this mechanism is complementary to, or independent of, CD4-mediated infection (Weiss, 1993(a)).

Following binding to the target cell it is likely that HIV-1 becomes internalised by direct fusion of HIV-1 envelope with the host cell membrane (Stein *et al.*, 1987; McClure *et al.*, 1988; Grewe *et al.*, 1990). A 17 amino acid (aa) region of the TM envelope glycoprotein gp41 has been implicated in the internalisation process (Qureshi *et al.*, 1990). Receptor-mediated endocytosis, typical of other retroviruses, has been documented for HIV-1 but is thought to be of minor significance (Grewe *et al.*, 1990). Following virus entry, reverse transcription of viral RNA occurs. HIV-1 replication differs from that of most other retroviruses in that much of the viral DNA persists in the unintegrated form which may contribute to the cpe of the virus (Shaw *et al.*, 1984). The fate of integrated provirus depends on the state of activation of the host cell. In non-activated cells infection is latent whereas in activated cells provirus is transcribed into genomic RNA and mRNA resulting in productive infection which is lethal to the cell (Ho *et al.*, 1987(a); Stevenson *et al.*, 1990).

A characteristic of retroviruses is genetic variability (1.1.4). This variability is believed to be largely a consequence of the error rate of HIV-1 RT (Ho *et al.*,

1987(a)). At more than 1 base per genome per cycle, the error rate of HIV RT is exceptionally high, even for a retrovirus (Preston *et al.*, 1988; Roberts *et al.*, 1988). This high mutation rate, particularly in *env* products (Leigh-Brown, 1988), gives rise to a population of closely related genomes or quasispecies (Wain-Hobson 1989). Variability is also seen between HIV-1 isolates at the biological level in terms of in vitro cell tropism, replication rate, syncytium-inducing capacity and cytopathicity. This variability in in vitro growth characteristics correlates with variability in *env* nucleotide sequence (Tersmette and Miedema, 1990).

1.2.4 Epidemiology

At the end of 1993, the World Health Organisation (WHO) estimated that 14 million adults and 1 million children had been infected with HIV worldwide since the start of the pandemic in the late 1970s (WHO, 1994(a)). The cumulative incidence of AIDS cases was put at over 3 million, of which over 75% occurred in developing countries (WHO, 1994(a)).

HIV is essentially a sexually transmitted disease which can also be spread vertically and by inoculation of contaminated blood and blood products. Arthropod transmission has been ruled out (Webb *et al.*, 1989). The different routes of transmission of HIV give rise to several at-risk groups; sexually active individuals and their offspring, intravenous (IV) drug users and recipients of contaminated blood and blood products. The global AIDS problem can be described as a series of overlapping epidemics involving each of these at-risk groups. The risk of sexual transmission is much greater for anal than for vaginal intercourse and increases with intercurrent venereal disease. IV drug users who become infected by using contaminated equipment represent a large and rapidly increasing proportion of the total cases. For example, in Edinburgh the incidence of HIV infection in IV drug users rose to 50% in 2 years (Des Jarlais and Friedman, 1987), in Bangkok from 1-43% in the space of a single year (Phanuphak *et al.*, 1989) and in the USA 25% of all AIDS cases have occurred in IV drug users (Friedland *et al.*, 1987). IV drug users are a major source of spread to the heterosexual population (Ellerbrock *et al.*, 1991). Individuals exposed to HIV "artificially" in contaminated blood almost always become infected (Colebunders *et al.*, 1991). Although this mode of transmission has been effectively eliminated by screening of blood products in developed countries, contaminated blood transfusion remains the third most common route for HIV transmission in Africa (Perriens and Piot, 1993). Babies born to HIV infected mothers have a 12-50% chance of becoming infected (Friedland and Klein, 1987; Blanche *et al.*, 1989; Katz *et al.*, 1989; Hirsch and Curran, 1990), the risk increasing with maternal immunosuppression. Maternal

transmission has been shown to occur *in utero*, peri- and postnatally (Friedland and Klein, 1987; Reviewed in Hirsch and Curran 1990) but the relative efficiency at each stage is unclear (Friedland and Klein, 1987). Virus can also be transmitted during breast feeding, particularly in acute infection where there is high level viraemia (Van De Perre *et al.*, 1991).

The HIV epidemic was originally recognised in homosexual men (Gottlieb *et al.*, 1981) and although this group still accounts for the majority of AIDS cases in industrialised countries the incidence of HIV infection in this group plateaued in the mid 1980s (Perriens and Piot, 1993). By far the greatest numbers of HIV seropositive individuals are presently found among the heterosexual communities of the developing world, particularly in Africa (Georges *et al.*, 1987). In sub-Saharan Africa it is estimated that 9 million people are HIV seropositive (WHO, 1994(a)) and the epidemic has not yet reached equilibrium there. In Cameroon there are fears of an exponential increase in the number of AIDS cases in the next few years (WHO, 1993). HIV-1 is also spreading into West Africa where estimates of prevalence are complicated by the presence of HIV-2 (WHO, 1994(b)).

It is in Asia, particularly south and southeast Asia, that the epidemic is presently increasing most rapidly. The rise of seropositivity in Asia from 0 to 1 million cases between 1987 and 1991 is a sobering demonstration of the capacity of HIV to spread within a population. These HIV infections were initially seen in IV drug users but the principal mode of transmission is now by heterosexual contact (Perriens and Piot, 1993).

1.2.5 Clinical signs

Infection with HIV-1 is associated with progressive immune dysfunction which eventually becomes manifest clinically. Patients suffer repeated episodes of illness and eventually die from one or a combination of AIDS-related illnesses. Recovery from HIV-1 infection has not been recorded.

An acute infectious mononucleosis-like primary phase syndrome associated with seroconversion may occur within a few weeks of HIV-1 infection (Cooper *et al.*, 1985; Feinberg, 1992). Signs include fever, nausea, arthralgia, myalgia and skin rash. Haematological changes include transient leucopenia, lymphopenia, monocytosis, thrombocytopenia and elevated ESR (Tindall and Cooper, 1991). During this primary phase HIV-1 can be readily isolated from PBMC and plasma, and p24 antigenaemia is often present (Cooper *et al.*, 1985; Cooper *et al.*, 1987). This stage is usually self-limiting, resolving within 2-3 weeks.

Patients then enter the "asymptomatic" phase which has been estimated to last for an average of 7-10 years, although disease progression among individuals is very variable (Curran *et al.*, 1988; reviewed in Moss and Bacchetti, 1989). p24 antigen is not usually detectable in plasma during this stage. Throughout this stage some patients have persistent generalised lymphadenopathy (PGL) characterised by palpable lymphadenopathy at 2 or more extrainguinal sites for longer than 3 months with no other identifiable cause.

Progressive changes in some haematological and biochemical parameters during the course of infection provide useful markers for disease staging. As infection proceeds the absolute and relative $CD4^+$ cell count decreases, β2 microglobulin levels increase and p24 antigenaemia recurs (Moss *et al.*, 1988; Fahey *et al.*, 1990; Janossy, 1991). There is also an increase in $CD8^+$ cells although this is also influenced by concurrent infections.

It is believed that the vast majority of HIV-1 infected individuals will eventually develop AIDS (Moss *et al.*, 1988). The first signs of decline (AIDS-related complex, ARC) include chronic fevers, night sweats, diarrhoea, weight loss, herpes zoster, oral candidiasis or hairy leukoplakia. In the AIDS stage there may be wasting syndromes, opportunistic infections, neoplasms, and nervous signs. Infections commonly associated with AIDS include *Pneumocystis carinii* pneumonia, mucosal candidiasis and disseminated cytomegalovirus (CMV) infection (Hirsch and Curran, 1990; Nelson *et al.*, 1990). Certain neoplasms are associated with AIDS particularly Kaposi's sarcoma (KS), non-Hodgkin's lymphoma and primary lymphoma of the brain (Schüpbach, 1989). HIV has not been shown to be directly oncogenic. The majority of HIV associated lymphomas are of B cell origin and provirus has not been detected in these tumours. There is evidence that an infectious agent may be involved in the aetiology of KS (Giraldo and Beth, 1986) possibly one which is sexually transmissible (Elford *et al.*, 1993). Neurological problems accompany HIV infection. Some of these such, as subacute encephalitis (AIDS dementia complex), are believed to be a primary effect of the virus. Secondary infections involving the CNS such as toxoplasmosis and cryptococcosis are also seen.

HIV-2 infection is also associated with immunodeficiency and a clinical syndrome similar to that described for HIV-1 although HIV-2 may be less virulent (Brun-Vezinet *et al.*, 1987; Wong-Staal, 1990).

1.2.6 Pathogenesis of HIV infection

Despite the intense worldwide research effort which has followed the isolation of HIV-1, the underlying factors responsible for the decline into AIDS are still not understood.

i) CD4$^+$ T lymphocyte depletion

Progressive depletion of circulating CD4$^+$ lymphocytes is the most consistent immunological abnormality seen in HIV-1 infection (Fahey *et al.*, 1990). Memory CD4$^+$ T cells (CD45RA$^-$, CD29$^+$) are specifically targeted (Schnittman *et al.*, 1990; Van Noesel *et al.*, 1990). These cells typically function as T helper cells (T_h) which have a central role in regulating the immune response. T_h regulate B cell growth and differentiation and modulate the activity of other effector cells including CTL, natural killer (NK) cells, macrophages and granulocytes. Elimination of T_h in HIV-1 infection therefore has a profound effect on immune function.

Many potential mechanisms have been proposed for the depletion of CD4$^+$ cells in HIV-1 infection (reviewed in Fauci, 1993; Rosenberg and Fauci, 1989). HIV-1 has been shown to be directly cytopathic for activated CD4$^+$ lymphocytes in vitro by various mechanisms including membrane damage during budding, syncytium formation, accumulation of unintegrated DNA and core proteins and intracellular gp120/CD4 binding (Ho *et al.*, 1987(a); Rosenberg and Fauci, 1989; Fauci, 1993). Indirect mechanisms of CD4$^+$ depletion may also occur. Uninfected CD4$^+$ lymphocytes may be killed by synctium formation with infected cells, or they may become coated with, or take-up and process, free gp120 making them targets for host immune responses such as antibody-dependent cell-mediated cytotoxicity (ADCC) and CD4$^+$ CTL-mediated lysis respectively (Lanzavecchia *et al.*, 1988; Siciliano *et al.*, 1988; Weinhold *et al.*, 1988). That HIV-infected cells may be more suceptible to superinfection by other pathogens has been shown in vitro with CMV (Ho *et al.*, 1987(a)). Lymphocytes dually infected with human herpesvirus-6 (HHV-6) and HIV-1 show accelerated HIV-1 expression and cell death in vitro (Lusso *et al.*, 1989). The extent to which these mechanisms occur in vivo is not known.

There is evidence from studies in vitro and in vivo that apoptosis may be a mechanism of lymphocyte loss in HIV infection (Ameisen and Capron, 1991; Gougeon *et al.*, 1993). Whether apoptosis specifically affects CD4$^+$ lymphocytes or

whether CD8+ cells are also affected is unresolved (Groux *et al.*, 1992; Meyaard *et al.*, 1992).

A role has been suggested for a putative superantigen in the pathogenesis of HIV-1 infection (Janeway, 1991). Superantigens can bring about widespread activation of T cells by their ability to bind specific Vβ chains of the T cell receptor (TCR) to conserved regions of MHC class II molecules. Superantigens induce stimulation and expansion of large numbers of T cells. The potential consequences of such activation include exhaustion, anergy or apoptosis of affected Vβ subsets, provision of further susceptible targets and, by maintaining the immune system in an activated state, suppression of the immune response to incoming antigens. A murine retrovirus has been shown to encode a superantigen that causes deletion of specific T cell subsets (Hügin *et al.*, 1991). If a putative HIV-1-associated superantigen operated by activating CD4+ cells allowing increased viral replication, then it would be expected that HIV-1 isolates would replicate more efficiently in T cells expressing certain Vβ chains. This has been shown (Laurence *et al.*, 1992). Also, if a superantigen were involved in the pathogenesis of HIV-1 infection then a different frequency of certain Vβ subsets would be expected in infected compared to uninfected individuals and this has been reported although results have not been consistent between studies. Imberti and colleagues (1991) used PCR and internal probe hybridisation to show that the Vβ repertoire was restricted in HIV seropositive individuals compared to seronegative controls whereas no difference was found in Vα repertoires. The HIV-1 infected individuals in this study all had advanced disease so that any effect of opportunistic infections on Vβ repertoire could not be ruled out. Also, unfractionated PBMC were examined so that the observed difference may have resulted from perturbations in the CD4:CD8 ratio. In a separate study, flow cytometric analysis was used to compare Vβ subsets in 16 healthy seropositive individuals with controls (Dalgleish *et al.*, 1992). A significant increase in Vβ5.3 was found in HIV-1 infected individuals. However, complete typing of the Vβ repertoire was not possible in this study due to limitations in the availability of monoclonal antibodies for this purpose and, again, CD4+ and CD8+ T lymphocyte subsets were not examined individually.

The MHC class II type of an individual is a major influence on TCR Vβ repertoire so that more meaningful comparisons of Vβ repertoire in an outbred population can be made between individuals of similar MHC background. Studies by Soudeyns and colleagues, (1993), took this factor into account by making comparisons between monozygotic twins discordant for HIV-1 infection and HIV-1 infected mothers and their infected and uninfected babies. They found significant alterations in the Vβ repertoire of HIV-1 infected subjects.

Although no superantigen has been identified in HIV, there is evidence for a viral superantigen effect in man since the nucleocapsid protein of rabiesvirus has been shown to have superantigen-like properties for human PBMC in vitro (Lafon *et al.*, 1992). More recently, an SIV isolate associated with acute disease in macaques, $SIV_{mmPBj14}$, has been shown to encode a superantigen (Chen *et al.*, 1994). Expansion of Vβ7 and Vβ14 T lymphocyte subsets has been found following exposure of lymphocytes to this virus both in vitro and in vivo.

Whatever the mechanism of $CD4^+$ cell depletion in HIV-1 infection it must be accompanied by a deficit in regeneration. HIV-1 may infect $CD4^+$ lymphocyte precursors or cells essential for their development. Myeloid progenitors in the bone marrow have been shown to be susceptible to HIV-1 in vitro (Folks *et al.*, 1988). Alternatively, exhaustion of the T_h precursor pool may occur. A decrease in numbers of $CD4^+$ cells alone cannot account for the eventual decline of HIV-1 seropositive patients. Other viruses which are cytopathic for $CD4^+$ cells in vitro, such as HHV6 which is endemic in Western populations, do not cause AIDS (Lusso *et al.*, 1988). Qualitative deficits in different populations of immune cells accompany HIV-1 infection and these functional abnormalities preceed a drop in $CD4^+$ cell number (Miedema *et al.*, 1988). For instance, T_h show decreased responses to soluble antigens (Lane *et al.*, 1985) and reduced proliferation to anti-CD3 monoclonal antibodies (Gruters and Miedema, 1992).

ii) HIV-1 infection of monocytes

HIV-1 also infects $CD4^+$ monocytes and macrophages (Collman *et al.*, 1990). It has been suggested that the wasting syndrome and CNS disease seen in HIV-1 infected patients are a consequence of macrophage infection since these features resemble those seen in maedi-visna infection of sheep, a macrophage-tropic lentiviruses (Weiss, 1993(b)). Monocytes are believed to function in virus persistence and dissemination to the brain and lungs (Koenig *et al.*, 1986; Haase, 1986; Gendelman and Meltzer, 1990). HIV-1 infected monocytes represent the major reservoir of virus in the brain and have been shown to secrete a neurotoxin in vitro which may be involved in CNS pathology (Giulian *et al.*, 1990). There is evidence that HIV-1 infected monocytes are defective in cytokine secretion and antigen presentation (Miedema *et al.*, 1988; Terpstra *et al.*, 1989). Antigen presentation in HIV-1 infection may be further disrupted by effects on dendritic cells and follicular dendritic cells (Knight *et al.*, 1992).

iii) B cell dysfunction

HIV-1 infection is accompanied by severe deficits in B cell function (Amadori and Chieco-Bianchi, 1990). Within 10 months of infection, reduced pokeweed mitogen (PWM)-induced immunoglobulin (Ig) synthesis can be detected (Terpstra *et al.*, 1989). This is followed by polyclonal activation of B cells leading to hypergammaglobulinaemia (Amadori *et al.*, 1989; Levy, 1989; Pahwa *et al.*, 1986; Schnittman *et al.*, 1986). Twenty-50% of the Ig which these activated B cells constitutively produce in vitro is specific for HIV-1 (Pahwa *et al.*, 1986; Amadori *et al.*, 1989). Antibody responses to both T-dependent and T-independent antigens are reduced in HIV-1-infected patients (Lane *et al.*, 1983; Ammann *et al.*, 1984; Teeuwsen *et al.*, 1987). Some aspects of B cell dysfunction may result from a general failure of immune regulation although other B cell abnormalities occur independently of deficits in T_h function and preceed the latter (Terpstra *et al.*, 1989). Specific effects of HIV-1 proteins, particularly gp120 (Schnittman *et al.*, 1986), up-regulation of cytokines such as IL6 by HIV-1 infected monocytes (Nakajima *et al.*, 1989) and B cell activation via HIV-1 activation of Epstein-Barr virus (EBV) (Schüpbach, 1989) have all been proposed as mechanisms involved in B cell dysfunction.

iv) Other mechanisms with a potential role in disease progression

Cytokines have been implicated in mediating many of the immunological abnormalities that occur in HIV-1 infection (Matsuyama *et al.*, 1991; Sinicco *et al.*, 1993). For example, IL6, IL1 and TNF-α are raised in some seropositive individuals and may be involved in B cell proliferation (Freeman *et al.*, 1989; Amadori and Chieco-Bianchi, 1990). More recently interest has focused on the activity of T_h subsets in HIV progression. There are 2 distinct T_h subsets, T_{h1} and T_{h2}, which were first descibed in mice (Mossmann and Coffman, 1989) and can be identified by the cytokines they produce. Based on resistance of mice lacking a T_{h2} response to murine AIDS (Kanagawa *et al.*, 1993) and cytokine profiles from lymphocytes from HIV-infected patients, it has been suggested that a switch from a predominant T_{h1} response to a T_{h2} response may be associated with progression to AIDS (Clerici and Shearer, 1993; Cohen, 1993). The mechanism of this loss is not understood but may involve decreased production of IL12. Addition of IL12 (a T_{h1} cytokine) can restore cell-mediated immune responses from HIV-seropositive individuals in vitro (Clerici *et al.*, 1993(a); Hall, 1994).

Clinical and immunological similarities between early AIDS and graft-versus-host disease have led to the suggestion that an autoimmune response is involved in the

pathogenesis of HIV-1 (Shearer, 1983). It has been proposed that HIV-1 gp120 mimics alloantigen causing activation of alloantigen specific lymphocytes which could account for many of the immunological abnormalities accompanying HIV-1 infection (Habeshaw *et al.*, 1992).

A role of has been suggested for viral variants in disease progression (Fenyö, 1993). Faster replicating, syncytium-inducing isolates (1.2.3) can more often be isolated from patients with advanced clinical disease. However, no clear correlation exists between clinical state and biological properties of the isolate (Tersmette *et al.*, 1988; Hirsch and Curran, 1990).

Another potential mechanism of disease progression is viral escape from host immune responses. Escape from neutralising antibodies has been shown but does not correlate with disease progression (Albert *et al.*, 1990; McCune, 1991). Possible CTL escape mutants have been described (Phillips *et al.*, 1991) and are discussed in detail below.

Following the initial peak of plasma viraemia, virus becomes much more difficult to detect in the periphery until the late stages of HIV-1 infection when clinical signs develop and plasma viraemia again becomes readily detectable. Quantitative competitive polymerase chain reaction (PCR) techniques have recently demonstrated that viraemia is present at low but fluctuating levels throughout the asymptomatic period (Piatak *et al.*, 1993). Using PCR for viral DNA and RNA in combination with *in situ* hybridisation it has recently been shown that, in contrast to the circulating T cells, virus can be readily detected in $CD4^+$ lymphocytes and macrophages in secondary lymphoid organs at all stages of infection. Active HIV-1 replication has been demonstrated in lymphoid tissues throughout the asymptomatic period (Pantaleo *et al.*, 1993), although not all infected cells in the germinal centres produce virus (Embretson *et al.*, 1993). Similarly in HIV-1 infected chimpanzees, which remain clinically healthy, active viral replication was detected in lymphoid tissue but not in PBMC, despite the fact that 1 in 10^3-10^4 PBMC contained proviral DNA (Saksela *et al.*, 1993). In addition, these latently infected cells could be induced to transcribe HIV-1 in vitro. HIV-1 therefore causes a persistent rather than a latent infection since low levels of viral replication can be detected at all stages. Lymph nodes may also be an important site for HIV-1 infection of $CD4^+$ cells. It has been proposed that HIV-1 may be trapped by follicular dendritic cells (FDC) and infect activated $CD4^+$ cells which migrate to the germinal centres in response to antigenic challenge (Fauci, 1993).

1.3 FELINE IMMUNODEFICIENCY VIRUS

1.3.1 Introduction

FIV was first isolated from cats in a rescue colony that were showing clinical signs suggestive of an underlying immunodeficiency but were seronegative for FeLV, a known immunosuppressive retrovirus of cats (Pedersen *et al.*, 1987). FIV infection in the cat has now been established as an important pathogen of domestic cats and as an excellent experimental model for human AIDS (Jarrett *et al.*, 1990; Gardner, 1991; Kindt *et al.*, 1992).

1.3.2 Structure and genomic organisation

FIV is a typical lentivirus with a single stranded RNA genome contained within a protein core surrounded by a viral envelope which contains the envelope glycoproteins. Mature FIV virions resemble those of HIV-1 but are slightly smaller (105nm x 125nm) and more ellipsoid (Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988).

Proviral DNA of several FIV isolates has now been cloned, in whole or in part (Talbott *et al.*, 1989; Olmsted *et al.*, 1989(a); Olmsted *et al.*, 1989(b); Philips *et al.*, 1990; Miyazawa *et al.*, 1991; Siebelink *et al.*, 1992; Rigby *et al.*, 1993). FIV has a typical lentivirus genome with *gag*, *pol* and *env* genes (1.1.3). The *gag* gene encodes the major core polyprotein precursor which is post-translationally cleaved to yield the CA, NC and MA proteins. The *pol* gene overlaps the 3' end of *gag* and is expressed as a GAG-POL polyprotein following ribosomal frameshifting (Morikawa *et al.*, 1992). The *pol* gene encodes the viral polymerase, RT, a PR which cleaves the GAG-POL precursor, a deoxyuridine triphosphatase (dUTPase) (Elder *et al.*, 1992) and an IN. The cleavage sites within the GAG and GAG-POL polyproteins have recently been identified (Elder *et al.*, 1993). *env* encodes the viral envelope proteins which are glycosylated and consist of SU and TM regions. Like other lentiviruses, the FIV genome also contains several small orf which may encode regulatory factors. For example, *rev* gene activity, similar to that in primate lentiviruses, has been reported for FIV (Kiyomasu *et al.*, 1991).

Nucleotide sequence analysis of FIV has allowed phylogenetic relationships to be established. FIV is more closely related to the ungulate lentiviruses equine infectious anaemia virus (EIAV) and maedi-visna virus than to the primate lentiviruses (Talbott *et al.*, 1989) and it is likely that these viruses diverged from a common ancestor at around the same time. Comparisons of *env* sequence data from different isolates of FIV have have been carried out. Rigby and colleagues (1993)

found similar genetic divergence among isolates except for a Japanese isolate, TM2 (Miyazawa *et al.*, 1991), which represents a distinct sequence subgroup. Sodora *et al.*, (1994) analysed sequence data from 12 North American isolates of FIV and identified 3 FIV subtypes, designated A, B and C, with the Japanese isolate serving as the prototype of their subtype B.

The major immunogenic proteins of FIV have been characterised by radioimmunoprecipitation (RIPA) and by immunoblot. Estimates of molecular weight of the component proteins vary slightly between experimenters (Steinman *et al.*, 1990; Egberink *et al.*, 1990) but are very similar to those for HIV-1 (1.2.2) so that the same nomenclature will be used here.

Antigenically, FIV is most similar to EIAV. There is reciprocal serological cross-reaction between the *gag* products of EIAV and those of FIV (Steinman *et al.*, 1990; Egberink *et al.*, 1990). The finding that anti-EIAV serum recognises FIV ENV was surprising since lentivirus envelope glycoproteins are highly variable, but this recognition was shown to be dependent upon glycosylation of the envelope backbone (Steinman *et al.*, 1990). Antisera from animals in the field to other non-feline lentiviruses do not cross-react with FIV (Yamamoto *et al.*, 1988). However, cross-reactive antibodies have been detected in both captive and free-ranging non-domestic cats including lions, cheetahs and pumas (Olmsted *et al.*, 1992). Several of these FIV-related viruses have been isolated and phylogenetic analyses suggest that each species harbours a distinct virus with interspecies transmission being a rare event. The sequence divergence between isolates from different felid species is often greater than that between HIV-1 and SIV. It is believed that FIV and related viruses have a common but distant evolutionary origin (Olmsted *et al.*, 1992; Barr *et al.*, 1993; Langley *et al.*, 1993). Horizontal transmission of these viruses has been established since natural seroconversion has been demonstrated in 2 free-roaming adult lions on sequential immunoblots (Brown EW, 1993). It is unclear at this stage whether FIV-related feline lentiviruses are pathogenic in their natural hosts. Further studies on the natural life-expectancy of each species, the presence of clinical signs in infected animals and analysis of T-lymphocyte subsets in infected an uninfected animals are necessary to establish this. In this regard it is worth noting that monoclonal antibodies which recognise CD4 and CD8 molecules in the domestic cat (fCD4 and fCD8) also recognise the puma homologues (Dr SJ O'Brien, personal communication).

1.3.3 Host cell range

Replication of FIV is essentially as described for other lentiviruses. The virus shows tropism for feline PBMC, monocytes and astrocytes in vitro. In addition, some strains grow in CRFK fibroblast cells (Yamomoto *et al.*, 1988; Brunner and Pedersen, 1989; Dow *et al.*, 1990; Brown *et al.*, 1991; Tozzini *et al.*, 1992). FIV has a characteristic cpe on activated lymphocytes in culture consisting of ballooning degeneration and syncytium formation (Pedersen *et al.*, 1987; Harbour *et al.*, 1988). In contrast to HIV-1, FIV has been shown to productively infect both $CD4^+$ and $CD8^+$ lymphocytes in vitro (Brown *et al.*, 1991). Recent efforts have concentrated on establishing the identity of the in vivo targets of FIV. Quantitative PCR of lymphocytes from peripheral blood and lymph node has shown that in acute infection, proviral burden is greatest in $CD4^+$ cells followed by B cells then $CD8^+$ cells, whereas in chronically infected animals B cells seem to be the major reservoir (English *et al.*, 1991; Dean *et al.*, 1993; English *et al.*, 1993). *In situ* hybridization and immunohistochemistry have been used to determine the tissue distribution of FIV at various stages post-infection. In early infection productively infected cells, predominantly T lymphocytes, are found in lymphoid organs suggesting that these cells are involved in early dissemination of virus (Beebe *et al.*, 1993). FIV RNA is also found in megakaryocytes in the bone marrow of acutely infected cats (Beebe *et al.*, 1992). During the primary stage of infection, there is a shift in the predominant cellular localisation of FIV from T lymphocytes to tissue macrophages (Beebe *et al.*, 1994). In terminally ill cats most virus is associated with macrophages and non-lymphoid cells (Beebe *et al.*, 1993).

FIV therefore exhibits a broader cell tropism than HIV. This difference may be a consequence of the wider distribution on feline cells of the cellular receptor for FIV which has recently been identified as the feline homologue of CD9 (Hosie *et al.*, 1993; Willett *et al.*, 1994). Like other lentiviruses, FIV seems to be very host specific. Humans in contact with the virus or with infected cats do not have serological evidence of exposure (Yamamoto *et al.*, 1989; Dr MJ Hosie, personal communication) and attempts to infect several cell types from man, dogs, sheep and mice have been unsuccessful (Yamamoto *et al.*, 1988). The significance of recent reports demonstrating FIV-induced syncytium formation in various human cells and stable, although non-productive, integration of the FIV genome into human lymphoid cells in vitro is unclear (Blair *et al.*, 1993; Tochikura *et al.*, 1993).

1.3.4. Epidemiology

FIV is endemic in domestic cat populations worldwide (Belford *et al.*, 1989; Bennett *et al.*, 1989; Hosie *et al.*, 1989; Ishida *et al.*, 1989; Shelton *et al.*, 1989(a); Yamamoto *et al.*, 1989). Retrospective serology has demonstrated FIV infection from 1968 (Shelton *et al.*, 1990(a)) but the virus is likely to have been present in domestic cat populations for far longer. In the UK the prevalence of FIV has been found to be 19% among sick cats and 6% in healthy cats. A higher prevalence rate has been recorded in Japan (Ishida *et al.*, 1988) and a lower prevalence in Germany, the Netherlands and Switzerland (Lutz *et al.*, 1988; Weijer *et al.*, 1988). Older, free-roaming male cats are particularly at risk from infection (Ishida *et al.*, 1988; Belford *et al.*, 1989; Bennett *et al.*, 1989; Hosie *et al.*, 1989; Shelton *et al.*, 1989(a); Yamamoto *et al.*, 1989). This observation reflects the natural mode of transmission, generally agreed to be biting, since this group of cats is most likely to exhibit territorial aggression. Consistent with this, FIV has been isolated from saliva of naturally and experimentally infected cats by virus isolation and PCR (Yamamoto *et al.*, 1988; Meers *et al.*, 1992; Matteucci *et al.*, 1993) and successful experimental transmission of FIV by a mock bite has been shown (Yamamoto *et al.*, 1989).

The extent of FIV transmission in multiple-cat households is unclear. Some studies suggest that in-contact cats are a low-risk population (Shelton *et al.*, 1989(a); Shelton *et al.*, 1990(a)) whereas others have reported a higher prevalence in members of multi-cat households than in the general population (Ishida *et al.*, 1988; Hosie *et al.*, 1989). Factors including virus strain and management conditions, such as increased stress and intercurrent disease which may affect virus shedding and suceptibility, could account for these discrepancies. Under experimental conditions, transmission of FIV between animals housed together is rare (Yamamoto *et al.*, 1988; Dandekar *et al.*, 1992).

Transmission of FIV from mother to kitten is an uncommon event (Callanan *et al.*, 1991; Hopper, 1991; Bennett and Smyth 1992; Wasmoen *et al.*, 1992;). The route of transmission in these cases is likely to be postnatal. *In utero* transmission has not been demonstrated although virus has been isolated from pooled thymocytes from an aborted litter from a cat in the acute stage of infection (Hopper *et al.*, 1993). The efficiency of maternal transmission may depend on the stage of infection. Attempts to isolate virus from colostrum or milk have generally not been successful (Yamamoto *et al.*, 1988; Callanan *et al.*, 1991; Hopper *et al.*, 1992) although virus has been isolated from the milk of queens in the acute phase of infection (Sellon *et al.*, 1994). The potential for transmission of FIV via milk has been confirmed by

experimental infection of neonates with orally administered virus (Sellon *et al.*, 1994). Transmission from naturally infected, asymptomatic queens has not been shown (Ueland and Nesse, 1992) but kittens have become infected from queens in the acute stage of experimental infection (Sellon *et al.*, 1994). Sexual transmission of FIV has not been documented although experimental FIV infection *per rectum* and *per vaginum* has been achieved (Harbour *et al.*, 1993; Moench *et al.*, 1993).

A strong linkage between infection with FIV and FeSFV has been found (Yamamoto *et al.*, 1989). This association may be due to similarities in the mode of transmission of these 2 viruses.

1.3.5 Clinical signs

Despite its closer evolutionary relationship to the ungulate lentiviruses, the clinical syndromes associated with FIV infection in the cat resemble those of HIV-1 infection in man rather than those of lentivirus infections of ungulates.

Experimentally, infection of kittens with FIV is often followed by a variable primary phase illness associated with seroconversion (Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988; Callanan *et al.*, 1992 (b)). Pyrexia, depression, leucopenia and generalised lymphadenopathy may be seen from 4-6 weeks post-infection. The primary phase of FIV has also been observed in a naturally infected kitten (Callanan *et al.*, 1991). These signs usually resolve in a few weeks with the exception of the lymphadenopathy which may persist for several months. Occasionally FIV-infected cats develop serious illness in the primary stage which may be fatal (Dr J Callanan, personal communication; George *et al.*, 1993). The severity of the primary phase may depend on the age at infection since it is often less severe in older cats (George *et al.*, 1993). Consistent with this, higher levels of virus can be isolated from PBMC and plasma of cats which are infected at an early age (Meers *et al.*, 1992).

Once any primary phase illness has resolved, cats enter the prolonged asymptomatic phase (Ishida *et al.*, 1989; Pedersen *et al.*, 1989; Barlough *et al.*, 1991) which probably lasts for several years (Pedersen and Barlough., 1991). This assumption is consistent with the epidemiological picture in naturally infected cats where clinical disease is generally seen in cats between 5 and 10 years of age (Hosie *et al.*, 1989; Pedersen *et al.*, 1989; Shelton *et al.*, 1989(a); Yamamoto *et al.*, 1989).

Clinical signs in naturally infected animals are non-specific and often chronic and recurrent in nature. The most commonly reported signs associated with FIV

infection include stomatitis, gingivitis, upper respiratory tract signs, diarrhoea, pyrexia, lethargy and weight loss (Hosie *et al.*, 1989; Ishida *et al.*, 1989; Yamamoto *et al.*, 1989; Sparkes *et al.*, 1993). Multiple cytopenias, similar to those found in HIV-seropositive humans have been found in symptomatic cats (Shelton *et al.*, 1990(b); Sparkes *et al.*, 1993). Neurological signs are seen in FIV infection, particularly behavioural changes such as roaming and occasionally motor deficits (Harbour *et al.*, 1988; Hosie *et al.*, 1989; Pedersen *et al.*, 1989; Shelton *et al.*, 1989(a); Yamamoto *et al.*, 1989). The neurotropic nature of FIV has been established (Dow *et al.*, 1990). FIV induced neuropathology consists of subcortical gliosis, perivascular mononuclear cell infiltration and discrete glial nodules and these lesions are found in animals without nervous signs (Dow *et al.*, 1990; Pedersen *et al.*, 1989; Hurtrel *et al.*, 1992). The encephalopathy associated with experimental FIV infection is analogous to HIV-1 paediatric encephalopathy (Podell *et al.*, 1993).

There is mounting evidence for an increased risk of neoplastic diseases in natural FIV infection, particularly lymphoproliferative diseases, in naturally (Shelton *et al.*, 1989(b); Yamamoto *et al.*, 1989; Shelton *et al.*, 1990(a); Hutson *et al.*, 1991) and experimentally infected cats (Yamamoto *et al.*, 1988; Callanan *et al.*, 1992(a)). Preliminary phenotyping of tumours from FIV seropositive cats has shown that these are predominantly B cell in origin. It has not been possible to demonstrate FIV proviral sequences in neoplastic cells from FIV-infected cats (Dr J Callanan, personal communication).

Co-infection with secondary and opportunistic agents is often a feature of secondary phase FIV infection. Toxoplasmosis, cryptococcosis, poxvirus infection, haemobartonellosis, demodectic and notoedric mange, candidiasis, cryptosporidiosis, mycobacterial infections and pseudomoniasis have all been reported (reviewed in Pedersen *et al.*, 1989; Bennett and Smyth, 1992; Callanan and Jarrett, 1993). Most surveys have found that the occurrence of dual FeLV/FIV-infected cats is no more common than would be expected from the prevalence for each virus (Hosie *et al.*, 1989; Ishida *et al.*, 1989; Shelton *et al.*, 1989(a); Sukura *et al.*, 1992). Pedersen has shown that experimental FIV infection of FeLV positive cats results in much more accelerated and severe disease than is seen in FIV infection alone (Pedersen *et al.*, 1990). Similarly, challenge of FIV-infected cats with FCV has been associated with more widespread clinical signs and longer periods of virus shedding than in FIV seronegative cats (Dawson *et al.*, 1991).

Secondary phase FIV-related illness has recently been reported in experimentally infected cats (Grail *et al.*, 1993; Matsumura *et al.*, 1993).

1.3.6 Pathogenesis

The pathogenesis of FIV infection shares many features with that of HIV (1.2.6). FIV infection is associated with a progressive depletion of circulating CD4$^+$ cells resulting in an inversion of the CD4$^+$/CD8$^+$ ratio (Ackley *et al.*, 1990; Novotney *et al.*, 1990; Barlough *et al.*, 1991; Tompkins *et al.*, 1991; Torten *et al.*, 1991; Bishop *et al.*, 1992(a); Hoffmann-Fezer *et al.*, 1992). Some workers have found that the decrease in the CD4$^+$/CD8$^+$ ratio is exacerbated by an increase in the number of circulating CD8$^+$ cells (Ackley *et al.*, 1990; Hoffmann-Fezer *et al.*, 1992). Willett and colleagues (1993) have described the expansion of a subset of CD8$^+$ lymphocytes, designated CD8$^{+(low)}$ since these cells express low levels of the surface marker, within a few weeks of infection. The significance of this finding is not yet known. In contrast, others have found no significant expansion of the CD8$^+$ population in FIV-infected cats (Novotney *et al.*, 1990; Torten *et al.*, 1991).

FIV infection results in functional deficits in the immune system affecting both T cell and B cell compartments. Numerous investigators have found decreased proliferative responsiveness to T cell mitogens and altered cytokine production by lymphocytes from FIV-infected cats compared to uninfected control cats (Lin *et al.*, 1990; Siebelink *et al.*, 1990; Barlough *et al.*, 1991; Torten *et al.*, 1991; Lawrence *et al.*, 1992). These changes can be detected very early after infection and are progressive. The ability of FIV-infected cats to mount a primary antibody response to T-dependent, but not to T-independent, immunogens becomes impaired (Dawson *et al.*, 1991; Taniguchi *et al.*, 1991; Torten *et al.*, 1991; Bishop *et al.*, 1992(a)). Secondary antibody responses to T-dependent antigens have been found to remain normal in these studies although a decline in memory T cell proliferation to recall antigen has been found from 19 weeks post-infection (Bishop *et al.*, 1992(b)).

These results suggest that the T cell dysfunction which accompanies FIV infection is likely to be due, at least in part, to the decrease in CD4$^+$ cell numbers which parallels these changes (Siebelink *et al.*, 1990; Barlough *et al.*, 1991). Similar mechanisms have been proposed to account for the CD4$^+$ cell decline in FIV infection as for that in HIV infection (1.2.6). For example, apoptosis may contribute to the CD4$^+$ cell decline (Bishop *et al.*, 1993). CRFK cells chronically infected with FIV are sensitive to TNFα-induced apoptosis (Ohno *et al.*, 1994).

It is interesting to note that, despite the much broader cell tropism of FIV compared to HIV-1, the lymphocyte subpopulation alterations which follow these infections are very similar and these viruses may have a common mechanism of pathogenesis.

B cell defects are also found in FIV-infected cats. Several groups have shown a decreased responsiveness to lipopolysaccharide (LPS) in infected animals (Siebelink *et al.*, 1990; Bishop *et al.*, 1992(a)). A recent study has shown that there is polyclonal B cell activation in FIV infection with the production of circulating antibodies to a broad range of antigens in addition to those of FIV (Flynn *et al.*, 1994). This accounts for the hypergammaglobulinaemia which has been detected in FIV-infected cats although cases of monclonal gammopathy have occasionally been recorded (Hopper *et al.*, 1989; Ackley *et al.*, 1990; Sparkes *et al.*, 1993). IL6 production is elevated in FIV-infected cats and may contribute to the polyclonal B cell activation (Ohashi *et al.*, 1992).

The participation of various co-factors in the pathogenesis of FIV have been suggested. FHV-1 has been shown to upregulate the FIV LTR and dually infected T lymphocytes have been identified in vitro (Kawaguchi *et al.*, 1991). Co-infection of FIV seropositive cats with FeSFV, which has not been shown to be pathogenic, does not seem to enhance FIV-associated disease (Zenger *et al.*, 1993). A study designed to determine the role of common feline pathogens in the immunological defects associated with FIV showed, paradoxically, that FIV-infected cats exposed to secondary agents had higher $CD4^+$ cell counts, higher $CD4^+/CD8^+$ ratios and greater mitogen-induced proliferation than those maintained in SPF conditions (Reubel *et al.*, 1993).

FIV GAG antigens have been detected in the lymph nodes of cats early after infection. GAG antigens were shown to be associated principally with either FDC or $CD4^+$ T cells depending on whether cats were infected with uncloned or cloned FIV (Toyosaki *et al.*, 1993). It will be interesting to determine by *in situ* hybridisation whether the FDC are productively infected with FIV. Characteristic lymph node changes are seen in FIV infection which are very similar to those seen in the PGL stage of HIV-1. These abnormalities include follicular hyperplasia, dysplastic follicles and increased numbers of intrafollicular $CD8^+$ cells and plasma cells (Yamamoto *et al.*, 1988; Callanan *et al.*, 1992(b)).

1.4 HOST IMMUNE RESPONSE TO HIV AND FIV

1.4.1 Immune response to HIV-1

Most HIV-1-infected individuals mount a comprehensive immune response to the virus, at least in the early stages of infection. Clearance of virus from the periphery following the initial peak of viraemia is generally thought to be due to host immune mechanisms. It is presumably due to a failure of host immunity after an extended asyptomatic period that plasma viraemia again becomes detectable and clinical signs develop. The more rapid progression to AIDS seen in HIV-infected infants compared to adults (Blanche *et al.*, 1989) may be a reflection of an immature immune system.

1.4.1.1 Humoral immune response to HIV-1

HIV-1 infected individuals produce antibodies to all of the structural and some of the regulatory proteins of the virus (Rosenberg and Fauci, 1989; Ho *et al.*, 1987(b)). Despite the functional deficits in T and B cells described above, seropositive individuals produce large quantities of virus neutralising antibodies early in infection (Albert *et al.*, 1990). Group-specific and type-specific neutralisation epitopes have been mapped on gp41 and gp120 respectively (Weiss RA *et al.*, 1986; Weiss *et al.*, 1988). An immunodominant epitope is located in the third variable region (V3) of the SU protein (Javaherian *et al.*, 1989). High-affinity, post-binding type-specific neutralising antibodies to V3 have been demonstrated (Nara *et al.*, 1991). The significance of neutralising antibodies in vivo is unclear (Goudsmit, 1993). In chimpanzees, neither passively administered nor actively induced neutralising antibodies have protected from challenge with HIV-1 (Prince *et al.*, 1988; Berman *et al.*, 1988) although in the latter study only low titres of neutralising antibodies were induced. There are conflicting data on the role of antibodies to viral core proteins. Some workers have found that anti-p24 antibody titres correlate negatively with disease progression (Cheingsong-Popov *et al.*, 1991) but others found that this was not significant particularly when corrected for the presence of p24 antigen which bound antibody (Moss *et al.*, 1988).

Sera from HIV-1 seropositive individuals can mediate ADCC, complement-dependent lysis of infected cells and cell fusion inhibition (CFI) in vitro (Blumberg *et al.*, 1987; Rivière *et al.*, 1989(a); reviewed in Rosenberg and Fauci, 1989; Goudsmit, 1993). Overall, evidence suggests that ADCC and CFI antibodies do not prevent disease progression or limit virus spread (Goudsmit, 1993).

1.4.1.2 Cellular immune response to HIV-1

i) T helper response

Many investigators have measured T_h responses to HIV-1 antigens from seropositive individuals by tritiated (^3H)-thymidine uptake or by IL2 production (Wahren *et al.*, 1987; Clerici *et al.*, 1989; Schrier *et al.*, 1989). Four synthetic ENV peptides have been identified which can be recognised by both T_h and class I MHC restricted CTL on direct isolation from HIV-1 seropositive patients (Clerici *et al.*, 1991) and this finding may be relevant to vaccination studies. Recognition of HIV antigens has occasionally been found in seronegative individuals (Nixon, 1992; Clerici *et al.*, 1992). The latter study provides evidence for priming of T cells in the absence of an antibody response. T helper responses to HIV-1 ENV peptides were detected in 5 seronegative, PCR negative homosexual men with recent sexual exposure to HIV-1, only 1 of whom subsequently seroconverted (Clerici *et al.*, 1992). Similar findings have been reported in macaques exposed to, but not persistently infected with, SIV (Clerici *et al.*, 1993(b)).

There is evidence for a selective defect in HIV-1 specific T_h responses as disease progresses, with responses to other viruses (CMV and herpes simplex) and mitogen (phytohaemagglutinin, PHA) being unaffected. This appears to be due to a functional defect in HIV-specific T_h responses rather than to low numbers of T_h cells since the addition of IL2 restores T_h proliferation to HIV-1 antigens (Wahren *et al.*, 1987).

ii) Suppression of HIV-1 replication by $CD8^+$ cells

$CD8^+$ cells can inhibit replication of HIV-1 in PBMC from HIV-1 infected individuals in vitro in a dose-dependent manner and by a mechanism independent of direct cellular cytotoxicity (Walker *et al.*, 1986; Kannagi *et al.*, 1988; Brinchmann *et al.*, 1990; Wiviott *et al.*, 1990; Mackewicz and Levy 1992). Other workers have demonstrated a similar phenomenon in the rhesus monkey/SIV_{mac} and chimpanzee/HIV-1 models (Tsubota *et al.*, 1989(a); Castro *et al.*, 1991). This suppression can be blocked by anti-CD8 monoclonal antibodies. A lag period after infection is required for induction, suggesting that clonal expansion of the effector population occurs. The question of whether the $CD8^+$ population responsible for virus suppression consists of CTL or is a separate subset of $CD8^+$ cells remains controversial. Koenig and colleagues (1991) found that NEF, ENV and GAG-specific CTL clones from seropositive donors, in addition to lysing HIV-1-infected targets, also inhibited replication of HIV-1 in freshly sorted, activated $CD4^+$ cells from infected patients suggesting that the same population of cells mediates both

lysis and suppression. Some workers have found that suppression is non-MHC-restricted, can occur across a semipermeable membrane and can be transferred in supernatant fluids (Walker and Levy, 1989; Brinchmann *et al.*, 1990; Mackewicz and Levy, 1992) suggesting that a soluble factor is involved. Others have found the effect to be MHC-restricted and to require cell to cell contact (Tsubota *et al.*, 1989(a)) whereas the results of Koeing and colleagues (1991) show MHC-restricted killing mediated by a diffusible factor. It is likely that some of these discrepancies are due to variations in experimental conditions between groups since different conditions may selectively promote the activity of distinct populations of effector cells or different functions of the same effectors. Walker and Levy (1989) found that there was individual variation in the production of the soluble inhibitory factor from $CD8^+$ cells but that where this was absent, inhibition of viral replication could still be achieved by cell contact. Different mechanisms may be operating in each case.

The inhibition of HIV-1 replication by $CD8^+$ T cells demonstrated by these experiments may be an important mechanism for the control of HIV-1 and SIV replication in vivo (Walker CM *et al.*, 1989; Walker, 1993) since a correlation has been found between clinical state, high $CD4^+$ cell count and $CD8^+$ anti-HIV-1 activity (Brinchmann *et al.*, 1990; Mackewicz *et al.*, 1991).

iii) Cytotoxic T-cell response to HIV-1

Features of cytotoxic T cells

CTL recognise and kill autologous cells which have been rendered "unusual" by infection with viruses or other intracellular pathogens or by neoplastic change. Early studies established that T lymphocytes can directly lyse virus-infected cells (Blanden, 1974). It is now known that CTL can exert their antiviral effects in a number of different ways including direct lysis (Cerottini and Brunner, 1974), apoptosis (Martz and Howell, 1989) or by the release of cytokines such as γ-interferon (Morris *et al.*, 1982), TNF-α and TNF-β (Jassoy *et al.*, 1993). Antigen recognition by CTL, in contrast to that by antibody, is MHC-restricted (Zinkernagel and Doherty, 1974). Virus-specific CTL are generally class I MHC-restricted, $CD8^+$ T lymphocytes.

Originally it was believed that CTL recognised whole viral proteins at the infected cell surface, until the specificities of cloned influenza specific CTL were mapped to internal virus proteins which are not found intact at the cell surface (Townsend *et al.*, 1984; Townsend *et al.*, 1986(a); Bennink *et al.*, 1987; Gotch *et al.*, 1987). Transfection of cells with fragments of the coding sequence for target antigens

allowed recognition of these cells by CTL (Townsend *et al.*, 1985). This implied that CTL recognise viral proteins which have undergone processing. That this is the case was shown when Townsend and colleagues were able to sensitise target cells for CTL recognition by the addition of synthetic peptides (Townsend *et al.*, 1986(b)). In conjunction with the characterisation of the TCR as the single recognition element for both antigen and MHC (Marrack and Kappler, 1986) and evidence for a physical association between a class II MHC product and a T cell epitope (Babbitt *et al.*, 1985), binding of peptide to class I MHC was strongly suspected. X-ray crystallographic analysis of human class I MHC structure (Bjorkman *et al.*, 1987(a); Bjorkman *et al.*, 1987(b)) revealed a potential peptide-binding groove on the outer surface of the molecule. The groove contained an electron density which was partially resolved as peptide (Madden *et al.*, 1991). Peptides associated with class I MHC molecules have common features i.e. 8-9 aa length, extended and directional orientation and haplotype specific anchoring residues (Van Bleek and Nathenson, 1990; Schumacher *et al.*, 1991; Madden *et al.*, 1991; Fremont *et al.*, 1992; Matsumura *et al.*, 1992). Consequently allele-specific peptide-binding motifs can be generated and have been successfully used to predict CTL epitopes from, for example, *Listeria monocytogenes* (Pamer *et al.*, 1991) lymphocytic choriomeningitis virus (LCMV) (Gairin and Oldstone, 1992) and influenza virus (Sutton *et al.*, 1993).

The peptides which associate with class I molecules are probably generated by the proteosome complex, a cytosolic protease with MHC-encoded subunits (reviewed in Howard and Seelig, 1993) although this has not been unequivocally proven (Arnold *et al.*, 1992; Momburg *et al.*, 1992). Two genes located in the class II region of mice, rats and humans encode proteins which are believed to act as ATP-dependent peptide transporters, (called TAP 1 and TAP 2 in humans i.e. "transporter associated with antigen processing") delivering peptides from the cytosol to the endoplasmic reticulum (ER) allowing assembly of class I /peptide /β_2 microglobulin complex which is transported to the cell surface (reviewed in Parham, 1992). It is still not clear whether peptides are "trimmed" to their final length in the ER after the assembly of class I molecules or if the final peptide is transported across the ER.

CD4+ lymphocytes recognise antigens in association with class II MHC molecules. Professional antigen presenting cells (APC) endocytose foreign proteins from the extracellular space which are processed via the exogenous route and are presented in association with class II MHC molecules at the cell surface (Neefjes and Ploegh, 1992). The peptide binding site on newly synthesised class II molecules is occupied by invariant chain which is degraded during intracellular transport and replaced by

peptides derived from endocytosed molecules. Recently, the intracellular location of class II MHC peptide binding has been identified as a unique endosome-related subcellular compartment (reviewed in Schmid and Jackson, 1994). The distinction between processing of endogenous antigens for presentation by class I MHC products and exogenous antigens for class II association is not absolute since exogenous antigens can be presented by class I molecules for recognition by CTL (Yewdell *et al.*, 1988; Barnaba *et al.*, 1990).

Immunological role of CTL

The role of CTL in clearing acute virus infections and controlling persistent infections is well established. CTL are generated early in virus infections, even before an IgM response (Yap *et al.*, 1978). In murine models the passive transfer of cloned virus-specific CTL, but not antibody, between mice of the same strain can clear influenza infection (Lin and Askonas, 1981). Mice that are T cell deficient cannot clear influenza virus (Wells *et al.*, 1981) although mice depleted of only CD4$^+$ cells can (Lightman *et al.*, 1987). Similarly CTL have been shown to mediate the elimination of virus in acute LCMV infection in mice (Byrne and Oldstone, 1984; Moskophidis *et al.*, 1987). Adoptive transfer of CD8$^+$ lymphocytes between mice has also been shown to protect against disease caused by CMV (Reddehase *et al.*, 1987), herpes simplex virus (Larsen *et al.*, 1983), Sendai virus (Kast *et al.*, 1986) and rotavirus (Offit and Dudzik, 1990). CTL have been shown to mediate the rejection of retrovirus-induced tumours in mice (Engers *et al.*, 1984).

Important in vitro observations suggest a protective role for virus-specific CTL. Firstly CTL can lyse virus-infected cells during the eclipse phase before infectious progeny are released (Zinkernagel and Althage, 1977). Secondly CTL can control replication of EBV (Moss *et al.*, 1978), HIV (Walker *et al.*, 1986), SIV (Kannagi *et al.*, 1988) and LCMV (Aebischer *et al.*, 1991) in vitro. Finally there is evidence in vitro that CTL may exert a selective pressure on virus replication. Escape mutation from CTL by LCMV has been shown in vitro (Aebischer *et al.*, 1991). Some viruses such as CMV (Gilbert *et al.*, 1993) and adenoviruses (Wold and Gooding, 1991) have evolved mechanisms for avoiding presentation of immunogenic proteins by class I MHC, presumably affecting recognition by CTL. In the latter case a delayed-early adenovirus protein, gp19K, prevents lysis of adenovirus infected cells by virus-specific CTL by preventing transport of class I molecules from the ER (Wold and Gooding, 1991).

In man CTL are responsible for the rapid clearance of influenza A virus following experimental intranasal exposure (McMichael *et al.*, 1983). The titre of pre-existing

antibody, although important in prophylaxis, has no effect on the infection. Similar studies by Quinnan *et al.* (1982) demonstrated that the ability to survive CMV in transplant recipients correlated with the ability to mount a CTL response to the virus. Virus-specific CTL responses control other persistent virus infections such as EBV (Rickinson *et al.*, 1980).

MHC class II restricted $CD4^+$ virus-specific CTL have also been described. Since these cells are generated after long term culture they are often regarded as an in vitro artefact. However, measles virus-specific $CD4^+$ CTL have been demonstrated in short-term culture and may have a role in vivo (Jacobson *et al.*, 1984). It has also been proposed that class II MHC-restricted CTL may function as suppressor cells in vivo (Lanzavecchia, 1989).

As well as their protective role CTL can contribute to immunopathology in certain circumstances. Mice which show a strong T cell response to LCMV develop a rapidly fatal neurological disease mediated by infiltration of virus-specific CTL into the CNS, whereas immunosuppressed mice tolerate the virus and survive (Buchmeier *et al.*, 1980). A T cell mediated hepatitis has also been described in LCMV infection (Zinkernagel *et al.*, 1986). An immunopathological role for CTL has been proposed in respiratory syncytial virus infection (RSV) (Cannon *et al.*, 1988), hepatitis B infection (Pignatelli *et al.*, 1987; Barnaba *et al.*, 1990) and mumps meningitis (Kreth *et al.*, 1982).

HIV-1 specific CTL

HIV-1 infection induces a strong CTL response with specificities for multiple epitopes within most of the HIV-1 proteins, both structural and regulatory (Plata *et al.*, 1987; Walker *et al.*, 1987; Autran *et al.*, 1988; Koenig *et al.*, 1988; Nixon *et al.*, 1988; Sethi *et al.*, 1988; Shepp *et al.*, 1988; Walker *et al.*, 1988; Chenciner *et al.*, 1989; Culmann *et al.*, 1989; Hoffenbach *et al.*, 1989; Koup *et al.*, 1989; Riviere *et al.*, 1989(b); Walker BD *et al.*, 1989; Torpey *et al.*, 1993). HIV-specific CTL have been generated from PBMC, lymph nodes, spleen, broncho-alveolar lavage fluid and CSF (Nixon and McMichael, 1991). There is mounting evidence to suggest that virus-specific CTL are responsible for suppressing the initial viraemia seen in HIV-1 infection and may play an important role in maintaining the asyptomatic period (Buseyne and Riviere, 1993; McMichael and Gotch, 1993). In addition to a putative protective role, HIV-1-specific CTL have also been implicated in mediating some of the lesions seen in HIV-1 infected patients (Sethi *et al.*, 1988; Autran *et al.*, 1991).

Non-MHC restricted HIV-specific cytotoxic effector cells have also been described but are thought to be of minor importance compared to the virus-specific CTL response. ENV-specific cytotoxic cells have been found in freshly isolated PBMC from HIV-1-infected patients (Weinhold *et al.*, 1988; Riviere *et al.*, 1989(b); Tyler *et al.*, 1989; McCheyney *et al.*, 1990) and armed killer cells and NK-like cells have been implicated as the effectors (Weinhold *et al.*, 1988; Tyler *et al.*, 1989).

HIV-1 specific, MHC-restricted CTL were first described in 1987 (Plata *et al.*, 1987; Walker *et al.*, 1987). Walker and colleagues showed HIV-1 GAG and ENV specific killing by circulating T lymphocytes. Plata and colleagues recovered effector cells by broncho-alveolar lavage and demonstrated lysis of HIV-1 infected targets by $CD8^+$ lymphocytes. These preliminary studies highlighted what has come to be recognised as a characteristic feature of the HIV-1-specific CTL response i.e. that cytotoxic activity can often be demonstrated from freshly isolated cells. This primary CTL activity seen in HIV infection is in contrast to most other viral infections where a restimulation step in vitro is required so that secondary responses can be detected. Similar activity has been described from directly isolated PBMC in HTLV-1 infection (Jacobson *et al.*, 1990) and from cryopreserved effectors in EBV infection (Strang and Rickinson, 1987). Primary HIV-specific CTL have been isolated from blood and broncho-alveolar lavage fluid from healthy HIV-1 seropositive donors by several groups with specificities for *env, gag, pol, nef, rev, vif* and *tat* gene products (Walker *et al.*, 1987; Walker *et al.*, 1988; Koup *et al.*, 1989; Riviere *et al.*, 1989(b); Torpey *et al.*, 1993). The frequency of circulating effector CTL is very high. Gotch and colleagues (1990) conservatively estimated that 1% of directly isolated PBMC from asymptomatic seropositive individuals may be specific for a HIV-1 GAG peptide and similar results have been shown by others (Hoffenbach *et al.*, 1989). The proportion of patients from whom lytically active CTL can be isolated directly varies greatly between studies: Koup *et al.* (1989) were able to detect HIV-1 ENV- and GAG-specific responses in 15 of 17 haemophiliac patients (88%) whereas Nixon *et al.*, (1988) were unable to demonstrate any fresh killing using a similar target cell system. Directly detectable CTL activity is seen particularly in the early asymptomatic phase (McMichael and Gotch, 1993) and is generally thought to result from persistent antigenic stimulation of the CTL compartment in the lymph nodes. Recent data showing continuous viral replication in the lymph nodes of asymptomatic individuals supports this hypothesis (Pantaleo *et al.*, 1993). Preliminary results suggest that the CTL response to HIV-2 has similar features to that against HIV-1. A CTL epitope was recently identified in HIV-2 GAG which was recognised by directly isolated $CD8^+$ effectors from 5 of 9 HIV-2 seropositive patients. These effectors did not recognise HIV-1 proteins but

the same peptide was recognised by lymphocytes from SIV-infected non-human primates (Gotch *et al.*, 1993).

Conventional restimulation and limiting dilution analysis (LDA) in asymptomatic seropositive individuals has highlighted a discrepancy between HIV-1-specific CTL precursor (pCTL) frequency and the very high levels of lytically active circulating HIV-1-specific CTL. Gotch and colleagues (1990) found the pCTL frequency to be around one hundred-fold lower than that for directly measurable CTL. However, even the precursor frequencies for HIV-1-specific CTL in healthy seropositive individuals are high (Hoffenbach *et al.*, 1989; Gotch *et al.*, 1990; Carmichael *et al.*, 1993) compared to those determined for most other virus infections (e.g., 1:30,000-1:500,000 in vesicular stomatitis virus infection in mice) and are comparable to the levels seen in CMV infection of humans (Borysiewicz *et al.*, 1988).

Most effectors, as expected for virus-specific CTL, are of the $CD8^+$ phenotype and restricted by class I MHC products. $CD4^+$ HIV-1-specific CTL with specificities for *env* and *gag* gene products have been isolated from the CSF and peripheral blood of infected patients (Sethi *et al.*, 1988; Littua *et al.*, 1992; Curiel *et al.*, 1993) and have been generated following various vaccination protocols (Nixon, 1992). The significance of $CD4^+$ CTL in vivo is not known. HIV-1 specific $CD4^+$ class II-restricted CTL can be generated from seronegative donors following repeated in vitro stimulation with HIV-1 gp120 (Siciliano *et al.*, 1988; Lanzavecchia *et al.*, 1988). More interestingly, $CD8^+$ class I restricted CTL can be generated following brief in vitro restimulation of PBMC from HIV-1 seronegative donors with specificities for ENV and NEF but not GAG (Langlade-Demoyen *et al.*, 1988; Chenciner *et al.*, 1989; Hoffenbach *et al.*, 1989; Nixon, 1992). The magnitude of the measurable CTL activity is comparable to a secondary response suggesting previous exposure to a cross-reacting antigen with the precursor frequency being similar to that for alloantigen-specific CTL (Hoffenbach *et al.*, 1989). HIV-1-specific pCTL frequencies in seronegative individuals are around ten-fold lower than those in healthy seropositive people suggesting that expansion of this pool occurs after infection. HIV-specific CTL activity has never been detected in unstimulated PBMC from seronegative individuals (Chenciner *et al.*, 1989; Hoffenbach *et al.*, 1989; Nixon, 1992).

HIV-1 CTL epitopes have been identified in the *gag, pol, env* and *nef* gene products (reviewed in Nixon *et al.*, 1992). The first human CTL epitope in HIV-1 was reported in 1988 (Nixon *et al.*, 1988). Using recombinant vaccinia viruses expressing HIV-1 GAG proteins, these workers found MHC class I restricted GAG-specific CTL in 13 of 15 seropositive individuals. Overlapping synthetic peptides

were then screened for their ability to sensitise target cells and a 15aa epitope in CA restricted by HLA-B27 was localised in 1 patient. A more direct but technically demanding method of identifying natural CTL epitopes is to elute peptides from class I molecules. Class I MHC molecules are immunoprecipitated from virus infected cells and the associated peptides are separated using reverse-phase high-performance liquid chromatography. Resulting fractions are tested for their ability to prime target cells for lysis by CTL (Rötzschke and Falk, 1991). Previous algorithms for the prediction of T cell epitopes have been found to be of limited value for HIV-1 (Nixon, 1992). Allele specific motifs, as described earlier, are likely to be more useful.

These experiments have identified many CTL epitopes in both structural and regulatory proteins of HIV (Nixon *et al.*, 1992). Seropositive individuals often have CTL with specificities for several of these epitopes (Culmann *et al.*, 1991; Lamhamedi-Cherradi *et al.*, 1992). This situation is in contrast to immune responses to other viruses such as influenza where CTL respond to only 1 or 2 epitopes in the whole virus (Bennink and Yewdell, 1988). A single HLA type may act as a restriction element for several epitopes in HIV-1 proteins (Walker BD *et al.*, 1989; Nixon and McMichael, 1991). Cross-restriction of HIV-1 CTL epitopes by more than one class I MHC molecule has also been shown in man and mice (Culmann *et al.*, 1991; Shirai *et al.*, 1992). This phenomenon is uncommon in other infections although it has been shown previously with influenza virus (Bodmer *et al.*, 1989). There is preliminary evidence for clustering of CTL epitopes within individual proteins which may reflect relative accessibility of areas of the protein for intracellular processing (Culmann *et al.*, 1991; Nixon and McMichael, 1991; Buseyne *et al.*, 1993 (a); McMichael and Gotch, 1993). Immunodominant areas have been identified in HIV which stimulate cross-reactive CTL (Takahashi *et al.*, 1992; Safrit *et al.*, 1994). CTL specific for a GAG epitope in HIV-1 also recognise the corresponding epitope in HIV-2 which differs by 5 aa (Nixon *et al.*, 1990).

It is quite clear, therefore, that infection with HIV stimulates a more powerful CTL response than that seen with most other virus infections as evidenced by high levels of primary circulating CTL in asymptomatic individuals and the large number of epitopes recognised by HIV-1-specific CTL. The question that then arises is whether the CTL response to HIV-1 infection serves a protective function in vivo.

Available evidence suggests that the appearance of HIV-1-specific CTL following infection correlates with the clearance of virus from the periphery. Virus-specific CTL responses characteristically occur early in infection (Yap and Ada, 1977). In SIV-infected rhesus monkeys circulating virus-specific pCTL are present from 4-6

days post-infection (Yasutomi *et al.*, 1993). There is unpublished evidence that the onset of HIV-1-specific CTL activity occurs when there is a rapid decline in HIV-1 replication and prior to seroconversion (Safrit *et al.*, 1993) suggesting that CTL may be more relevant than humoral immunity to clearance of virus from the periphery.

HIV-1-specific CTL activity remains high during the asymptomatic period (Plata *et al.*, 1987; Walker *et al.*, 1987; Autran *et al.*, 1988; Nixon *et al.*, 1988; Walker *et al.*, 1988; Hoffenbach *et al.*, 1989; Carmichael *et al.*, 1993). Work by Koup and colleagues in asymptomatic haemophiliacs showed that those patients with more vigorous CTL responses were those from whom it was most difficult to culture virus from PBMC (Koup *et al.*, 1989). Although this association was not statistically significant it suggests a role for CTL in controlling virus replication. In SIV, the available data suggest that a CTL response may predict a better clinical outcome (Bourgalt *et al.*, 1993). In animal models, Koenig *et al.* (1991) showed that virus replication could be suppressed in HIV-1 infected hu-PBL-SCID mice by adoptively transferred HIV-1 NEF-specific CTL clones in a class I MHC-restricted manner.

Several groups have shown that HIV-1-specific CTL activity declines as the disease progresses (Hoffenbach *et al.*, 1989; Joly *et al.*, 1989; Gruters *et al.*, 1990; Pantaleo *et al.*, 1990; Cheynier *et al.*, 1992) although patient numbers are small. Several groups have found deficits in both primary and secondary anti-HIV-1 CTL implying a central defect (Hoffenbach *et al.*, 1989; Plata *et al.*, 1989; Gotch *et al.*, 1990; O'Toole *et al.*, 1992) although others have measured high levels of secondary responses in advanced disease suggesting impaired activation and/or proliferation of memory cells in vivo (Rivière *et al.*, 1889(b); Grant *et al.*, 1992; Buseyne and Rivière, 1993; Buseyne *et al.*, 1993(b)). Impaired proliferation of memory cells as a mechanism for declining anti-HIV cytotoxicity is also suggested by the results of 2 groups (Ho HN *et al.*, 1993; Watret *et al.*, 1993) who showed that, despite an overall decrease in CD8$^+$ cells with progression, CD8$^+$ cells expressing activation (HLA-DR) and memory markers (CD45RO) remained elevated. Failure of proliferation may be a reflection of the fall in CD4$^+$ cell numbers since IL2 has been shown to restore cell-mediated immune responses in certain circumstances (Rook *et al.*, 1983; Gruter *et al.*, 1990) suggesting that this defect is at the level of T cell help. There is debate as to whether disease progression is accompanied by a generalised impairment of CTL function (Rook *et al.*, 1985; Shearer *et al.*, 1985) or if only HIV-1-specific clones are affected (Pantaleo *et al.* 1990; Carmichael *et al.*, 1993). Results of a cross-sectional study by Carmichael *et al.* (1993) suggest the latter since the CTL response to another persistent virus, EBV, was maintained. A

similar study by Rook *et al.* (1985) suggested that both CTL and NK responses to CMV are lower in AIDS patients although seronegative homosexuals were used for comparison and no seropositive asymptomatics were included. A longitudinal study by Shearer *et al.* (1985) showed that there was a progressive decline in the influenza-specific CTL response in seropositive homosexuals while levels of allospecific-CTL activity were maintained. To elucidate the nature of the impairment in cellular immunity it will be important to determine, with controlled longitudinal studies, whether there is a selective defect in the HIV-specific CTL response.

No clear correlation has been found between HIV-specific CTL activity and markers of disease progression such as $CD4^{+}$ cell counts, antigenaemia or viral load (reviewed in Buseyne and Rivière, 1993) although an association between the level of primary HIV-1-specific CTL activity and $CD8^{+}$ lymphocyte counts has been found (Grant *et al.*, 1992). A higher relative risk of progression to ARC/AIDS was noted in a cohort of 38 seropositive individuals with no primary anti-GAG CTL at presentation (Buseyne and Rivière, 1993). In agreement with this result, others have found that the majority of long-term survivors have primary GAG-specific CTL (Gotch *et al.*, 1992; Watret *et al.*, 1993).

One of the central questions in HIV-1 immunology is whether the association between the decline in CTL activity and clinical deterioration is a causal one. An area of intense investigation is the reason for the decline. As discussed above, the loss of CTL responses may be secondary to the $CD4^{+}$ immunodeficiency. Gotch *et al.*, (1990), have suggested that the high levels of primary HIV-1-specific CTL activity seen in most seropositive individuals represent a terminally differentiated population and that the response becomes exhausted. This theory is consistent with the specific loss of anti-HIV-1 CTL activity. Another possibility is that pCTL become targets for HIV-1 infection and are killed by viral or host immune factors. Productive in vitro infection of $CD8^{+}CD4^{-}$ PBMC from SIV_{mac} infected rhesus monkeys and HIV-1 infected humans has been demonstrated (Tsubota *et al.*, 1989(b)) but only after long-term culture so that it is unlikely to be significant in vivo. In man, infection of $CD8^{+}$ cells with HHV6 induces CD4 expression permitting HIV-1 infection of these cells in vitro (Lusso *et al.*, 1991). Alternatively, HIV-1 infection of $CD4^{+}/CD8^{+}$ precursors in the bone marrow, which has been demonstrated in vitro, may prevent regeneration of CTL (Lunardi-Iskandar *et al.*, 1989; McMichael and Gotch, 1993). In addition, active suppression of CTL may be occurring. Recombinant and synthetic HIV-1 ENV proteins have been shown to be immunosuppressive for normal lymphocytes in vitro (Nair *et al.*,

1988). A population of $CD8^+$ suppressor cells have been identified which mediate broad suppression of CTL activity (Joly *et al.*,1989).

Further preliminary evidence for a putative protective role for CTL in HIV-1 infection comes from studies on babies born to HIV-1-infected mothers. These babies have a 12-50% risk of becoming infected with HIV-1 (Friedland and Klein, 1987; Blanche *et al.*, 1989; Katz *et al.*, 1989; Hirsch and Curran, 1990). In 2 separate reports, virus-specific CTL activity has been identified in 4 babies, suggesting that exposure to HIV-1 had occurred, but HIV-1 infection could not be detected by virus isolation or PCR for virus DNA or RNA. Once maternal antibody had declined, the babies became seronegative (Cheynier *et al.*, 1992; Rowland-Jones *et al.*, 1993). In one case the effectors were shown to be class I MHC-restricted (Cheynier *et al.*, 1992). If these cases represent genuine recovery then virus-specific CTL may have contributed. There has been an anecdotal report of a similar phenomenon in a seronegative adult (Cohen, 1992).

It has been suggested that resurgence of viral activity associated with decline in clinical state may be due to the emergence of CTL escape mutants. HIV-1 mutates rapidly in vivo (see 1.2.3) so that the effects of a selective pressure will be amplified. Neutralising antibody has been shown to exert a selective pressure on the emergence of HIV-1 variants in vitro (McKeating *et al.*, 1989). Viral variants that can escape recognition by CTL in vivo have only been described in mice transgenic for a TCR specific for an epitope in LCMV (Pircher *et al.*, 1990). A pre-requisite for immune escape from CTL is that all progeny from a single cell should contain the escape mutation. Consistent with this, it has been shown that many circulating HIV-1 infected cells contain a single provirus (Simmonds *et al.*, 1990). Johnson *et al.* (1992 and 1993) showed that a single aa substitution in a highly conserved 8 aa epitope in ENV can abrogate lysis of target cells by a CTL clone supporting the hypothesis that HIV-1 sequence variation may serve as a mechanism of immune escape. Preliminary evidence for the existence of CTL escape mutants in HIV-1 infection has been obtained (Phillips *et al.*, 1991). Longitudinal studies in 3 HIV-1 positive patients with HLA B8 show that the dominant CTL response changes with time. Three epitopes restricted by HLA B8 were identified in HIV-1 and the dominant epitope varied with time in each patient. Proviral DNA from these patients was sequenced and corresponding variant epitope peptides synthesised. Some of these variant epitopes were not recognised by the patients' CTL. If these viral variants are true CTL escape mutants then this finding implies that HIV-1-specific CTL are exerting a selection pressure on virus which is further evidence that CTL have a beneficial role in HIV-1 infection. It also suggests a mechanism by which the containment of virus may eventually fail, i.e. the accumulation of CTL

escape mutants in an individual may eventually outstrip the ability of the immune system to keep pace. In the same study, 3 patients with HLA B27 had stable CTL responses over time. The authors suggest that variation in epitopes presented by this HLA type may be incompatible with virus survival. If this is so then disease progression should vary between different HLA types. Consistent with these data, the A1-B8-DR3 haplotype has been associated with rapid progression in 3 separate studies (Steel *et al.*, 1988; Mallal *et al.*, 1990; Kaslow *et al.*, 1990).

In contrast to these findings, Chen *et al.* (1992) found no evidence of CTL escape mutations in an immunodominant epitope in GAG in 3 SIV_{mac} infected rhesus monkeys despite the fact that this response was lost in 1 animal by 2 years post-infection.

An immunopathological role has been suggested for virus-specific CTL in HIV-1 infection. As discussed above, $CD4^+$ class II MHC-restricted CTL may be involved in killing of "innocent bystanders", i.e. uninfected $CD4^+$ cells which have taken up and processed free gp120. Suppression of ENV-specific CTL has been suggested as an immunotherapy, particularly since HIV-infected chimpanzees, which do not suffer immunodeficiency, do not have ENV-specific CTL (reviewed in Nixon, 1992). Virus-specific CTL may be involved in localised inflammatory lesions in seropositive patients (Plata *et al.*, 1989; Plata, 1992). $CD8^+$ CTL targeted to HIV-1 infected alveolar macrophages are thought to mediate a lymphocytic alveolits (Autran *et al.*, 1988; Autran *et al.*, 1991) and CTL have been readily isolated from the CSF of patients with neurological disorders (Sethi *et al.*, 1988; Jassoy *et al.*, 1992). The destruction of lymph node architecture that follows HIV-infection is associated with the infiltration of $CD8^+$ cells although there is debate as to whether these cells have a cytotoxic function (Devergne *et al.*, 1991; Parmentier *et al.*, 1991; Tenner-Racz *et al.*, 1993).

1.4.2 Immune response to FIV

1.4.2.1 Humoral response to FIV

Cats experimentally infected with FIV generally seroconvert within 2-9 weeks of infection (Yamomoto *et al.*, 1988; Hosie and Jarrett, 1990). The time to seroconversion may be affected by the virus dose in the infecting inoculum since earlier seroconversion has been seen with higher doses of virus (Hosie and Jarrett, 1990). Antibodies to CA are detected first followed by antibodies to MA, GAG precursor and SU. Antibodies which inhibit FIV RT can be detected in infected animals but only reach significant levels 1-2 years post-infection (Fevereiro *et al.*, 1991(a)).

The presence of circulating antibodies is generally accepted to indicate persistent infection with FIV since it has always been possible to isolate virus from seropositive animals. Although it is an uncommon finding, virus has been isolated from seronegative cats both in the field and experimentally (Hopper, 1991; Yamamoto *et al.*, 1988; Lutz, 1990). There are a number of possible explanations for this finding including delayed seroconversion, which has been described for HIV-1 infected humans (Ranki *et al.*, 1987), and shut-down of antibody production in the terminal stages. Interestingly FIV genome-positive, seronegative cats have been found among animals in-contact with FIV seropositive cats (Dandekar *et al.*, 1992). PBMC from these cats could not be induced to produce infectious virus by mitogen stimulation suggesting that provirus was defective although genome positivity could be transferred to naive cats by blood and bone marrow. The significance of these findings is unclear at this stage and long-term follow up of these animals will provide further information. Rare HIV-1-seropositive asymptomatic individuals have been described who have become seronegative but remain latently infected with HIV-1 since provirus can still be detected in PBMC by PCR (Farzadegan *et al.*, 1988).

The role of antibodies in immunity to FIV is not known. Neutralising antibodies have been detected in FIV-infected cats from 1 month post-infection (Fevereiro *et al.*, 1991(b); Tozzini *et al.*, 1992). A major neutralising domain has been identified in ENV V3 (Lombardi *et al.*, 1993; De Ronde *et al.*, 1994). Broad cross-neutralisation has been demonstrated indicating that group-specific neutralisation epitopes exist (Fevereiro *et al.*, 1991(b); Tozzini *et al.*, 1993). Antigenic differences have been detected between 4 FIV isolates, previously shown to be equally divergent by *env* nucleotide sequence analysis (Sodora *et al.*, 1994; Rigby *et al.*, 1993), on the basis of neutralisation (Mr R. Osborne, personal communication). FIV escape from neutralising antibody has been demonstrated in vitro using a molecular clone grown in the presence of feline neutralising serum (Siebelink *et al.*, 1993). Escape was associated with a single aa change in the V5 region of ENV.

Anti-ENV antibodies, including virus neutralising antibodies, may be important in vaccine protection from FIV. A paraformaldehyde inactivated virus vaccine and an infected cell vaccine have been shown to protect over 90% of immunised animals from homologous and heterologous challenge (Yamamoto *et al.*, 1993). The correlates of this protection are not yet known. Protected cats generally had high levels of antibodies to ENV although not all cats with high levels of anti-ENV antibodies were protected. Kittens born to vaccinated queens have been protected from low-dose challenge by maternal antibodies (Pu *et al.*, in preparation). Passive

protection from low-dose IP homologous FIV challenge has been achieved by immunisation of cats with sera containing high levels of neutralising antibodies. These sera were obtained from cats vaccinated with either inactivated virus or infected cells or from FIV-infected cats (Hohdatsu *et al.*, 1993). Although passive protection from FIV infection has been achieved, large quantities of immune serum were infused both prior to and post-challenge so the relevance of this finding to natural immunity is unclear.

1.4.2.2 Cellular immune response to FIV

i) Suppression of FIV replication by CD8$^+$ cells

CD8$^+$ T lymphocyte suppression of FIV replication in CD4$^+$ cells and macrophages in vitro has been described (Jeng *et al.*, 1993 (a)). This suppression has been seen in cultures from cats in the asymptomatic stage of FIV infection but not in the acute or late stage, or from seronegative cats. The suppression was not MHC-restricted, did not depend on lysis of CD4$^+$ cells and could be transferred in supernatants. Attempts to identify the soluble mediator involved by RT-PCR of CD8$^+$ depleted and non-depleted cultures showed no involvement of IL2, 4, 6, 10, IFNα, IFNγ or TNFα (Jeng *et al.*, 1993(b)).

ii) Cytotoxic T-cell response to FIV

The persistence of FIV infection in the face of a neutralising antibody response, the role of CTL in controlling other persistent virus infections and circumstantial evidence that the CTL response to HIV has a protective function have focused attention on the CTL response to FIV. However, to date there has been only a single published report demonstrating the induction of FIV-specific cytotoxic effector cells by experimental FIV infection (Song *et al.*, 1992). Rather than a lack of interest in this area, this paucity reflects the diffficulties associated with measuring feline CTL. Using T-lymphoblastoid cells as targets, Song and colleagues demonstrated FIV-specific cytotoxicity in restimulated PBMC from 7-9 weeks post-infection and also in chronically infected cats. Killing was restricted to autologous targets and no killing could be demonstrated by similarly prepared PBMC from FIV-seronegative cats. It was necessary to use ^{111}In, rather than ^{51}Cr, to label target cells so that less target cells could be used thereby achieving suitable effector:target ratios. The phenotype of the effector cells was not determined in that study.

The main aim of the present study was to design an assay system capable of reliably and reproducibly detecting FIV-specific CTL from cats experimentally infected with FIV. The availability of such an assay system is an essential prerequisite for the determination of the role of FIV-specific CTL in FIV-infection.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cells

2.1.1.1 Lymphocytes

Feline PBMC were isolated from peripheral blood as decribed in section 2.2.1. Q201 is a line of feline ovalbumin-specific T cells (Hosie, 1991; Willett *et al.*, 1991).

2.1.1.2 Fibroblasts

Feline renal fibroblasts (provided by Dr J Christie) were a short term line obtained by unilateral nephrectomy of an experimental cat. RK13 cells are a rabbit kidney fibroblast line (Beale *et al.*, 1963) (provided by Dr M Mackett). WS HeLa cells are a fibroblast line derived from a human cervical epithelial carcinoma (Scherer *et al.*, 1953) (provided by the Institute of Virology, University of Glasgow). BHK 21 C13 are a Syrian hamster kidney fibroblast line (Macpherson, 1963) (provided by Dr B Blacklaws, University of Edinburgh). Vero cells are an African Green monkey kidney cell line (reference in Thompson, 1994) (provided by Dr F Thompson, Department of Veterinary Parasitology, University of Glasgow). Human thymidine kinase deficient TK^{-}143 cells are derived from an osteosarcoma (Rhim *et al.*, 1975) (provided by Dr M Browning, John Radcliffe Hospital, Oxford, UK). AH927 fibroblasts are a feline embryo derived line (Rasheed and Gardner, 1980).

2.1.2 Cell culture media

Media described as "10%" contained 10% foetal calf serum (FCS, Gibco, UK), 2mM glutamine (Gibco, UK), 100 U/ml penicillin (Gibco, UK) and 10μg/ml streptomycin (Gibco, UK). Lymphocytes were grown in complete RPMI (10% RPMI (Gibco, UK) supplemented with 5×10^{-5} M 2-mercaptoethanol (Sigma)). Feline bone marrow fibroblasts were grown in 10% MEM alpha (MEM alpha containing ribonucleosides and deoxyribonucleosides, Gibco, UK). Feline renal fibroblasts and AH927 cells were grown in 10% DMEM (Gibco, UK). Human TK^{-}143 cells, RK13, WS HeLa, BHK 21 C13 and Vero cells were grown in DMEM containing 5% FCS, 2mM glutamine, 100 U/ml penicillin, 10μg/ml streptomycin. Feline skin fibroblasts cells were grown in complete MEM alpha (10% MEM alpha supplemented with human recombinant epidermal growth factor at 10ng/ml (Sigma, UK)).

2.2 METHODS

2.2.1 Isolation of PBMC

Peripheral blood samples were taken by jugular venipuncture with or without sedation, into preservative free heparin (pfh) (Monoparin, CP Pharmaceuticals Ltd, Wrexham UK) in RPMI 1640 at a final concentration of 10 U/ml. PBMC were separated by density gradient separation as follows. Blood was diluted with an equal volume of phosphate buffered saline (PBS) and gently layered onto an equal volume of Lymphoprep, S.G. 1.077, (Nycomed, Norway) in a universal container. Samples were centrifuged at 1200 rpm for 20 minutes with the brake in the "off" position. PBMC were aspirated from the interface and washed in 50ml of PBS.

2.2.2 Cell culture

Cells were grown at 37°C in an atmosphere of 5% CO_2 in air in sterile plastic tissue culture flasks or plates, the latter requiring a humidified incubator.

Adherent cells were subcultured by removing the medium, rinsing the cells in versene (NaCl 40g, KCl 1g, Na_2HPO_4 5.75g, KH_2PO_4 1g, EDTA 1g in 50ml 1% phenol red), then incubating with 0.01% trypsin in versene at 37°C for 5 minutes. Cells were washed off into medium, recovered by centrifugation and then resuspended in fresh medium.

2.2.3 Cell storage

Cells were stored in a mixture of 10% DMSO, 45% medium and 45% FCS in 1.8ml cryotubes (Nunc, InterMed). PBMC and lymphoblasts were frozen to -100°C in a controlled rate cell freezer (Planer Kryo 10, Series II) and then transferred to liquid nitrogen vapour phase. Other cell types were frozen to -70°C in polystyrene containers overnight and then transferred to liquid nitrogen vapour phase.

2.2.4 Generation of lymphoblasts

PBMC were resuspended in complete RPMI supplemented with 7.5μg/ml concanavalin A (ConA; C-0412 Sigma, UK) at 2 x 10^6/ml. After 24-48 hours, cells were washed and resuspended in complete RPMI supplemented with 100 U/ml human recombinant IL2 (human rIL2) (kindly provided by Dr J Nunberg, Cetus Corporation). Cultures were examined daily, fed with fresh culture medium and split as necessary to maintain a cell concentration of 2 x 10^6 cells/ml.

2.2.5 Measurement of FIV p24 antigen by ELISA

FIV p24 antigen was detected in tissue culture supernatants using an ELISA kit (PetChek Feline Immunodeficiency Virus Antigen Test Kit, IDEXX). The kit was used according to the manufacturer's instructions. The sensitivity limit of this assay is 0.2ng/ml (Tilton *et al.*, 1990)

2.2.6 Production of FIV/GL14 stock

A stock of the Glasgow-14 isolate of FIV (FIV/GL14), was grown from the original isolate (Hosie, 1991). FIV/GL14 was isolated from an aged, entire male domestic longhair cat originating from the Colwyn Bay area of Wales, UK. This cat originally presented with pyrexia, weight loss and lymphadenopathy (Hosie, 1991; Rigby *et al.*, 1993).

A 30ml culture of Q201 cells (2.1.2) at 2 x 10^6 cells/ml, was infected with a 1ml aliquot of FIV/GL14 tissue culture supernatant. After 24 hours the cells were recovered by centrifugation, washed in complete RPMI and resuspended in fresh medium. The culture was expanded by the addition of uninfected Q201 cells over an 11 day period. Culture supernatants were tested for FIV antigen by ELISA. On day 11 the optical density (O.D.) reading for the ELISA exceeded the maximum for the reader and supernatants were harvested by centrifugation. The fluid was filtered through a 0.45µm filter to remove cell debris and stored in 1ml aliquots at -70°C.

2.2.7 Immunoblotting

Immunoblotting (Western blotting) was performed as described by Hosie and Jarrett (1990). Briefly, cell lysates were boiled with 1X reducing sample buffer (3X reducing sample buffer; 3ml 0.625 M Tris HCl pH 6.8, 3ml 20% SDS in ddH$_2$O, 1.5ml 2-mercaptoethanol, 3ml glycerol, 300µl 0.2% bromophenol blue, 200µl ddH$_2$O, stored at -20°C) for 3 minutes. Samples were separated on 10% polyacrylamide gels (2 x 10^6 cells/lane) using a Biorad Mini-Protean II Cell system. Proteins were transferred to nitrocellulose membranes and the remaining binding sites were blocked with 0.5% non-fat milk powder (NFMP) in Tris-buffered saline (TBS) for 2 hours. Nitrocellulose blots were washed twice for 10 minutes in TBS/0.05% Tween-20 and then washed overnight at 4°C on a rocking platform. Blots were incubated with pooled high-titre serum from cats experimentally infected with FIV (P1) diluted 1:10 in 1% NFMP and 0.5% Tween-20 in TBS for 2 hours. After 3 x 10 minute washes in 0.05% Tween-20 in TBS, blots were incubated for 1 hour with biotinylated protein A (Amersham, UK)

diluted 1:1000 in 1% NFMP and 0.5% Tween-20 in TBS. After further washing blots were incubated for 1 hour with avidin-alkaline phosphatase (Biorad) diluted 1:1000 in 1% NFMP and 0.5% Tween-20 in TBS. The blots were washed again then developed in the dark with substrate (33μl 5-bromo-4-chloro-3-indolyl phosphate (Sigma, UK), 66μl nitro blue tetrazolium (Sigma, UK)) in 10ml of AP buffer (100mM NaCl, 5mM MgCl, 100mM Tris, pH 9.5).

2.2.8 Preparation of FIV/GL14 positive antigen control for immunoblotting

FIV/GL14 infected Q201 cells from which a stock of FIV/GL14 was grown (2.2.6) were used to prepare positive control samples for immunoblotting. The cell pellet was resuspended in 150μl of lysis buffer. Serial two-fold dilutions of crude lysate were made in PBS. Samples were boiled for 3 minutes with 1X reducing sample buffer then analysed by immunoblot as described (2.2.7). The immunoblot blot was stained with P1 serum, which had been preabsorbed with uninfected Q201 cells on a rotating platform at 4°C overnight (Figure 2.1). A positive control was prepared by diluting the lysate 1:4. The lysate was stored at -20°C.

Figure 2.1

Titration of FIV/GL14 infected T cell lysate by immunoblotting

Feline T cell line Q201 was infected with FIV/GL14 and a lysate prepared for use as a positive control for FIV antigens on immunoblots. Lanes a-g contain the following dilutions of crude lysate; 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 respectively. A positive control was prepared by diluting the lysate 1:4.

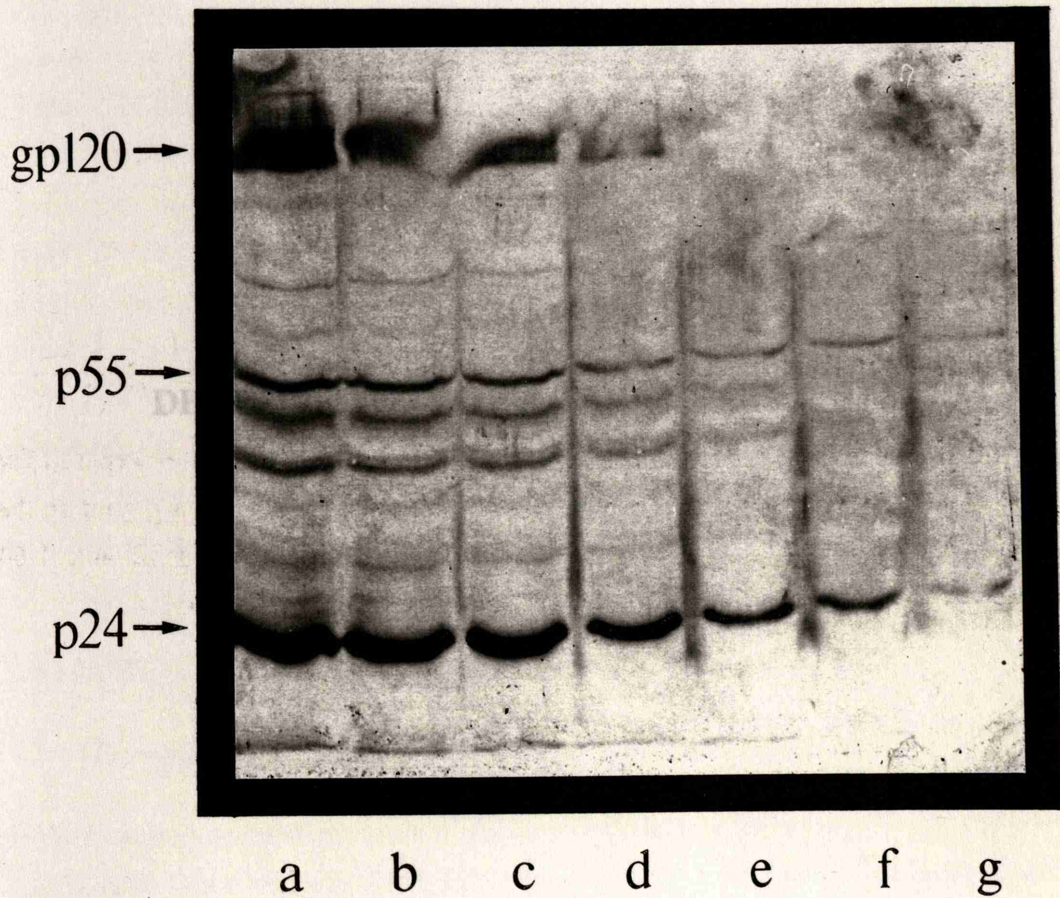


Figure 2.1

CHAPTER 3

DEVELOPMENT OF A TARGET CELL SYSTEM FOR AN ASSAY TO DETECT FELINE CYTOTOXIC EFFECTOR CELLS

3.1 INTRODUCTION

The activity of cytotoxic effector cells is detected by their ability to kill target cells. A useful system for detecting target cell lysis is to measure the release of a radioactive isotope from suitably labelled cells. The activity of the culture medium can be measured and is proportional to the number of target cells killed. By manipulating the target cell system it is possible to elucidate the nature of the cytotoxicity in terms of antigen-specificity and MHC-restriction.

This chapter describes the development of an autologous, FIV-GAG expressing, ^{51}Cr labelled target cell system for detecting feline cytotoxic effector cells generated in vivo in response to FIV infection. The class I MHC-restricted nature of the CTL response necessitates the use of autologous target cells since MHC-typing for this species is not yet available. PBMC, renal fibroblasts, bone marrow fibroblasts and skin fibroblasts were all considered for use as target cells in the assay. These cell types were compared on the basis of their availability and in vitro growth characteristics since routine immortalisation of feline cells is not yet possible. Using these criteria, skin fibroblasts were selected as the most suitable target cell for the assay. Of the target cell types considered, only PBMC are readily infectable by FIV. Antigen expression in fibroblasts was, therefore, achieved by using a recombinant FIV/vaccinia virus. A convenient and effective method of ^{51}Cr labelling of target cells was developed involving overnight incubation of fibroblast monolayers *in situ*.

3.2 MATERIALS AND METHODS

3.2.1 Harvesting and growth of feline bone marrow fibroblasts

Bone marrow samples were obtained by needle aspiration biopsy from the femur. Adult cats were anaesthetised with an injectable steroid anaesthetic (Saffan, Pitman-Moore Ltd. 0.5ml/kg by rapid IV injection). With the animal in lateral recumbency an area over the hip-joint was clipped and prepared aseptically. At a point midway between the head of the femur and the greater trochanter a sterile biopsy needle (Jamshidi disposable modified Illinois sternal/iliac bone marrow aspiration needle, 15 gauge, Baxter Healthcare Corporation, Berkshire, UK) was passed through the skin and advanced towards the trochanteric fossa with the long axis of the needle parallel to that of the femur. Manipulation of the joint aided in localisation of the trochanteric fossa. Penetration of the cortex was achieved by moderate pressure and rotation of the needle. The stylet was removed, a 10ml syringe was attached and negative pressure was applied until the aspirate was seen in the barrel. Rotation or slight withdrawal of the needle was sometimes necessary

to obtain the sample. The biopsy was transferred into 10ml of MEM alpha containing 10U/ml of preservative-free heparin.

The cells were separated by centrifugation at 1000 rpm for 10 minutes. The cell pellet was resuspended in 25ml 0.83% ammonium chloride at 37°C for 4 minutes to lyse the red blood cells (rbc). Centrifugation was repeated and the pellet was inspected to ensure that complete lysis of the rbcs had occurred. Ammonium chloride lysis was repeated if necessary. Cells were washed 3 times in plain MEM alpha and then resuspended in 30ml of 10% MEM alpha in a 175cm^2 tissue culture flask. Primary fibroblast cultures were incubated for approximately 7 days until they became confluent and were then split into 5 x 175cm^2 flasks. Second passage fibroblasts were stored when confluent.

3.2.2 Harvesting and growth of feline skin fibroblasts

Cats were anaesthetised and prepared as described for bone marrow biopsy (3.2.1). A sterile disposable 0.5cm diameter skin biopsy punch was used to take a full thickness skin biopsy from the area over the hip joint. The biopsy was transferred into MEM alpha containing antibiotics (100 U/ml penicillin and 10µg/ml streptomycin). The biopsy site was disinfected but left unsutured.

The biopsy was washed several times in MEM alpha containing antibiotics. Hypodermal fat was removed using a sterile scalpel, then the biopsy was sectioned perpendicular to the plane of dissection and cut into several pieces. Sequential digestions were performed by incubating the skin fragments with 10ml of 2.5% trypsin solution (Gibco, UK) diluted 1:10 with PBS in a conical flask at 37°C for 10 minutes with magnetic stirring.

Each digest was added to an equal volume of complete MEM alpha in a universal container and cells were recovered by centrifugation at 1400 rpm for 10 minutes. The cells were resuspended in 4ml of complete MEM alpha and incubated in 25cm^2 flasks. Primary explant cultures were split when subconfluent, after approximately 12 days, into 3 x 175cm^2 flasks. At confluence, each 175cm^2 flask was split into 5 and the cells were stored when the third passage reached confluence.

3.2.3 Generation of recombinant FIV-vaccinia virus

A recombinant vaccinia virus containing the *gag* gene of FIV/GL14, vFIV/GL14-*gag*, was prepared. The *gag* gene of FIV/GL14 was cloned by Dr M Rigby (Department of Veterinary Pathology, Glasgow, UK). The *gag* gene and part of the RT region of the *pol* gene of FIV/GL14 (residues 588-2633) was amplified by PCR

from genomic DNA of infected F422 cells (Rickard *et al.*, 1969) using primers corresponding to the prototype Petaluma isolate of FIV (FIV/PET). The fragment was gel purified, cloned, sequenced and subcloned into the vaccinia shuttle vector pSC11 (Chakrabarti *et al.*, 1985; Figure 3.1).

The vFIV/GL14-*gag* recombinant virus was generated by Dr M Mackett (Paterson Institute for Cancer Research, Manchester, UK). Cells infected with wild-type vaccinia virus (vWT), were transfected with the pSC11-FIV/GL14-*gag* construct. The wild type strain of vaccinia virus was Western Reserve, a laboratory strain derived from the New York City Board of Health vaccine strain by passage in mouse brain. Recombinant progeny were plaque purified on the basis of $TK^{-}\beta gal^{+}$ phenotype (Chakrabarti *et al.*, 1985). The principle of the generation of recombinant vaccinia viruses is summarised in Figure 3.2.

3.2.4 Growth of recombinant vaccinia virus

Following preliminary screening of RK13, WS HeLa, BHK 21 C13, Vero and human $TK^{-}143$ cell lines, the latter were found to give the highest yield of virus and these cells were subsequently used to grow virus stocks. Human $TK^{-}143$ cells were screened on arrival for contamination by *Mycoplasma* species (*M.arginini*, *M.hyorhinis*, *A.laidlawii* and *M.orale*) using a commercial ELISA kit (Mycoplasma Detection Kit, Boehringer Mannheim Biochemica). The assay was used according to the manufacturer's instructions. These cells were found to be free from contamination by these *Mycoplasma* species. Human $TK^{-}143$ cells were grown in 5% DMEM and subcultured 1:10 weekly.

Ten $175cm^2$ flasks were seeded with 2×10^7 $TK^{-}143$ cells overnight to give confluent monolayers. One ml of vFIV/GL14-*gag* stock was thawed rapidly and added to 49ml of PBS/1% FCS. The medium was removed from the monolayers and replaced with 5ml of the virus in PBS/1% FCS. Incubation was carried out at room temperature for 1 hour on a flat surface to ensure even distribution of the virus inoculum. Twenty ml of 5% DMEM was then added to each flask. Flasks were incubated at 37°C and the cultures were harvested 48-72 hours later.

Adherent cells were scraped into the medium and recovered by low speed centrifugation in universal containers at 4°C. Each cell pellet was resuspended in 1ml of hypotonic buffer (10mM Tris, 1mM EDTA, pH 9) at 4°C and the suspensions were combined.

Two methods of releasing virus from the cells were used. In the first method, cells were disrupted with a tissue homogeniser. Nuclei were pelleted by

centrifugation at 750g (2000 rpm) for 10 minutes and the supernatant fluid was retained. The pellet was resuspended in 10ml of hypotonic buffer, homogenisation and centrifugation were repeated and the supernatants were combined. On one occasion the first and second virus yields were not combined but assayed separately to assess the usefulness of repeating the procedure. Alternatively, virus was released by 3 rapid freeze-thaw cycles. Virus was stored at -70°C in 1ml and 100μl aliquots.

A single vFIV/GL14-*gag* stock (batch number 004) was used for all the experiments described in this thesis.

3.2.5 Titration of recombinant vaccinia virus

3.2.5.1 Plaque assay

Four 6-well, 1.5cm diameter tissue culture plates (Falcon 3046, Becton Dickinson) were seeded with 4×10^5 fibroblasts (TK^-143, RK13, WS HeLa, BHK 21 C13, Vero, AH927, feline bone marrow fibroblasts or feline skin fibroblasts) in 2ml of medium to give confluent monolayers after overnight incubation. Ten-fold virus dilutions were prepared in PBS/1%FCS from 10^{-2} to 10^{-12}. The medium was removed from the monolayers and 1ml of each virus dilution was added to duplicate wells. One ml of PBS/1%FCS was added to mock-infected control wells. After 1 hour, the virus inoculum was carefully removed and 2ml of 5% DMEM was added to each well for a further 48 hours. The plates were left undisturbed during this period to avoid secondary plaque formation. The cell sheets were fixed and stained with 0.5% crystal violet in 10% formalin for 30 minutes at room temperature. Plates were washed with tap water and allowed to dry.

To calculate the infectivity of the stock a monolayer with approximately 100 macroscopically visible plaques was selected. The number of separate plaques was counted in duplicate wells and the mean value was calculated. The infectivity of the stock was calculated in plaque forming units (pfu)/ml as follows;

$$\text{Infectivity of virus stock} = \left(\frac{A}{B}\right) \times \frac{1}{C} \text{ pfu/ml}$$

Where;

A = mean number of plaques

B = virus dilution

C = volume of inoculum (ml)

3.2.5.2 Infectious centre assay

Six-well, 1.5cm diameter tissue culture plates were seeded with 4 x 10^5 TK^-143 cells in 2ml of culture medium to give confluent monolayers overnight. These were the indicator cells. Two wells of a 6-well, 1.5cm diameter tissue culture plates were seeded with 4 x 10^5 feline bone marrow fibroblasts or feline skin fibroblasts in 2ml of culture medium to give confluent monolayers overnight. The medium was removed from the monolayers and 1ml of a 10^{-3} dilution of vFIV/GL14-*gag* in PBS/1%FCS or PBS/1%FCS alone was added for 1 hour. The inoculum was removed and 2ml of culture medium was added to each well for a further 2 hours. The cells were removed with trypsin (2.2.2), washed 5 times with culture medium and counted. Dilutions of vFIV/GL14-*gag* infected fibroblasts were prepared in PBS/1%FCS containing 100, 50 or 25 cells/ml. Medium was removed from the indicator cells and 1ml inocula of each dilution of infected fibroblasts were added. To control for residual non-cell associated virus, duplicate infected fibroblasts which had been killed by freezing and thawing were seeded on to indicator cells. One ml of mock infected cells at 100 cells/ml in PBS/1%FCS was added to a second control well. The procedure was then continued as for the plaque assay (3.2.5.1).

3.2.6 FIV-GAG expression in vFIV/GL14-*gag* infected fibroblasts

3.2.6.1 Immunoblotting

The following fibroblast cells were screened by immunoblotting for FIV-GAG expression following infection with vFIV/GL14-*gag*; AH927, WS HeLa, human TK^-143 cells and feline renal, bone marrow and skin fibroblasts. The time course of GAG expression was determined for these cell types. The virus inoculum required for expression of the product was titrated in feline skin fibroblasts.

Six-well, 1.5cm diameter tissue culture plates were seeded with 4 x 10^5 fibroblasts/well in 2ml of appropriate medium to form monolayers overnight. Five monolayers were infected with vFIV/GL14-*gag* in 1ml PBS/1% FCS at 10 pfu/cell. The remaining monolayer was mock infected with 1ml PBS/1% FCS only. After 1 hour (time zero), the virus inoculum was replaced with 1ml of culture medium . At 30-90 minute intervals thereafter the medium was removed from an infected well and the cells were harvested into 0.5ml 3X reducing sample buffer (2.2.7) using the plunger of a disposable 1ml syringe as a cell scraper. The control well was harvested at the final time point. Samples were placed in a boiling water bath for 3 minutes and were then stored at -20°C. Antigens were resolved on a 10%

polyacrylamide gel and examined by immunoblotting as described previously (2.2.6).

Using a modification of the above procedure, vFIV/GL14-*gag* was titrated on feline skin fibroblasts in 6-well plates. The following dilutions of vFIV/GL14-*gag* (batch 004) were prepared in PBS/1%FCS; 1:50, 1:100, 1:200, 1:500, 1:1000. Skin fibroblasts were incubated with 1ml of each virus dilution for 1 hour. One well contained PBS/1%FCS alone as a control. The inoculum was replaced with complete MEM alpha and incubation was continued for a further 2.5 hours. The cells were harvested as described above and were examined by immunoblotting.

3.2.6.2 Immunocytochemistry

Feline skin fibroblast cells were seeded into 96-well flat bottomed tissue culture plates at 1 x 10^4 cells/well in 100μl of complete MEM alpha and allowed to form monolayers overnight. The medium was removed and cells were incubated with 50μl of the following dilutions of vFIV/GL14-*gag*; 1:100, 1;200, 1:400, 1:800, or vWT; 1:100, 1:200, in 2% MEM alpha. Some cells were incubated with 2% MEM alpha alone as a control. After incubation for 1 hour, the inoculum was replaced with complete MEM alpha and the plates were incubated for a further 2 hours. The medium was removed with a multichannel pipette and then by inverting the plate on absorbent paper. The cells were fixed with 1% 0.5M acetic acid in ethanol at room temperature for 30 minutes. The fixative was removed and 100μl of 5% normal goat serum (Scottish Antibody Production Unit, Carluke, UK) in PBS was added to each well for 1 hour at 4°C to block non-specific antibody binding.

Normal goat serum at a concentration of 0.5% in PBS was prepared as a wash solution. Fixed monolayers were washed 3 times and then incubated for 1 hour at 37°C with two-fold dilutions (starting at 1:1000) of ascitic fluid containing anti-FIV p24 monoclonal antibody 1E2 (kindly provided by Dr N Pedersen, Davis, California, USA) in wash solution. Control wells were incubated with dilutions of an irrelevant, isotype-matched (IgG1) control anti-FIV-ENV monoclonal antibody, vpg67 produced by Mr T Dunsford in this department, or with wash solution alone. Cells were washed 3 times and then incubated with a 1:2500 dilution of biotinylated horse anti-mouse IgG (Sigma, UK) for 15 minutes at 37°C. The cells were washed as before and then incubated with streptavidin-peroxidase (1:2000) (Vector) for 15 minutes at 37°C. After washing, the substrate (0.04% amino ethyl carbazole in 0.05M acetate buffer, pH 4.9 and 0.08% 30 volume H_20_2) was added and colour development was allowed to proceed at room temperature for approximately 20-30 minutes. The reaction was stopped by immersing the plate in water. Cell sheets were counterstained with Meyer's haematoxylin for 10 minutes.

Plates were dried and the cell sheets were examined by light microscopy using a blue filter. The proportion of skin fibroblasts expresssing GAG was assessed by counting the number of positive cells in 50 adjacent cells from 3 wells.

3.2.7 ^{51}Cr labelling of target cells

3.2.7.1 ^{51}Cr labelling of fibroblasts in suspension. Effect of vaccinia infection on spontaneous and maximum isotope release.

Feline bone marrow and skin fibroblasts at fourth passage were trypsinised at confluence (2.2.2) and washed in 10% MEM alpha or complete MEM alpha, respectively. The cells were resuspended at 1 x 10^6/ml and 1ml of each cell type was placed into 2 polystyrene tubes (Falcon 2054, Becton Dickinson, UK). The cells were centrifuged at 1200 rpm for 5 minutes, the medium was discarded and the cell pellet was resuspended in 25µl of vFIV/GL14-*gag* (representing 7.5 pfu/cell calculated from virus infectivity for human TK^-143 cells). Virus adsorption was carried out at 37°C for 1 hour. The cells were washed in plain MEM alpha and were then resuspended in 25µl of MEM alpha, or MEM alpha containing 50µCi ^{51}Cr sodium chromate (Amersham, UK). Cells were incubated at 37°C and agitated every 15 minutes. After 1 hour the cells were washed 3 times with MEM alpha in a universal container then resuspended at 1 x 10^5/ml in 10% MEM alpha (bone marrow fibroblasts) or complete MEM alpha (skin fibroblasts). The cells were dispensed at 100µl/well into round-bottomed 96-well plates. A volume of 100µl of medium or 5% Triton X-100 (BDH, Poole, UK) was added to quadruplicate cultures and the plate was incubated for a further 4 hours.

At the end of the incubation period, 125µl samples of culture supernatant were harvested from each well synchronously into a 96-well holding plate using a multichannel pipette. A volume of 100µl of each sample was then transferred individually into LP3 tubes (Denley Instruments Limited, UK) and the activity of the samples was measured in a gamma counter (Minaxi gamma, Packard, Berkshire, UK) for 1 minute. Mean and standard deviation values were calculated for each assay condition.

3.2.7.2 ^{51}Cr labelling of fibroblasts monolayers. I. Effect of vaccinia infection on spontaneous and maximum isotope release.

Confluent skin fibroblast monolayers at second (cat F23) and seventh passage (cat A142) were trypsinised, washed and seeded into round-bottomed 96-well tissue culture plates at 1 x 10^4 cells/well in complete MEM alpha. ^{51}Cr was added at 1µCi/well in 50µl of complete MEM alpha. Following an overnight incubation,

skin fibroblast monolayers were washed 4 times with complete MEM alpha. The cells were incubated with vFIV/GL14-*gag* diluted 1:200 in 2% MEM alpha or 2% MEM alpha alone for 1 hour. The virus inoculum was replaced with complete MEM alpha for a further 2 hours. The medium was removed and fresh complete MEM alpha or 10% Triton X-100 was added for a further 4 hours to determine the spontaneous and maximum isotope release from the cells, respectively. Quadruplicate cultures were used. Cultures were contained on a single assay plate and incubated for 4 hours. Harvesting of the culture medium was carried out as described above (3.2.7.1).

3.2.7.3 ^{51}Cr labelling of fibroblasts monolayers. II. Effect of ^{51}Cr concentration on spontaneous and maximum isotope release.

Third passage skin fibroblasts from 2 cats were seeded into 96-well round-bottomed tissue culture plates at 1 x 10^4 cells/well in complete MEM alpha. Fibroblasts were incubated overnight with ^{51}Cr at 25, 50, 100 or 200μCi/10^6 cells. After washing the targets 4 times in complete MEM alpha, 50μl of a 1:100 dilution of vFIV/GL14-*gag* was added to each well for 1 hour. The inoculum was replaced with complete MEM alpha and incubation was continued for a further 2 hours. The medium was replaced with either complete RPMI or 10% Triton X-100 and the cultures incubated for a further 4 hours. Harvesting was carried out as described above (3.2.7.1).

3.3 RESULTS

3.3.1 In vitro culture of feline bone marrow and skin fibroblasts

Bone marrow and skin biopsies were taken from experimental cats to generate fibroblasts for use as target cells in cytotoxicity assays. Both cell types could be maintained in culture. Skin fibroblasts grew faster and recovered more quickly and consistently from storage than bone marrow fibroblasts.

3.3.2 Growth of vaccinia viruses

Recombinant and wild-type vaccinia viruses produced a characteristic cpe on the human TK^-143 cells. After inoculation of fibroblast monolayers, infected cells began to round-up within a few hours and disruption of the monolayer could be appreciated by direct visualisation within 24 hours. By 48-72 hours post-infection widespread diruption of the monolayer had occured with many cells floating free in the medium (Figure 3.3).

3.3.3 Titration of vaccinia virus-infected cells by plaque assay

Vaccinia cpe was detected as discrete plaque formation on all non-feline permanent cell lines (human TK^{-}143, RK13, WS HeLa, BHK 21 C13 and Vero cells) at higher virus dilutions in the plaque assays (Figures 3.4, 3.5 and 3.6). The infectivity of vFIV/GL14-*gag*, batch 004, which was grown in human TK^{-}143 cells, was 3×10^8 pfu/ml for TK^{-}143 cells. The infectivity of this batch was also determined in RK13 and BHK 21 C13 cells by plaque assay (Table 3.1) and was found to be very similar to that in human TK^{-}143 cells.

Similar yields of vaccinia viruses were obtained when harvesting was carried out using homogenisation (2.8×10^8 pfu/ml) or freeze-thawing (2.6×10^8 pfu/ml). When the yield of virus from the first and second rounds of homogenisation were compared it was found that the second round contained the same amount of virus as the first (3.1×10^8 pfu/ml), but the virus from the second harvest gave larger plaques in the assay.

Discrete plaques were not seen when the feline cell line AH927, short term feline bone marrow or feline skin fibroblast explant cultures were used as indicator cells in the plaque assay. Mock-infected monolayers had a mottled, blotchy appearance on staining and although at lower virus dilutions cpe could be detected as an overall thinning of the monolayers, the distinction between infected and uninfected monolayers at higher dilutions was not clear. It was considered that overgrowth of cells during the period of the assay may have obscured viral plaque formation. For this reason, the concentration of serum was decreased from 10% to 5% and then 2% to slow the rate of cell replication but this change had no effect on the result.

3.3.4 Titration of vaccinia virus-infected cells by infectious centre assay

Infectious centre assays were carried out to attempt to determine the infectivity of vFIV/GL14-*gag* for feline fibroblasts using human TK^{-}143 cells as indicator cells. In these assays, the indicator cells sustained widespread cpe at all infected cell dilutions and individual plaques could not be discerned. Control preparations containing killed infected cells also produced this effect. Monolayers of indicator cells seeded with mock-infected fibroblasts remained intact.

3.3.5 Expression of GAG in vFIV/GL14-*gag* infected fibroblasts determined by immunoblotting

The time course of GAG expression following FIV/GL14-*gag* infection was determined by immunoblotting in the following fibroblast cells; AH927, WS HeLa, human $TK^{-}143$ cells and feline renal, bone marrow and skin fibroblasts. Recombinant GAG could be detected as a band at approximately 55K on immunoblots by 1.5 hours after virus adsorption in all cell types tested. This band was not seen in uninfected cells or cells infected with vWT (Figures 3.7 and 3.8).

vFIV/GL14-*gag* (batch 004) was titrated in skin fibroblasts to determine a suitable dilution for infecting target cells in cytotoxicity assays. Expression of recombinant GAG was detected in skin fibroblasts by immunoblotting even when virus was used at the highest virus dilution of 1:1000 (Figure 3.9).

3.3.6 Expression of GAG in vFIV/GL14-*gag* infected fibroblasts determined by immunocytochemistry

A 2-step, indirect staining method using a biotinylated second antibody and peroxidase-labelled streptavidin was used to identify GAG-expression in vFIV/GL14-*gag* infected skin fibroblasts in 96-well plates. Skin fibroblasts expressing GAG appeared reddish/brown by light microscopy. When the first antibody, 1E2, was used at 1:4000, a clear differentiation between GAG expressing cells and background was achieved. Where target cells were infected with 50μl of a 1:100 dilution of vFIV/GL14-*gag* it was not possible to determine the proportion of GAG positive cells due to disruption of the monolayer. Using a 1:200 or 1:400 dilution of vFIV/GL14-*gag*, between 70% and 80% of the cells were positive. Those infected with 50μl of a 1:800 dilution of vFIV/GL14-*gag* contained 60% positive cells.

3.3.7 ^{51}Cr labelling of fibroblasts

3.3.7.1 Effect of vaccinia virus infection prior to ^{51}Cr labelling in suspension on ^{51}Cr release.

Suspensions of bone marrow and skin fibroblasts were infected with vFIV/GL14-*gag* or left uninfected, then labelled with ^{51}Cr at 50μCi/10^6 cells. Spontaneous isotope release from uninfected bone marrow and skin fibroblasts was 35% or 30% respectively of the maximum release. However, in vaccinia-infected fibroblasts absolute counts were much higher than in uninfected cells and spontaneous isotope release was 51% or 63% of the maximum release in bone marrow and skin fibroblasts respectively (Figure 3.10).

3.3.7.2 Effect of vaccinia virus infection after ^{51}Cr labelling of monolayers on ^{51}Cr release

Skin fibroblasts were incubated overnight in 96-well plates with 100μCi $^{51}Cr/10^6$ cells. Cells were either infected with vFIV/GL14-*gag* or left uninfected and spontaneous and maximum isotope release over a 4 hour period was determined. The results are presented in Figure 3.11. The mean maximum isotope release from infected skin fibroblasts was in the same range as that from uninfected cells. However, the background ^{51}Cr release was higher from vFIV/GL14-*gag* infected cells compared to uninfected cells. The background ^{51}Cr release was higher from A142 skin fibroblasts (seventh passage) than from F23 cells (second passage); 8.2% compared with 6.9% for uninfected fibroblasts and 36.3% compared with 19.6% for infected cells.

3.3.7.3 Effect of ^{51}Cr concentration on spontaneous and maximum isotope release.

Skin fibroblasts from 2 cats were incubated with 25, 50, 100 or 200μCi $^{51}Cr/10^6$ cells overnight. The cells were then infected with vFIV/GL14-*gag* and spontaneous and maximum isotope release over a 4 hour period was determined. The results are shown in Figure 3.12. Increasing the concentration of ^{51}Cr generally resulted in an increase in the spontaneous and maximum isotope release from the fibroblasts. The dose of ^{51}Cr associated with the lowest level of background release was achieved using 50μCi $^{51}Cr/10^6$ cells in cat A but using 100μCi $^{51}Cr/10^6$ cells in cat B (Figure 3.13).

Figure 3.1

pSC11

pSC11 is a shuttle vector for the insertion of foreign genes into vaccinia viruses. It contains flanking vaccinia virus sequences from the left (TKL) and right (TKR) sides of the thymidine kinase (TK) gene, thus directing insertion of FIV-*gag* into this non-essential vaccinia virus locus. Expression of FIV-*gag* is driven by the vaccinia virus promoter p7.5K. pSC11 contains a second promoter p11K and the following restriction sites; *Bam*HI, *Sma*I and *Eco*RI. The vector also contains two genes concerned with the selection of recombinant viruses; a gene for ampicillin resistance (AMPr) and the *LacZ* gene which encodes β-galactosidase. Arrows indicate the direction of transcription.

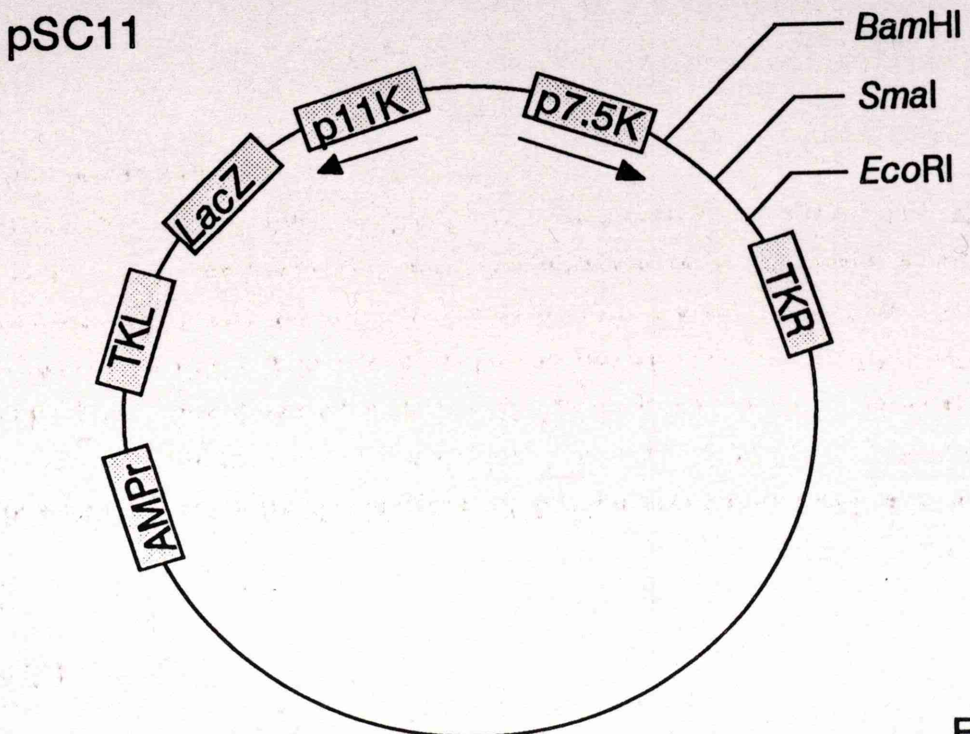


Figure 3.1

Figure 3.2

Generation of recombinant vaccinia viruses

Unlike the DNA of other DNA viruses, naked vaccinia virus DNA is non-infectious. Therefore, rather than simply cleaving the genome, re-ligating with a foreign gene then transfecting suceptible cells to form recombinant virus particles, foreign gene insertion relies on a process of homologous recombination. A permissive cell line is infected with vWT and transfected with the plasmid vector pSC11 containing the *gag* gene of the Glasgow 14 isolate of FIV. A small number of homologous recombination events occur whereby FIV/GL14-*gag* is inserted into the TK locus of the parent vaccinia virus. Recombinant viruses are then selectable on the basis of their TK-βgal+ phenotype.

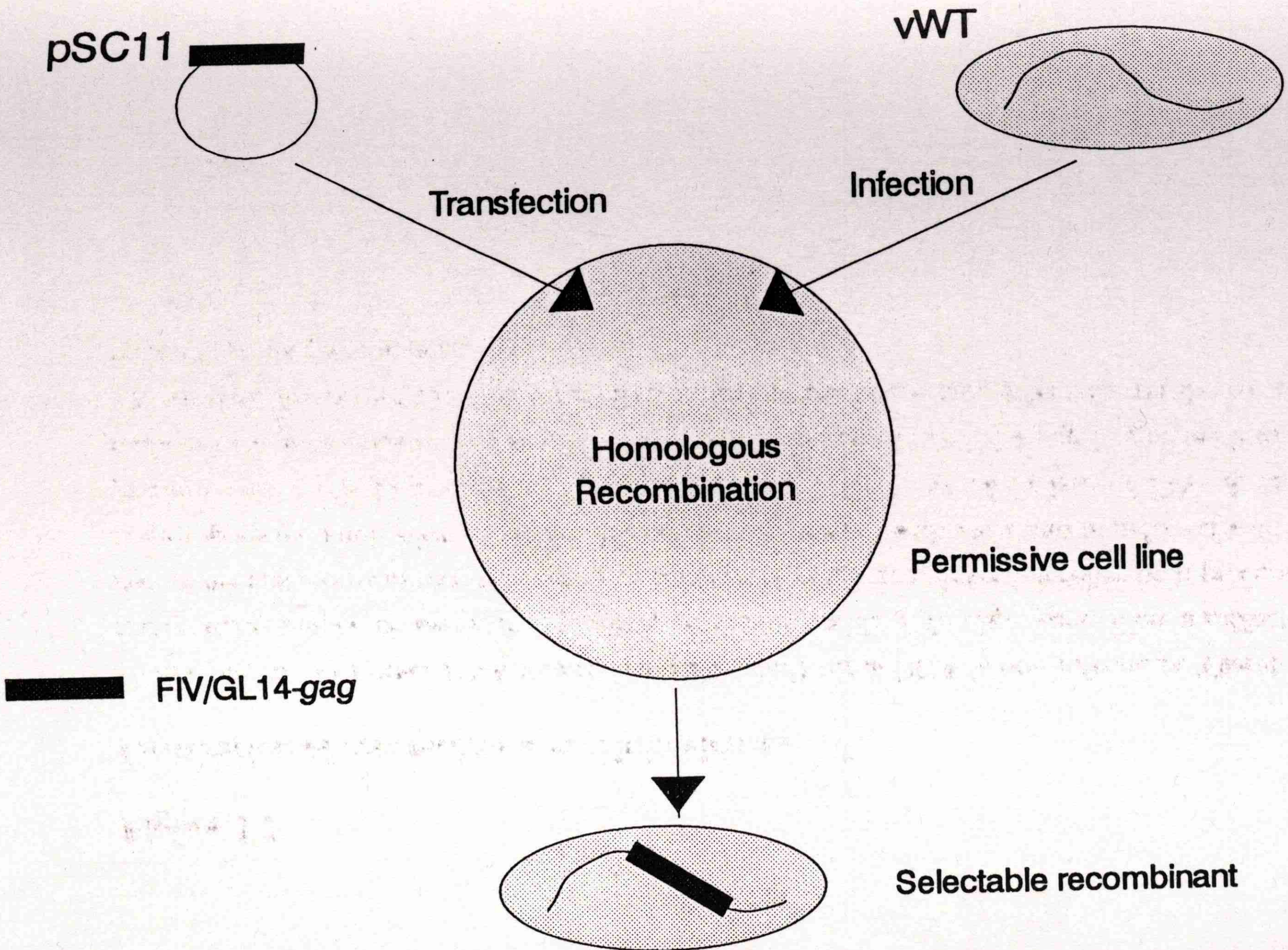


Figure 3.2

Figure 3.3

TK$^-$143 cell monolayer 48 hours after infection with vaccinia virus

Vaccinia virus cpe is widespread with rounding-up of infected cells and consequent disruption of the monolayer (x 40).

Figure 3.3

Figure 3.4

Titration of vaccinia viruses by plaque assay

Completed plaque assay (x 1). Details of the method are described in the text (3.2.5.1). Briefly, confluent monolayers of Vero cells were inoculated with the following dilutions of vFIV/GL14-*gag* 10-5(a), 10-6(b), 10-7(c), 10-8(d), 10-9(e) in PBS/1%FCS or with PBS/1%FCS alone (mock infected)(f). After 48 hours the cell sheets were fixed and stained with 0.5% crystal violet in 10% formalin, washed with tap water and allowed to dry. The infectivity of this batch of virus for Vero cells is approximately 2 x 108 pfu/ml.

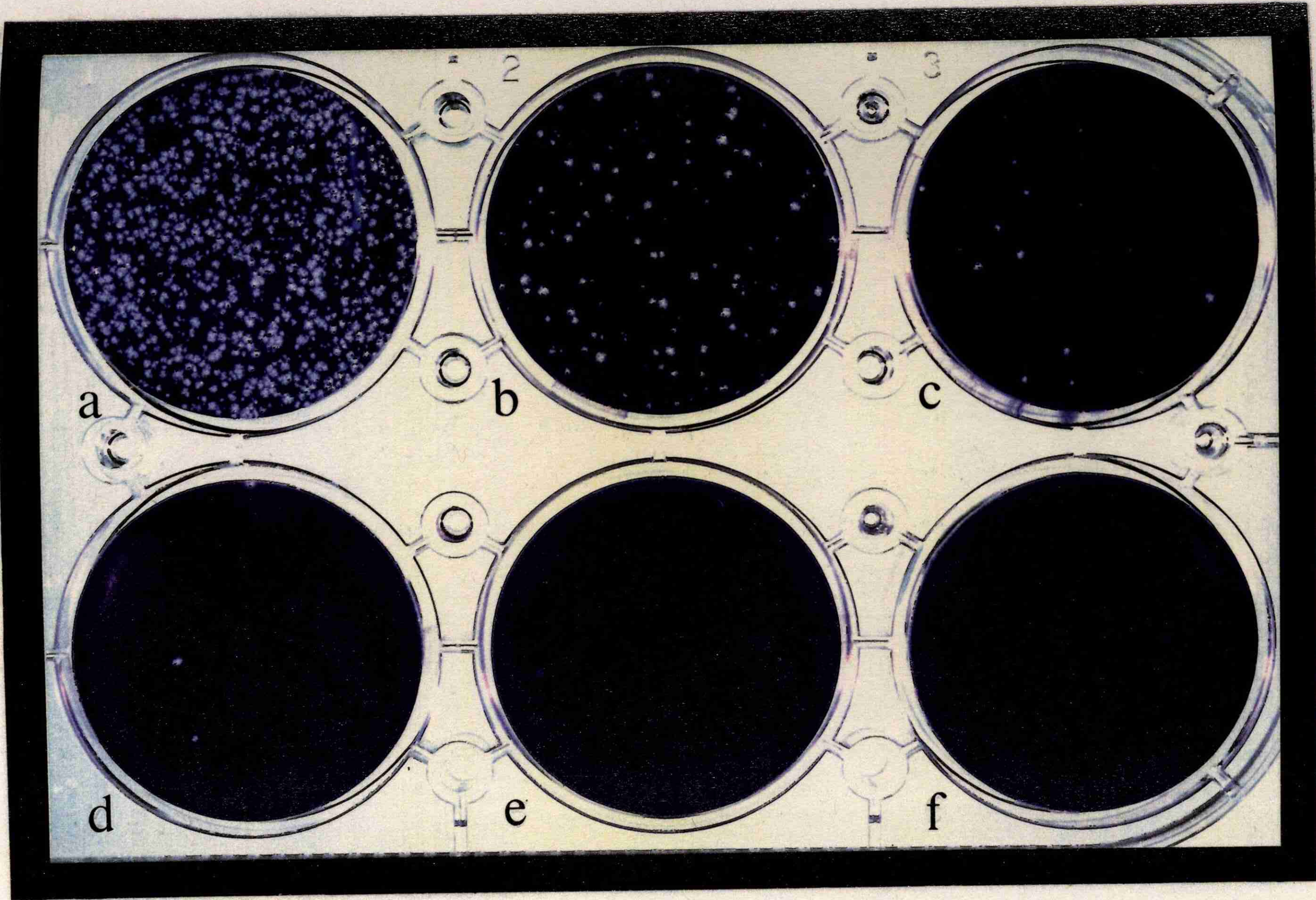


Figure 3.4

Figure 3.5

Vaccinia virus plaque formation on Vero cells (low power)

Single well from Figure 3.4 infected with 10^{-5} dilution of vFIV/GL14-*gag* (x 2) to show plaque morphology.

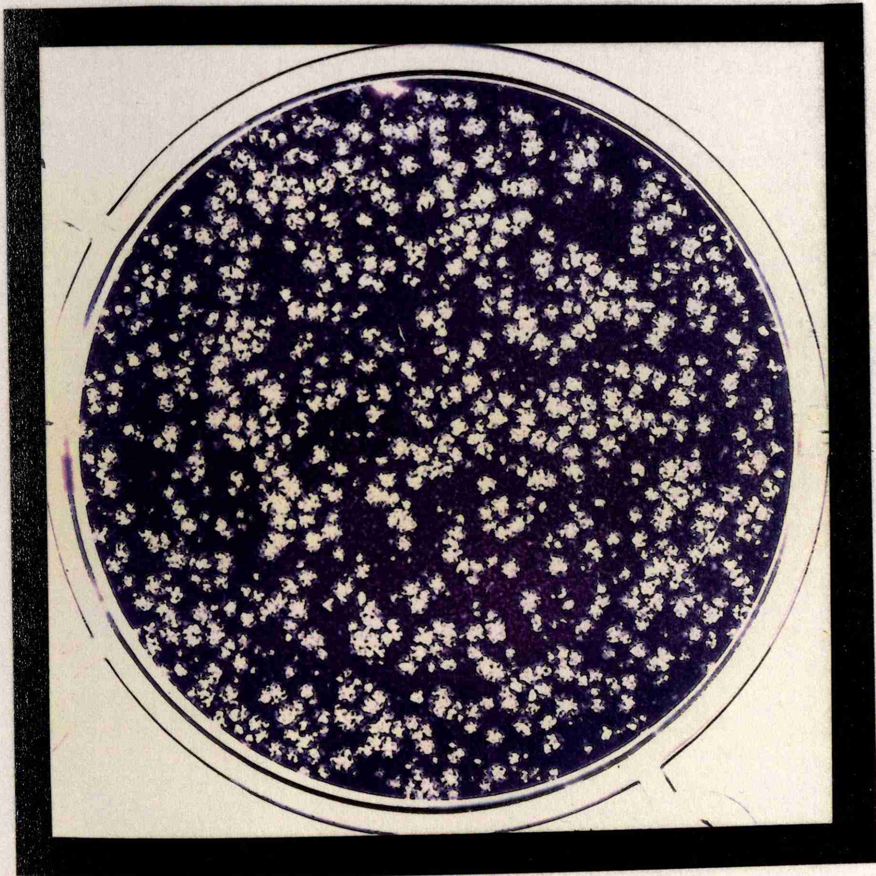


Figure 3.5

Figure 3.6

Vaccinia virus plaque formation on Vero cells (high power)

Cellular proliferation occurs at the edges of the lesion resulting in a thickened appearance. This may be due to attachment of virus to the cell surface receptor for epidermal growth factor which is believed to be the host-cell receptor for vaccinia virus (Eppstein *et al.*, 1985) (x 50).

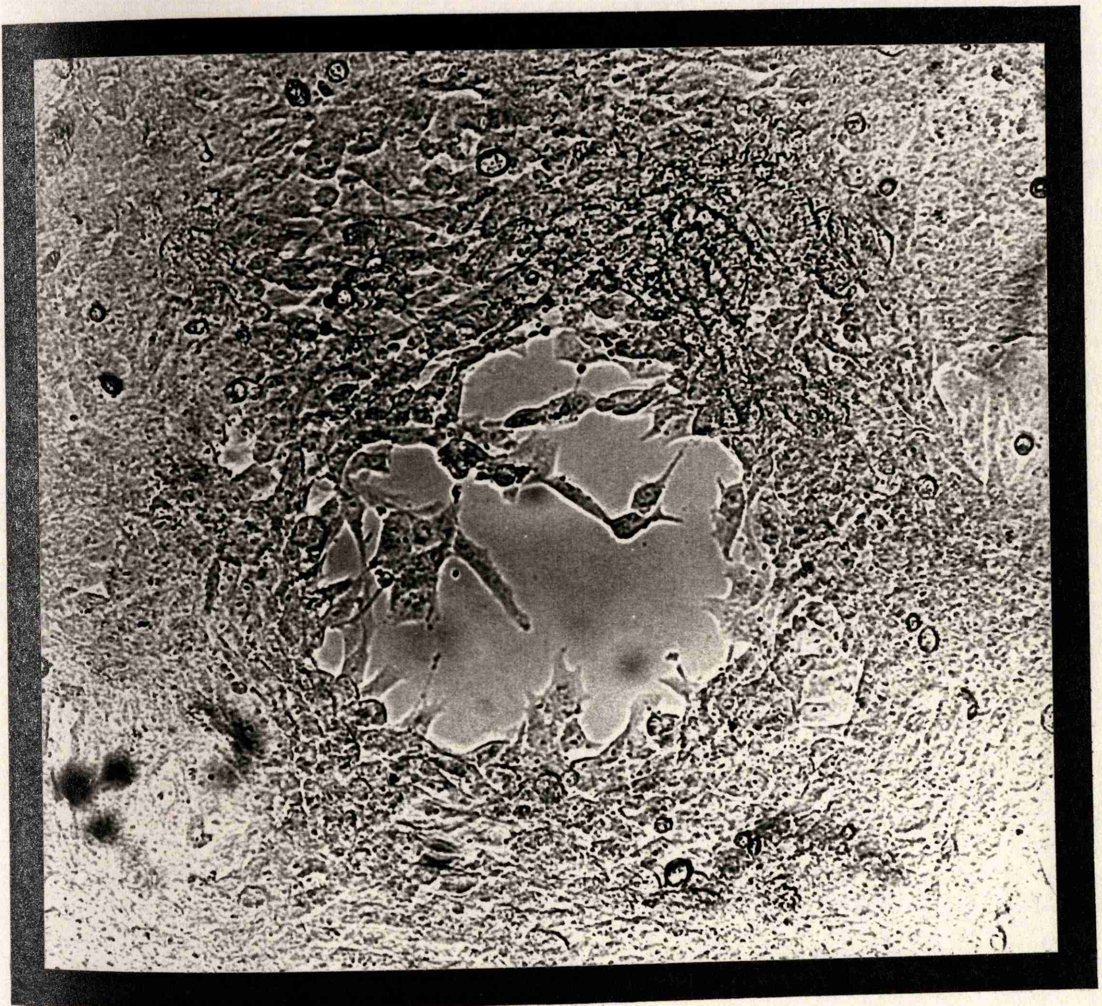


Figure 3.6

Figure 3.7

Expression of FIV-GAG in feline renal fibroblasts infected with vFIV/GL14-*gag*, demonstrated by immunoblotting

The GAG precursor can be seen as a band at approximately 55K from 1.5 hours after virus adsorption in cells infected with vFIV/GL14-*gag* (lanes a-c). This band is not seen in cells infected with vWT (lane d).

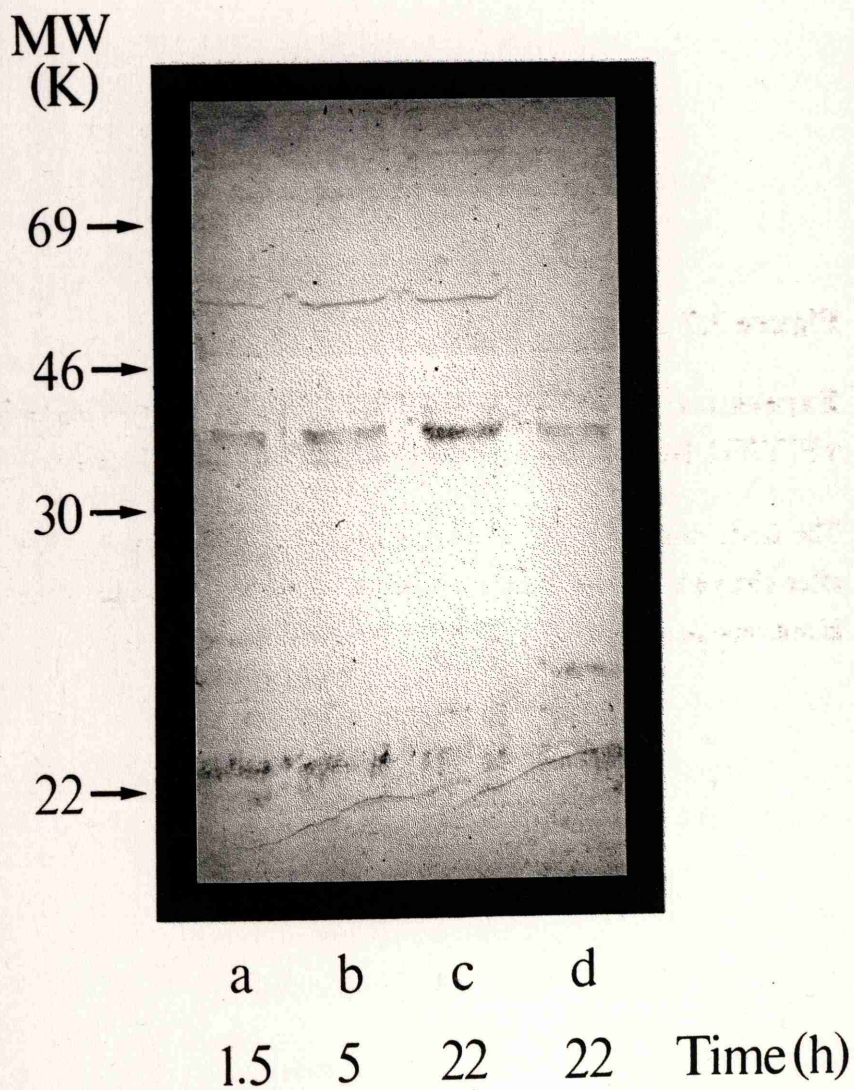


Figure 3.7

Figure 3.8

Expression of FIV-GAG in feline skin and bone marrow fibroblasts infected with vFIV/GL14-*gag*, demonstrated by immunoblotting

(a) FIV/GL14 infected Q201 cells (positive control).

(b) Bone marrow fibroblasts, mock-infected.

(c) Bone marrow fibroblasts, 150 minutes post-infection.

(d) Skin fibroblasts, mock-infected.

(e) Skin fibroblasts, 150 minutes post-infection.

(f) Skin fibroblasts, 120 minutes post-infection.

(g) Skin fibroblasts, 90 minutes post-infection.

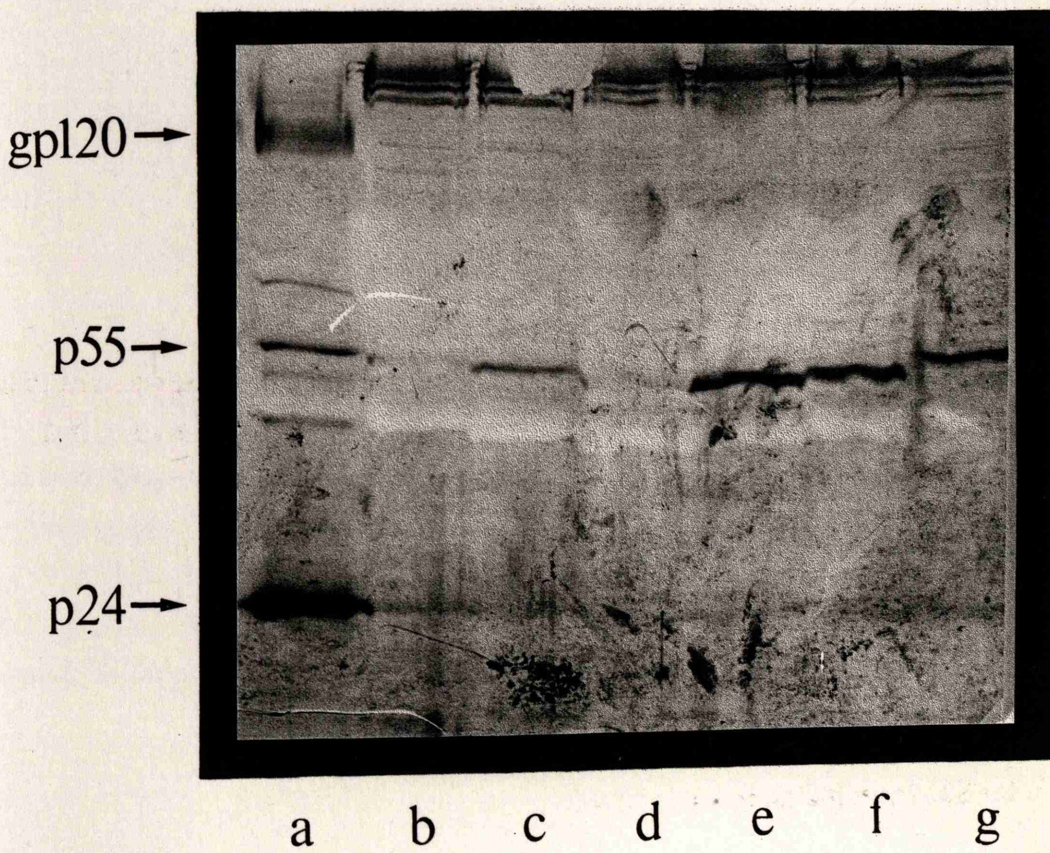


Figure 3.8

Figure 3.9

Titration of vFIV/GL14-*gag* on feline skin fibroblasts

Feline skin fibroblasts were infected with dilutions of vFIV/GL14-*gag* for 1 hour and then incubated for a further 2.5 hours. GAG (p55) expression is demonstrated by immunoblot. Lanes c-g contain cells infected with the following dilutions of vFIV/GL14-*gag*; 1:1000, 1:500, 1:200, 1:100, 1:50. Lane a contains mock infected skin fibroblasts (negative control). Lane b contains FIV/GL14 infected Q201 cells (positive control).

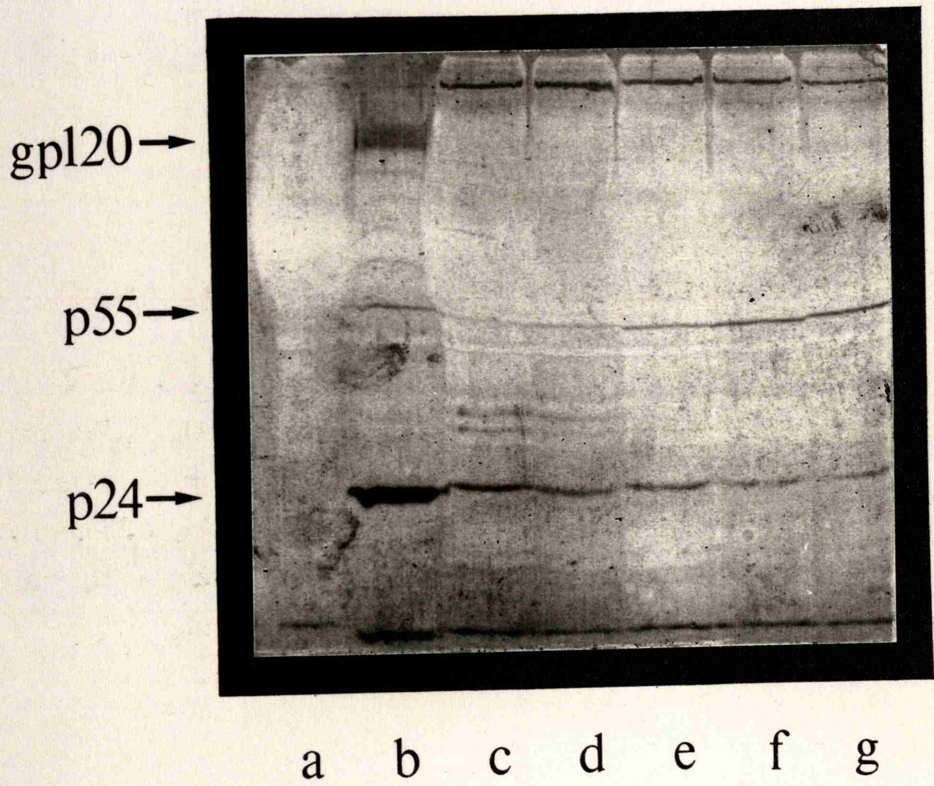


Figure 3.9

Figure 3.10

Spontaneous and maximum isotope release from fibroblasts infected with vaccinia virus then labelled with ^{51}Cr in suspension

Figures indicate background ^{51}Cr release.

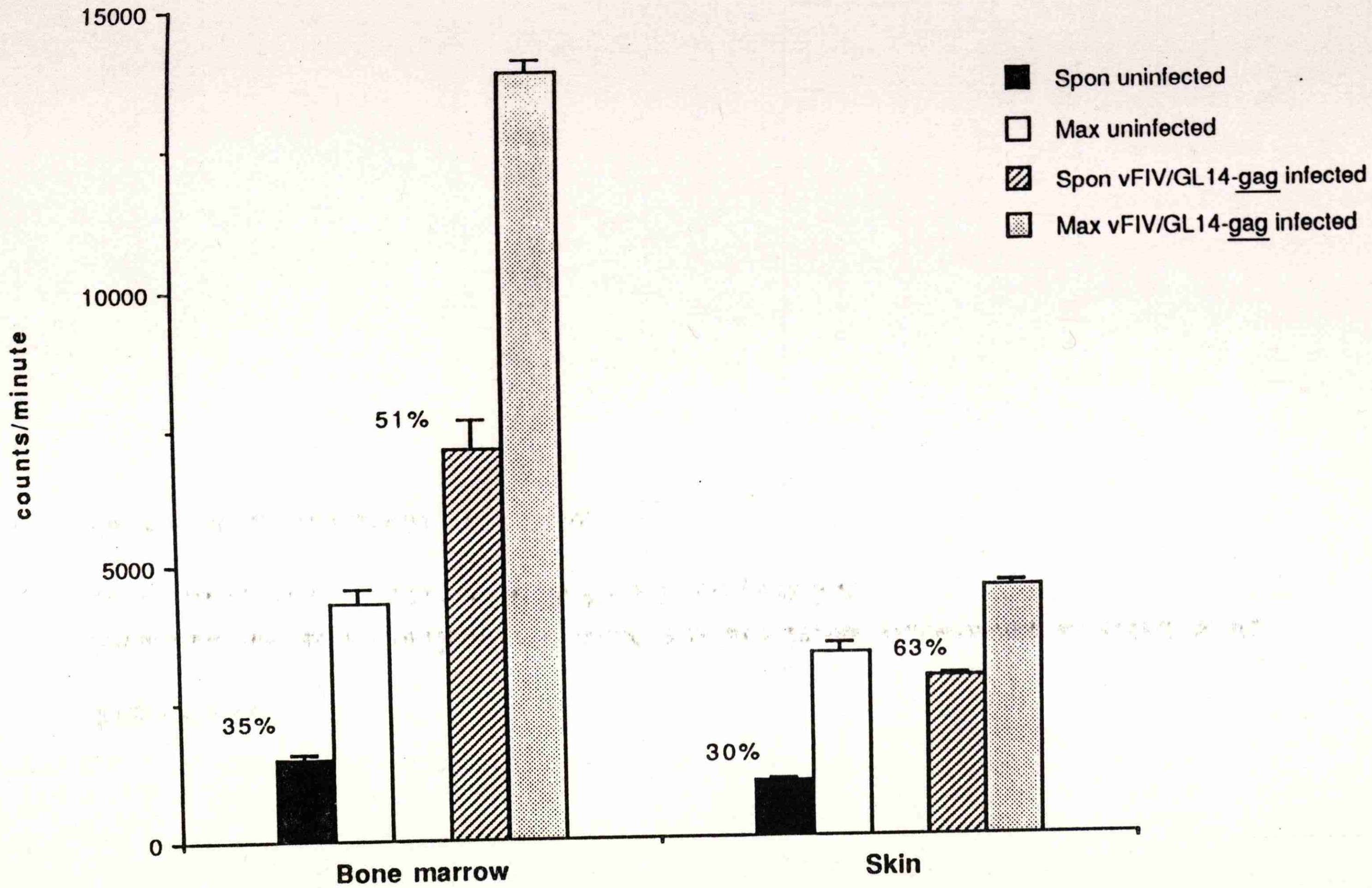


Figure 3.10

Figure 3.11

Spontaneous and maximum isotope release from fibroblasts labelled with ^{51}Cr overnight then infected with vaccinia virus

Figures indicate background ^{51}Cr release.

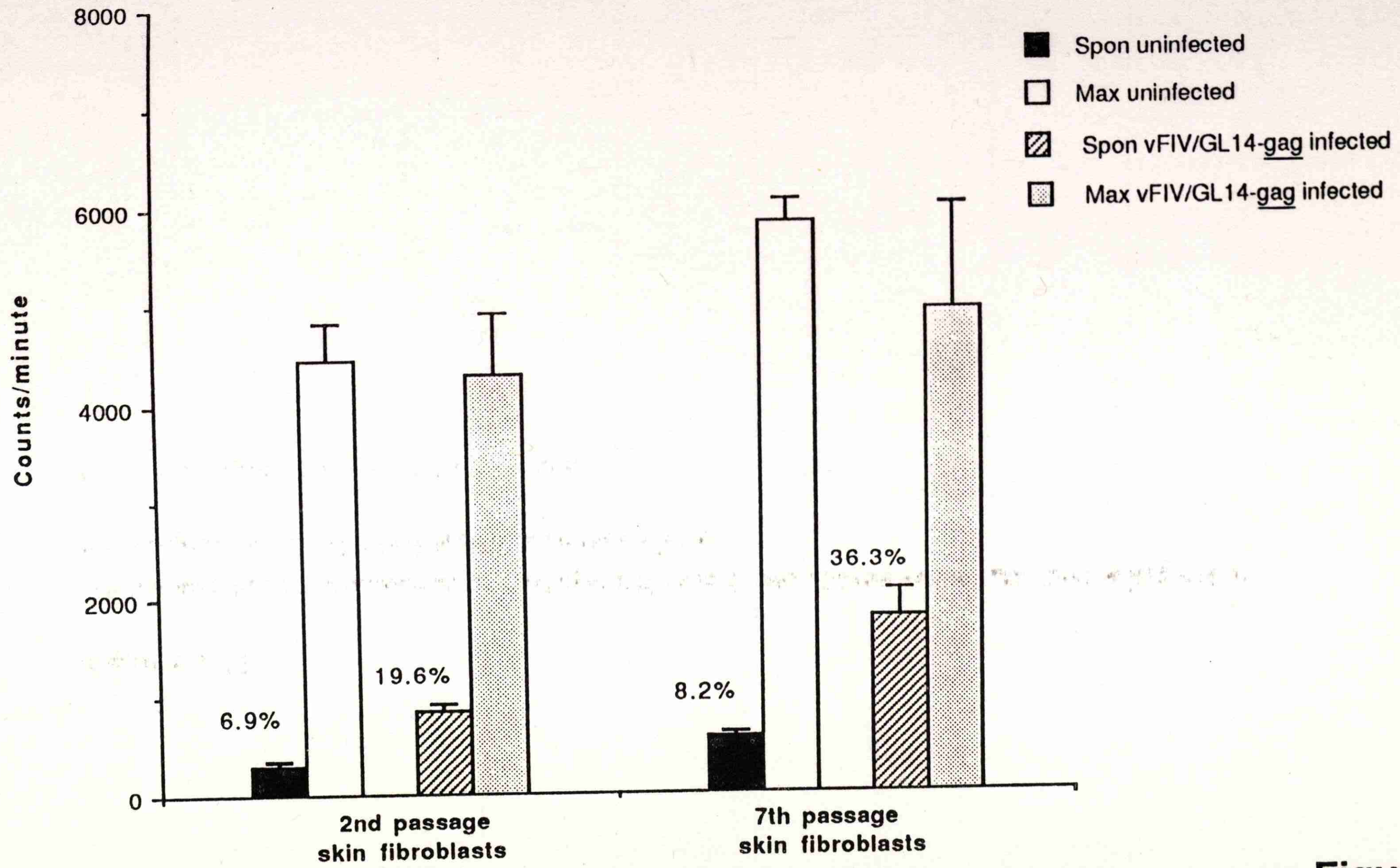


Figure 3.11

Figure 3.12

Effect of ^{51}Cr titration on spontaneous and maximum isotope release from vaccinia virus infected skin fibroblasts monolayers

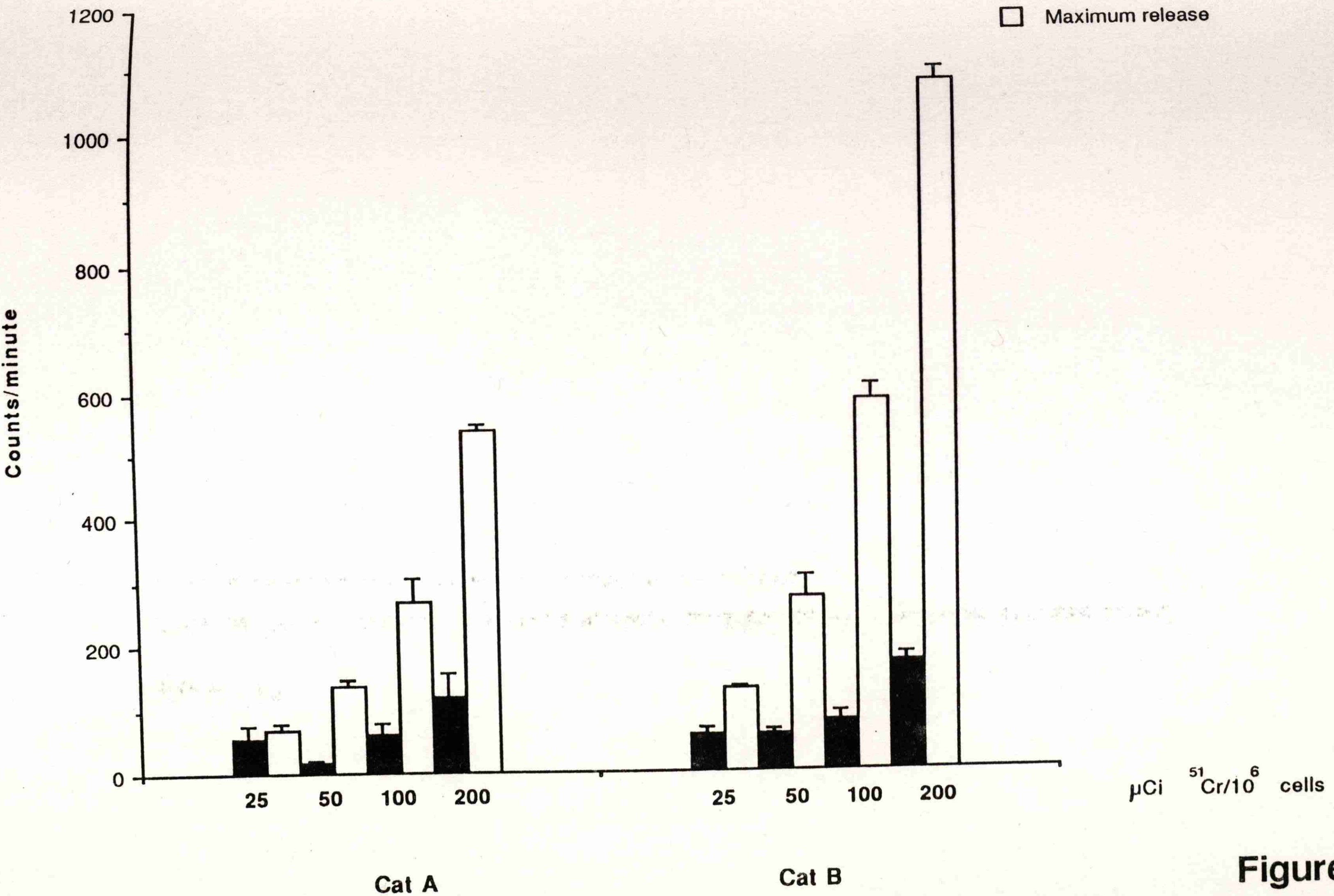


Figure 3.12

Figure 3.13

Effect of ^{51}Cr titration on background release from vaccinia virus infected skin fibroblasts monolayers

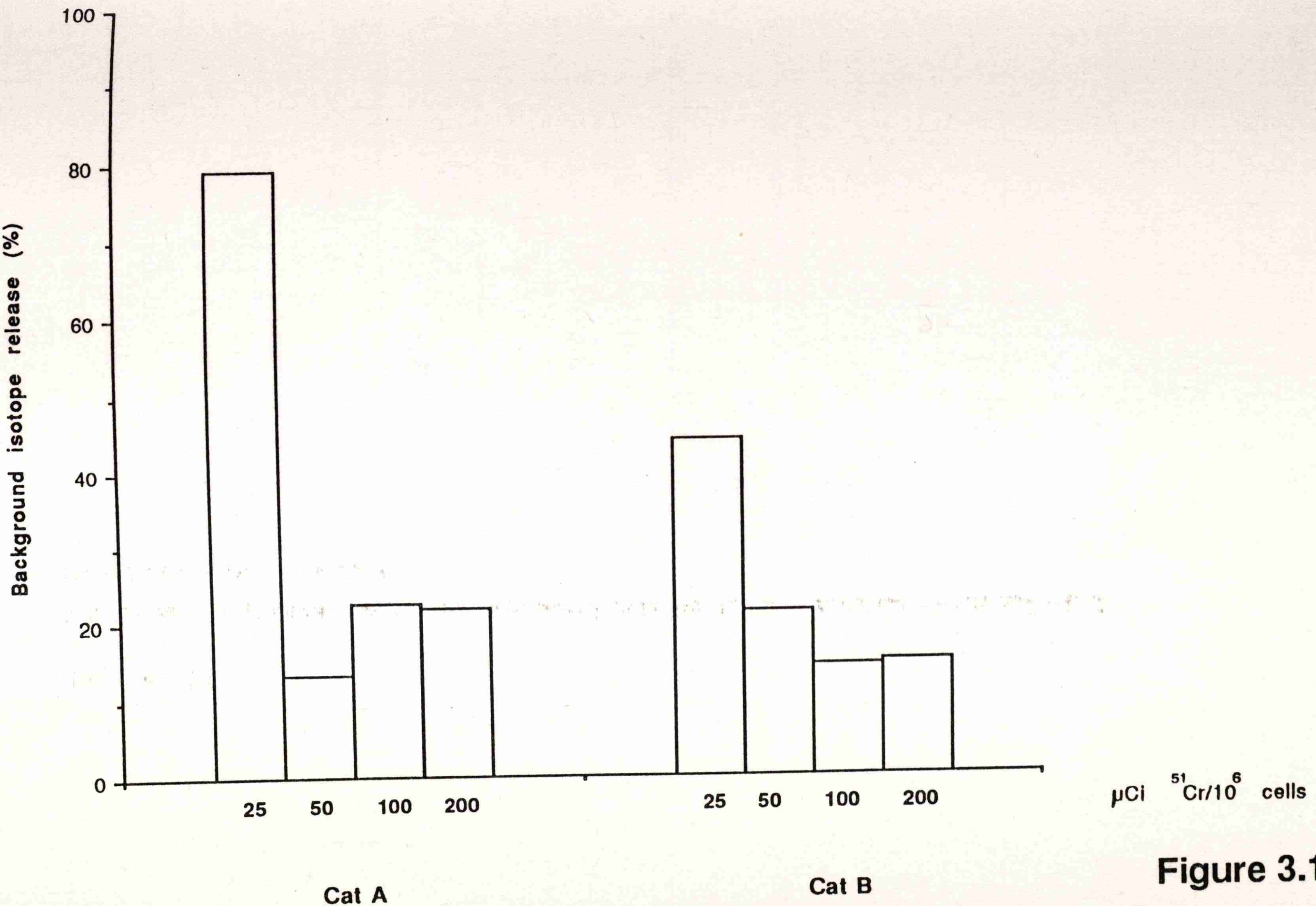


Figure 3.13

Table 3.1

Infectivity of recombinant vaccinia virus for different cell lines determined by plaque assay

pfu = plaque forming units. For details see text.

Cell line	Infectivity of FIV/GL14-gag (batch 004)
human TK-143	3.0×10^8 pfu/ml
BHK 21	2.5×10^8 pfu/ml
RK 13	2.1×10^8 pfu/ml

Table 3.1

3.4 DISCUSSION

There were several considerations governing the choice of target cells for the cytotoxicity assay. CTL-mediated lysis is characterised by MHC-restriction so that target cells must be MHC-matched to the effectors in the assay. MHC-typing is not yet available for the cat, although characterisation of MHC loci and alleles of the domestic cat is in progress (Winkler *et al.*, 1988; Yuhki *et al.*, 1989; Yuhki and O'Brien, 1993). Until MHC-typing is routinely available for this outbred population, the requirement for MHC-matched tissues must be fulfilled by using autologous target cells.

When selecting a cell type to use as a target, the capacity to grow well in tissue culture conditions was considered to be of paramount importance. Firstly, fragile and dying target cells "leak" ^{51}Cr raising the background level of isotope release in the assay. Secondly, replication of target cells in vitro reduces the number of biopsies required thus improving the welfare of the cats. Thirdly, target cells must be stored between assays so that the capacity to survive storage is essential.

In contrast to human and simian systems where herpesviruses (EBV and Herpesvirus papio respectively) are used routinely to immortalise B cells, providing a continuous source of autologous B-lymphoblastoid cell lines as targets for the assay (Miller *et al.*, 1971; Rabin *et al.*, 1977), no reliable transforming agent for feline cells in vitro has been identified. Hoshino and colleagues (1984) reported immortalisation of feline PBMC using HTLV-1 but in the absence of further reports and the potential hazards associated with working with HTLV-1 this possibility was not pursued here. The potential use of feline sarcoma virus (FeSV), an acute transforming retrovirus, was considered since FeSV isolates have been shown to be capable of transforming fibroblasts in vitro (Hardy, 1981; Rasheed *et al.*, 1982; Ziemiecki *et al.*, 1984; Dr C Grant, personal communication). However, investigations into the use of an agent for routine transformation of target cells proved ultimately to be unnecessary (see below).

Cell types considered for use as targets to detect FIV-specific cytotoxic cells were PBMC, renal fibroblasts, bone marrow fibroblasts and skin fibroblasts. Others have also used alveolar macrophages (Plata *et al.*, 1987), PHA lymphoblasts (Nixon, 1992) and murine tumour cells (P815) transfected with MHC and target antigen genes (Hoffenbach *et al.*, 1989; Chenciner *et al.*, 1989) to successfully detect HIV-specific CTL. The potential use of the latter in the cat awaits full molecular characterisation of feline MHC alleles. PBMC represent the most "natural" target since FIV has a broad tropism for PBMC, but not for fibroblasts, in vivo (English *et al.*, 1993). Feline PBMC have been used by other workers as targets (Song *et al.*,

1992) and PHA lymphoblasts have been used to detect HIV-1-specific CTL (Nixon, 1992). However, survival of PBMC in culture was found to be very variable often with high levels of cell death, particularly following storage (see Chapter 5). Therefore the use of fibroblasts as targets was investigated since these cells grew readily in culture. Initial investigations into the suitablity of renal fibroblasts as target cells were carried out. Renal fibroblasts grew well in culture, recovered well from storage and expression of FIV GAG could be detected following vFIV/GL14-*gag* infection of these cells (Figure 3.7). Renal fibroblasts have been used previously as targets in feline cytotoxicity assays (Tham *et al.*, 1987(a); Tham *et al.*, 1987(b)). However, unilateral nephrectomy is necessary to obtain these cells and it was considered that this invasive procedure should be avoided if possible, particularly since this would preclude the use of the assay in naturally FIV-infected cats which were showing clinical signs, for ethical reasons. Fibroblasts derived from bone marrow and skin were investigated for their suitabilty as targets. Skin fibroblasts were the final choice for targets for the assay based on superior growth characteristics, particularly the rapid and reliable recovery from storage by these cells. The minimally invasive biopsy procedure used to harvest skin fibroblasts was an added advantage. Skin fibroblasts have previously been employed as target cells for human (Quinnan *et al.*, 1982; Shepp *et al.*, 1988) and caprine (Lichtensteiger *et al.*, 1993) CTL assays.

In order to measure MHC class I restricted responses viral antigens must generally undergo endogenous processing in the target cells. This can be readily achieved by virus infection of target cells and this method has been used for detecting CTL with specificities for influenza and CMV (Nixon, 1992). Although some strains of FIV are able to infect certain feline fibroblast cell lines in vitro (Yamamoto *et al.*, 1988; Phillips *et al.*, 1990; Rigby *et al.*, 1993) fibroblasts are not known to become infected with FIV in vivo. The ability of FIV/GL14 to infect short term fibroblasts cell lines was not investigated here. Instead a recombinant vaccinia virus was used to achieve viral antigen expression in target cells since this method allows rapid, reliable, high level protein expression in a wide range of cell types (Mackett *et al.*, 1985; Moss, 1991). The use of recombinant technology also provides the potential to study CTL responses to individual proteins so that the viral targets of CTL activity can be identified. It has been shown for influenza virus specific CTL that effectors which lyse targets infected with recombinant virus invariably recognise influenza virus infected targets confirming the validity of this approach (Mills *et al.*, 1989).

The *gag* gene was chosen for insertion into the initial recombinant. The rationale behind this strategy was that GAG-specific CTL were considered the most likely

population to be readily measurable, an important consideration when designing the assay system. Internal viral proteins are often major targets for CTL responses (Rouse *et al.*, 1988) and anti-GAG CTL are a frequently detected in asymptomatic HIV-seropositive individuals (Lamhamedi-Cherradi *et al.*, 1992). Also since retroviral core proteins are generally well conserved, by using GAG as our target viral protein we might be able to use this recombinant to detect CTL raised against other laboratory and field isolates.

Initial problems experienced in growing the recombinant viruses were solved by using human TK-143 cells. Dounce homogenisation was generally used to harvest the virus although rapid freeze thawing was used for small volumes (<3ml). Similar yields were obtained using these 2 methods.

Plaque assay was not a suitable method for determining the infectivity of vFIV/GL14-*gag* in feline fibroblasts since no discrete plaques were seen when these fibroblasts were used as indicator cells. Although at lower dilutions, vaccinia virus cpe could be appreciated as an overall thinning of the monolayer, there was no obvious cut-off point so that titration by end-point dilution of viral cpe was not possible. Where vaccinia infectivity could not be determined by plaque assay, an alternative method had to be found because, despite the fact that there was little variation in infectivity for the cell lines used here (Table 3.1), the infectivity of a vaccinia stock can vary up to five-fold when assayed on different cell lines (Dr M Mackett, personal communication) so the infectivity of a batch for one cell type cannot be inferred from that obtained in another cell type.

Infectious centre assays were performed in an attempt to determine the infectivity of vFIV/GL14-*gag* for feline fibroblasts using TK-143 cells as indicator cells. The rationale was that progeny virus from each infected fibroblast would be seen as a dicrete plaque on the indicator cells. The results of this technique are not directly comparable to those of the plaque assay since infectious centre assays quantify the proportion of cells productively infected at a given dilution of stock virus which may be less than the total number of infected cells. This technique was unsuccessful in this study since free infectious virus was not removed from infected fibroblasts despite repeated washing. This was indicated by widespread viral cpe on indicator cells seeded with either test or control killed infected fibroblasts, but not those seeded with uninfected fibroblasts. A possible solution may have been to incubate infected cells with antibody to vaccinia virus to neutralise any remaining extracellular virus.

In the absence of data on the infectivity of vFIV/GL14-*gag* for target cells, expression of the GAG product was used to titrate virus. From the results of

immunoblotting it was decided to use vFIV/GL14-*gag* batch 004 at a dilution of 1:200 to infect target cells for the assays (Figure 3.9). Based on the infectivity of the vFIV/GL14-*gag* stock for human TK^{-}143 cells, the dose of virus used to infect the skin fibroblast target cells represents 7.5 pfu/cell. Streptavidin-peroxidase immunocytochemistry confirmed the suitability of this virus dose. Determination of the percentage of target cells expressing GAG product in the cytotoxicity assays may underestimate the proportion of cells which are potentially lysable by CTL since processed GAG antigens may appear at the cell surface before GAG antigens are detectable by immunocytochemistry. Using product expression in target cells to titrate virus is more relevant to their future use in the assay than infectivity but the calculation of infectivity allows for comparison between experimenters.

The standard method for labelling targets to detect murine and human CTL is with ^{51}Cr (Brunner *et al.*, 1968). Labelling of target cells with ^{51}Cr is carried out by incubating with $Na_2{}^{51}CrO_4$ (sodium chromate) which enters the cells by passive diffusion and binds to cytoplasmic proteins, retarding spontaneous leakage. In most cases the ^{51}Cr is reduced from $^{51}Cr^{6+}$ to $^{51}Cr^{3+}$. The latter enters and leaves cells much more slowly than the former so that ^{51}Cr liberated from target cells during the assay is not significantly reincorporated by undamaged cells (Clark, 1991). The objectives of target cell labelling are to achieve the highest uptake and the lowest leakage of isotope from the smallest number of target cells. There is an intrinsic error in counting radioactive decay and this error decreases as the absolute counts increase, hence high maximum counts are favoured. The background level of ^{51}Cr release, which is used to determine the validity of the assay (Chapter 5), is calculated by expressing the spontaneous release as a percentage of maximum release so that spontaneous release should be as low as possible. Low numbers of target cells in the assays are desirable to maximise the lymphocyte:target (L:T) ratio. The conditions for ^{51}Cr labelling which gave the highest maximum release and lowest spontaneous release from target cells were systematically investigated. To this end target cells were incubated for 4 hours (the intended duration of the cytotoxicity assays) in detergent or in medium. In later investigations, cells from which spontaneous ^{51}Cr release was being measured were incubated in complete RPMI rather than complete MEM alpha, since this condition mimicked the intended assay procedure more closely since effector cells would be added in RPMI.

When fibroblasts were labelled in suspension, the maximum isotope release from vaccinia virus infected cells was much greater than from the same number of uninfected cells (Figure 3.10). This was surprising since the final concentration of detergent used (2.5%) would be expected to lyse all the cells (Taylor *et al.*, 1987).

In the light of the results from infectious centre assays, where it was shown that repeated washing was inefficient at removing vaccinia virus from the cell surface, it may be that extracellular virus is responsible for the retention of ^{51}Cr by vaccinia virus infected cells. This problem was solved by labelling cells with ^{51}Cr before vaccinia virus infection. A convenient way to do this was by overnight incubation of fibroblasts with the isotope. This method had the added advantage of spreading the workload for each assay over 2 days. Although incubation of cells overnight prior to the assay may have resulted in multiplication or loss of cells, thus affecting L:T ratio in the assays, it was considered that any error here would be relatively constant and acceptable. Uninfected feline skin fibroblasts, both second and seventh passage cells, gave very low background ^{51}Cr release when labelled in this way (Figure 3.11). vFIV/GL14-*gag* infection had the effect of increasing background ^{51}Cr release from the target cells. Inspection of vFIV/GL14-*gag* infected targets revealed rounding of cells and disruption of the monolayer after approximately 2 hours whereas uninfected monolayers remained intact throughout the incubation period. It is likely that the virus inoculum causes cell damage which is reflected as higher spontaneous ^{51}Cr release from infected cells. Higher dilutions of virus may reduce the background activity while still allowing sufficient GAG expression in the target cells (Chapter 5).

Skin fibroblasts from cat A142 (seventh passage) gave higher absolute counts than F23 (second passage) (Figure 3.11). This result is not an effect of inter-plate variation since a single assay plate was used in this experiment. This result may be due to individual variation and/or the difference in passage number of the fibroblasts but these possible causes cannot be distinguished from the results of this experiment. Individual variation in isotope release was shown in a separate experiment (Figures 3.12 and 3.13). Results for cytotoxicity assays are calculated in percentage terms to counteract the effect of individual variation, allowing comparison between animals within an assay. Expressing results as a percentage also allows interassay comparisons to be made. It was decided to use skin fibroblasts at the lowest passage number possible for the cytotoxicity assays in case high passage number contributed to the higher background release from A142 skin fibroblasts in the previous experiment (Figure 3.11).

The concentration of ^{51}Cr required to label fibroblasts was titrated. In general, increasing the concentration of ^{51}Cr resulted in an increase in both spontaneous and maximum isotope release from the fibroblasts (Figure 3.12). The lowest values for background ^{51}Cr release were achieved using 50 or 100μCi ^{51}Cr/10^6 cells depending on the animal (Figure 3.13). It was decided to use 100μCi/10^6 cells for

labelling target cells in the cytotoxicity assays since this concentration gave higher absolute counts and therefore lower error, as noted earlier.

Other workers have found it necessary to use the more active radioisotope indium-111 (^{111}In) to label target cells to achieve acceptable levels of isotope release from small numbers of cells so that the L:T ratio can be increased where effector cells are limiting (Brown *et al.*, 1989; Song *et al.*, 1992). Although the availability of effector cells was potentially a limiting factor in our experiments, the use of this more hazardous isotope was avoided here since excellent labelling of skin fibroblast targets could be achieved with ^{51}Cr.

The observations made in these experiments suggested a protocol for overnight ^{51}Cr labelling of target cells followed by vFIV/GL14-*gag* infection which was used in the cytotoxicity assays (Chapter 5). The suitability of the target cell system devised here was confirmed in Chapter 5.

CHAPTER 4

DEVELOPMENT OF AN ANTIGEN PRESENTING SYSTEM FOR THE IN VITRO GENERATION OF SECONDARY FIV-SPECIFIC CTL

4.1 INTRODUCTION

Virus-specific cytotoxic effector cells have been derived from the lymph nodes, spleen and PBMC of virus-infected mice and humans (Rouse *et al.*, 1988; Clark, 1991). Cytotoxic activity from freshly isolated cells is usually low or indetectable as most effector cells are in the precursor state. pCTL require restimulation in vitro to generate secondary virus-specific CTL responses which are more readily detected in the CTL assay. The conditions necessary to generate secondary CTL responses vary between systems (Dunlop and Blanden, 1976; Koszinowski and Simon, 1979; Nixon *et al.,* 1988; Van Baalen *et al.*, 1993; Rouse *et al.*, 1988). In general, this is a two-stage process involving firstly activation, followed by expansion and differentiation (Figure 4.1). The activation of pCTL is generally antigen-dependent, requiring presentation of appropriately processed antigen by class I MHC molecules, although non-specific mitogen activation has been successful in some circumstances (Zeidner *et al.*, 1993). Activated pCTL upregulate the expression of cell surface receptors for IL2 and other cytokines. The addition of these cytokines triggers clonal expansion and maturation into cytotoxic effector cells.

In this study cytotoxic effector cells were derived from circulating PBMC from cats primed in vivo by experimental FIV infection. This chapter describes the development of an antigen presenting system for generating secondary FIV-specific CTL from circulating precursors. Due to the requirement for class I MHC presentation of FIV antigens to pCTL, various types of virus-infected "stimulator" cells were prepared. Special considerations in the FIV system are firstly, the availability of lymphocytes. Blood sampling from experimental cats must not exceed 10% of the blood volume each month. It was anticipated that most of the available lymphocytes from infected cats would be used as the bulk effector population in order to achieve high L:T ratios in cytotoxicity assays. Stimulator cells must, therefore, be prepared either from lymphocytes taken prior to experimental infection or from the same blood sample as the effector cells. FIV-infection is often accompanied by leucopenia (Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988; Callanan *et al.*, 1992 (b)) so the efficient use of available lymphocytes is particularly important. Secondly, FIV can infect both $CD4^+$ and $CD8^+$ lymphocytes in vitro resulting in decreased cell viability (Brown *et al.*, 1991). FIV must be inactivated in the final preparation since the cytotoxic and helper populations are potentially suceptible to viral cpe. Similarly, vFIV/GL14-*gag* is cytopathic for lymphocytes and must be inactivated.

Stimulator cell preparations consisted of mitogen-activated PBMC from FIV-infected cats or SPF-derived PBMC which were infected with either FIV or with vFIV/GL14-*gag* in vitro. Antigen expression was investigated in these different cell types and their potential suitability for the generation of lytically active effectors from circulating FIV-specific pCTL is discussed.

4.2 MATERIALS AND METHODS

4.2.1 FIV antigen expression following mitogen stimulation of PBMC from FIV-infected cats

PBMC were isolated from 3 adult male cats infected with FIV/PET at 19 weeks post-infection (2.2.1). Cells were cultured in complete RPMI supplemented with 100 U/ml human rIL2 in 1.5ml cultures in 24-well tissue culture plates. Cells were stimulated with ConA at 5 or 10 μg/ml for 24, 48 or 72 hours, washed and resuspended in complete RPMI with 100 U/ml human rIL2. A fraction of PBMC were left unstimulated as a control. Cells were maintained at 2 x 10^6/ml throughout the experiment. Supernatant fluids were harvested on days 9 and 13 after isolation and stored at -20°C prior to FIV p24 antigen detection by ELISA (2.2.5). On day 13, cells were harvested into lysis buffer for immunoblotting (2.2.7). Immunoblots were stained with P1 serum (2.2.7) which had been preabsorbed with Q201 cells at 4°C overnight to reduce background reactivity due to anti-cellular antibodies.

4.2.2 Preparation of FIV-G14 infected, paraformaldehyde fixed lymphoblasts

Lymphoblasts were generated from SPF cats E3, F22 and F23, as described in Chapter 2 (2.2.4). On day 6, 40ml cultures of each lymphoblast line were inoculated with 5ml of FIV/GL14 stock (2.2.6). After 24 hours, cells were washed and resuspended in complete RPMI at 2 x 10^6/ml. Cultures were monitored daily and maintained by feeding and adding fresh cells as necessary. From 4 days after in vitro infection supernatant fluids were tested for FIV p24 production. When the cultures were found to be positive for FIV p24 antigen, cells were recovered by centrifugation and washed twice in 20ml of PBS. Cells were then fixed by resuspending in 1ml of 0.1% paraformaldehyde in PBS in polystyrene round-bottomed tubes (Falcon 2054, Becton Dickinson, UK) and incubating overnight. These tubes were used in preference to universal containers as the latter resulted in cell loss due to adhesion of fixed cells to the container. Fixed lymphoblasts were then washed 6 times in 3ml of PBS, resuspended in RPMI containing 20% FCS and stored in liquid nitrogen vapour phase. Samples of FIV-infected cells pre- and post-fixation and fixed uninfected cells were analysed by immunoblotting as described above.

4.2.3 Preparation of vFIV/GL14-*gag* infected, paraformaldehyde fixed lymphoblasts

Lymphoblasts were generated from SPF cats E3, F23 and F22 (2.2.4). After 4-6 days of culture, lymphoblasts were washed twice in 20ml of PBS and viable cell counts performed. Cells were recovered by centrifugation and infected with vFIV/GL14-*gag* at 10 pfu/cell in 2% RPMI in a total volume of 50µl-100µl for 1 hour at 37°C (Note that the infectivity of vFIV/GL14-*gag* stock was determined on human TK-143 cell line, see Chapter 3). A portion of cells was left uninfected as a control. At the end of virus adsorption period, cells were washed 4 times in PBS then incubated for a further 2.5 hours at 37°C. This incubation period has previously been shown to be sufficient for p55 expression in fibroblast cells (Chapter 3). After washing in PBS, viable cell counts were performed by trypan blue dye exclusion. Two million infected and uninfected cells were pelleted and resuspended in 10µl of lysis buffer for immunoblot analysis. The remaining cells were fixed in 0.1% paraformaldehyde overnight then washed in PBS as described above (4.2.2.). The cells were counted, a further sample (2×10^6 cells) was taken for immunoblotting and the remainder were stored in liquid nitrogen in RPMI with 20% FCS or at 4°C in complete RPMI. Cell lysates were analysed by immunoblotting as described above.

In addition, a sample of vFIV/GL14-*gag* infected lymphoblasts was prepared as above except that the incubation with virus was allowed to continue for 24 hours before fixation to determine whether cleavage of the GAG polyprotein precursor could be detected.

4.3 RESULTS

4.3.1 FIV antigen expression in ConA activated PBMC from FIV-infected cats

Culture fluids were harvested from ConA-activated PBMC after 9 and 13 days of culture and tested for the presence of FIV p24 by ELISA. The results are summarised in Table 4.1. By 13 days of culture all but 1 sample tested positive for FIV. At the end of the experiment (day 13), cells were harvested for immunoblot analysis. A band at 55K was found to correspond to a positive ELISA result in all cases.

4.3.2 FIV antigen expression in lymphoblasts following FIV-infection in vitro and fixation

Lymphoblasts were generated from SPF cats and infected with FIV/GL14 in vitro. Culture fluids were positive for FIV p24 on ELISA from 5 days post-infection and early viral cpe could be seen as syncytia formation at this stage.

On immunoblot, a virus-specific band at 24K was seen in FIV-infected, fixed lymphocytes (Figure 4.2). Other virus-specific bands in the FIV-infected cell preparations were weak or absent. A second band, running just behind p24, was seen in fixed, infected lymphocytes. This band was absent from the negative and positive control lanes.

4.3.3 vFIV/GL14-*gag* infection and fixation of lymphoblasts

Feline lymphoblasts were infected in vitro with vFIV/GL14-*gag*. More than 95% of the cells were viable by trypan blue exclusion at the end of the incubation with virus. Cells were then fixed, washed and counted. Approximately 80% of the starting population was present in the final preparation.

An immunoblot of cell lysates taken at different stages of preparation is shown in Figure 4.3. A band was seen at 55K (p55) in lanes containing both fresh and fixed vFIV/GL14-*gag*-infected lymphoblasts. The appearance of this protein band on immunoblot was unaffected by fixation since it ran at the same level as p55 in the positive control lane which was prepared from an unfixed, FIV-infected feline lymphocyte line, Q201 (2.2.8). Recombinant protein represented the principal component in lanes containing vFIV/GL14-*gag* infected cells. The cellular protein bands seen in the uninfected control cells were much reduced in infected cell preparations. A band at approximately 24K in fixed, infected cells was not virus-specific since it was also seen in uninfected cells.

Overnight incubation of vFIV/GL14-*gag* infected lymphoblasts did not result in cleavage of p55 into its component proteins as evidenced by a single virus specific band at 55K on immunoblot (Figure 4.4).

Figure 4.1

Two-stage model of the generation of secondary CTL from resting precursor cells

The maturation of pCTL into cytotoxic effector CTL requires 2 signals. Activation is achieved by interaction of the T cell receptor/CD3 complex with the appropriate antigen presented by class I MHC molecules. Activated cells express cell surface receptors for IL2 and other cytokines. The addition of IL2 and possibly other molecules triggers the expansion and differentiation of activated CTL into lytically active cytotoxic effector cells.

Two-stage model of the generation of secondary CTL from resting precursors

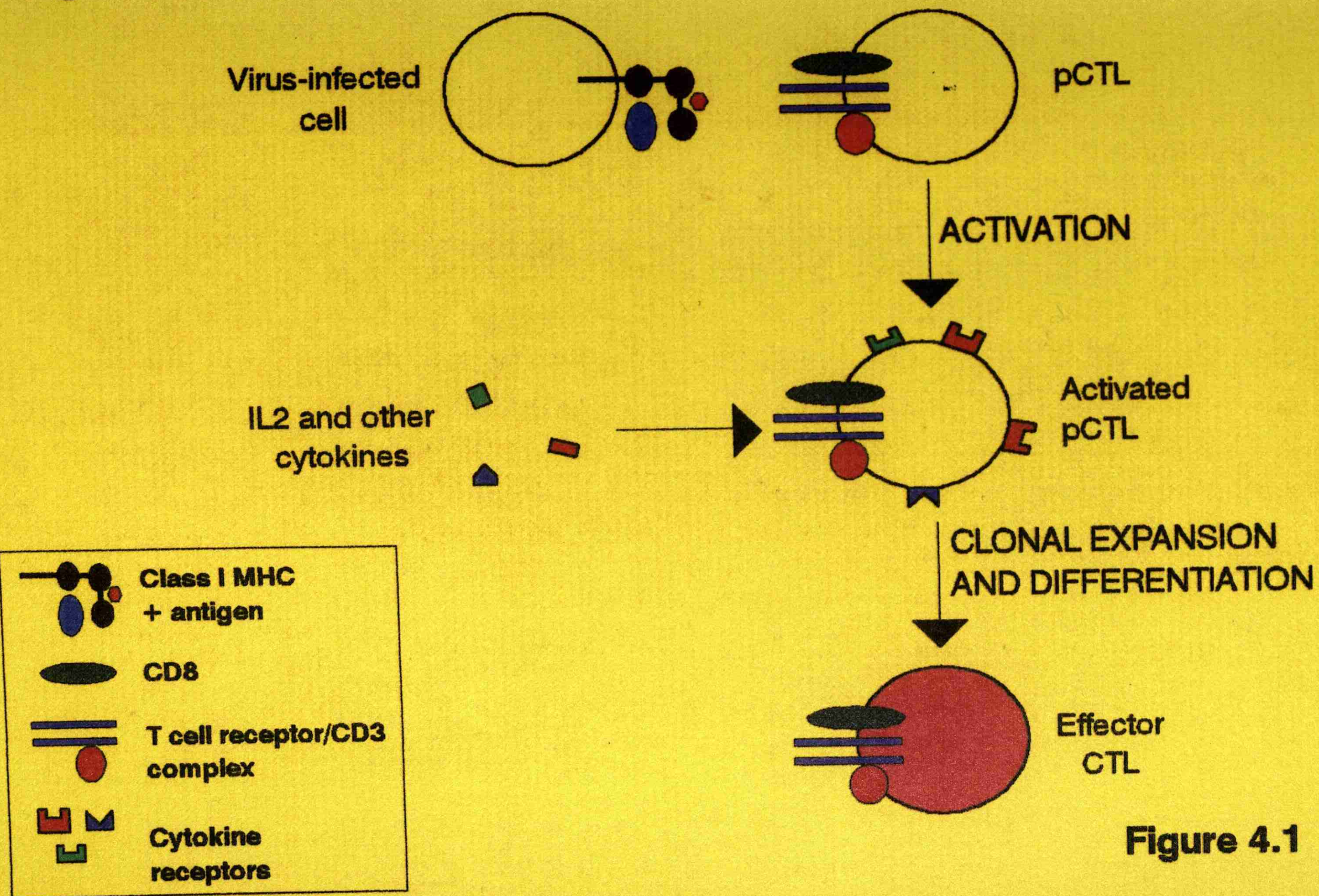


Figure 4.1

Figure 4.2

Virus antigen expression in lymphoblasts infected with FIV in vitro

Lanes a and b contain FIV/GL14 infected, fixed lymphoblasts from SPF cats F23 and E3 respectively. Lane c (negative control) contains uninfected, fixed lymphoblasts. Lane d (positive control) contains FIV/GL14 infected Q201 cells. A FIV specific band is seen at 24K in lanes a and b. The band travelling immediately behind it is believed to be an artefact of fixation.

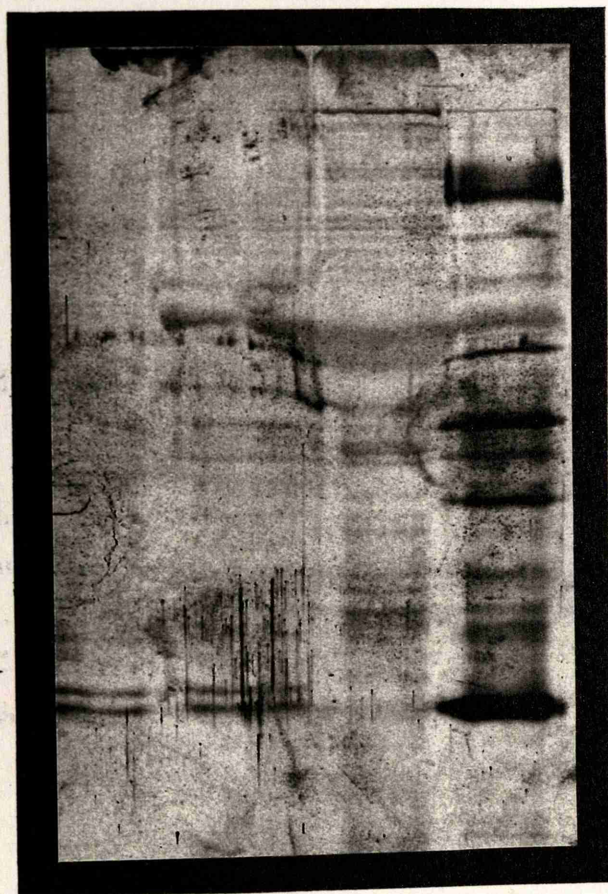


Figure 4.2

Figure 4.3

FIV GAG expression in lymphoblasts infected with vFIV/GL14-*gag* in vitro

Lane a (negative control) contains uninfected lymphoblasts. Lanes b, c and d contain fresh, vFIV/GL14-*gag*-infected lymphoblasts. Lanes e, f, g and h contain fixed, vFIV/GL14-*gag*-infected lymphoblasts. Lane i (positive control) contains FIV/GL14 infected Q201 cells. Recombinant FIV-GAG is seen as a band at 55K in fresh and fixed vFIV/GL14-*gag*-infected lymphoblasts, lanes b-h. Normal cellular protein synthesis is dramatically reduced in vaccinia virus-infected cells.

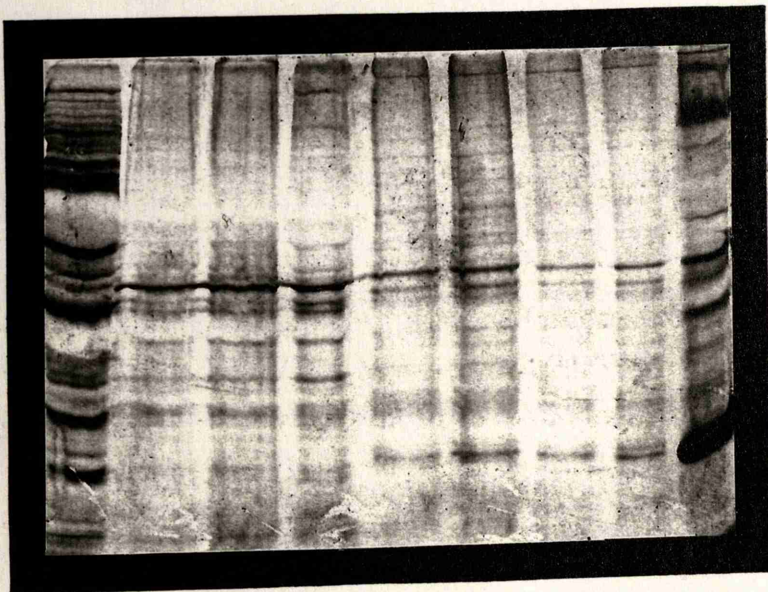


Figure 4.3

Figure 4.4

FIV GAG expression in lymphoblasts following extended incubation with vFIV/GL14-*gag* in vitro

Extended incubation with vFIV/GL14-*gag* was carried out to determine if cleavage of p55 into GAG products occurred. Lane a (positive control) contains FIV/GL14 infected Q201 cells. Lane b is empty. Lane c contains lymphoblasts infected with vFIV/GL14-*gag* for 3 hours prior to fixation. Lane d contains the same lymphoblasts infected with vFIV/GL14-*gag* for 24 hours prior to fixation. Lane e (negative control) contains uninfected lymphoblasts. The only virus specific band in lanes c and d is at 55K showing that the GAG product is not cleaved in vFIV/GL14-*gag*-infected lymphoblasts after a 24 hour infection.

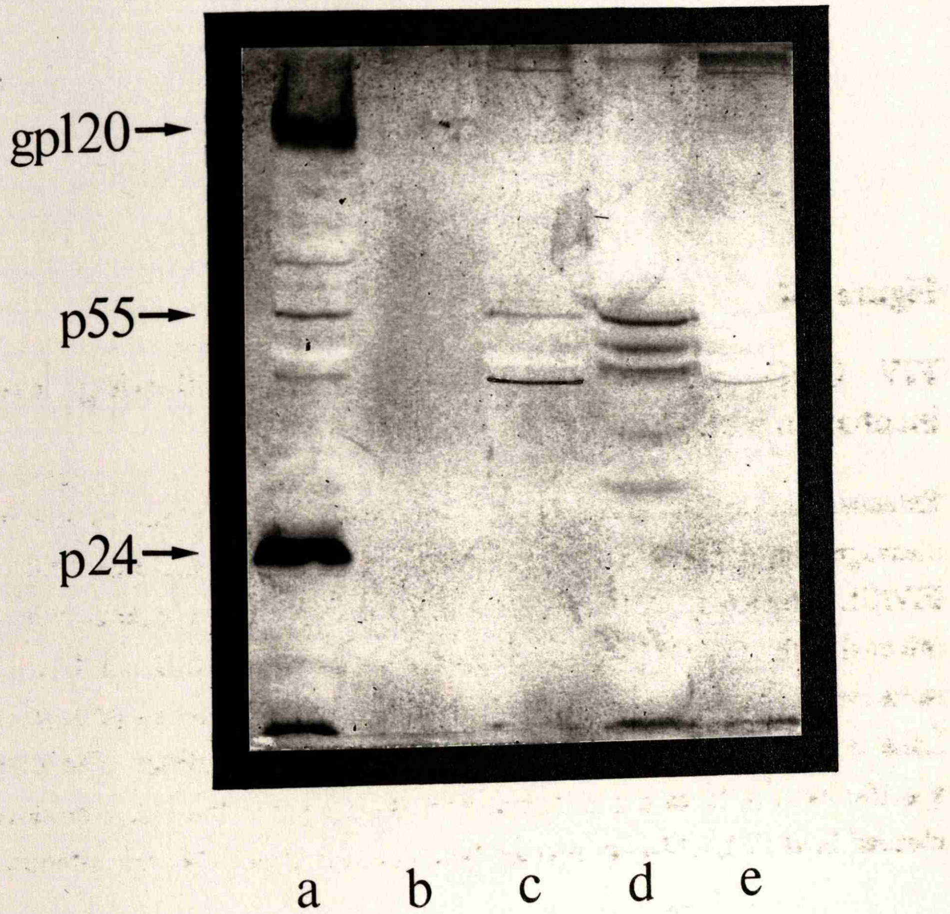


Figure 4.4

Table 4.1

FIV antigen production by PBMC from FIV-infected cats following ConA stimulation

PBMC from FIV/PET infected cats were stimulated with ConA to determine the optimum concentration and duration of stimulation for early antigen expression. FIV p24 antigen expression was detected in culture fluid by ELISA. Absorbance values were measured at 650nm.

- = absorbance < assay cutoff

+ = absorbance > assay cutoff

++ = absorbance > 2x assay cutoff

NT = not tested

Sample	ConA (µg/ml)	Duration of ConA stimulation (hours)	p24 ELISA 9 days	p24 ELISA 13 days
A83	5	24	-	+
"	5	48	-	+
"	5	72	-	++
"	0	-	-	++
A84	10	24	+	++
"	10	48	-	++
"	10	72	-	++
"	5	24	+	++
"	5	48	-	++
"	5	72	+	++
"	0	-	-	+
A85	10	24	-	+
"	10	48	-	+
"	10	72	-	-
"	5	24	+	+
"	5	48	-	+
"	5	72	-	+
"	0	-	-	NT

Table 4.1

4.4 DISCUSSION

The aim of the experiments described in this chapter was to devise a suitable source of endogenously processed FIV antigens for the stimulation of pCTL in vitro. Mitogen activated, autologous lymphoblasts have been used successfully for the stimulation of secondary HIV-specific CTL responses from circulating pCTL from HIV-infected humans (Nixon *et al.,* 1988). The possibility of using this technique to stimulate secondary FIV-specific CTL responses was investigated. The results suggested that the onset of FIV p24 expression following ConA activation depended not on the stimulation protocol, but rather on the individual animal. Lymphoblasts from cat A85 produced virus most readily and unstimulated cells from this cat performed at least as well as mitogen activated cells from all 3 animals. Even after 13 days in culture, FIV p24 could not be detected in all cultures. Prolonged incubation of lymphocytes is undesirable because it likely to result in decreased cell viability. In addition, the time required to generate stimulator cells by mitogen activation would preclude the harvest of stimulator and effector cells from the same blood sample.

The pattern of FIV production following mitogen stimulation was determined for lymphoblasts only from cats infected with FIV/PET and may be different for other FIV isolates. However, it was intended to devise a method of stimulator cell production which could be applied to a range of FIV isolates since the assay would be used in naturally-infected cats. In addition, FIV/PET-specific CTL responses are of particular interest since an experimental vaccine based on this isolate has been shown to be protective against homologous challenge (Yamamoto *et al.,* 1991; Yamamoto *et al*., 1993).

The reason for individual variation in the onset of virus production following mitogen stimulation of PBMC is not clear. Although isolation of FIV generally becomes more difficult after the first few weeks of infection (Dr MJ Hosie, personal communication), animals in this experiment were all at the same stage of infection. The observed differences may reflect individual differences in the immune response to the virus. In particular a strong cell mediated immune response may delay virus production in vitro. $CD8^+$ cells have been shown to suppress HIV, SIV and FIV replication in vitro (Walker *et al.,* 1986; Kannagi *et al.,* 1988; Brinchmann *et al.,* 1990; reviewed in Mackewicz and Levy 1992; Jeng *et al.*, 1993(a) and 1993(b)).

A given set of conditions does not allow us to predict when lymphocytes will be producing virus so that stimulator cells prepared by mitogen activation would have to be repeatedly tested for virus production. For this reason, coupled with the

prolonged culture periods, the use of mitogen activated autologous PBMC from infected cats as stimulator cells was not considered further.

In an attempt to standardise antigen presentation by stimulator cells, it was decided to investigate the use of lymphoblasts infected in vitro with FIV/GL14. FIV inactivation was achieved by paraformaldehyde fixation. Paraformaldehyde has been used for the inactivation of FIV in protective FIV vaccines at 1.25% (Yamamoto *et al.*, 1991) although virus is inactivated by a 0.1% paraformaldehyde solution (Dr MJ Hosie, personal communication). There are several precedents for the use of fixed cells for in vitro stimulation of lymphocytes (Koszinowski and Simon, 1979; Lightbody and Kong, 1978; Dunlop and Blanden, 1976; Bubbers and Henney, 1975; Van Baalen *et al.*, 1993). Others have used irradiation for this purpose (Song *et al.*, 1992). In the present study, fixed, FIV-infected lymphoblasts could be prepared by 13 days from the time of isolation. Again, the time taken to generate these cells precludes the production of stimulators from the same blood sample as the effector cells. The preparation of stimulator cells in batches prior to experimental infection was considered. However, as noted by others (Song *et al.*, 1992; Dr MJ Hosie, personal communication), it was necessary to add fresh cells every few days to maintain FIV-infected cultures. Therefore, multiple bleeds were necessary to maintain lymphoblasts until p24 positivity was achieved and final yields were low so that these cells would have to be produced in batches rather than as a single stock.

Semi-quantitative immunoblot analysis suggests that the level of FIV protein expression in lymphoblasts prepared in this way was low. Only the capsid protein p24 was consistently present although other virus specific bands, particularly p55 were sometimes seen. A second band which migrated just behind p24 in SDS-PAGE was seen in fixed, FIV infected samples. The reason for this artefact is not clear but it may be that fixation alters the pH such that a second species of this protein is produced which travels more slowly.

The preparation of fixed, vFIV/GL14-*gag*-infected lymphoblasts for use as stimulator cells was investigated. Preliminary experiments showed that directly isolated PBMC did not produce p55 detectable on immunoblot after exposure to vFIV/GL14-*gag*. However, lymphoblasts infected with the recombinant produced p55 readily, while cellular protein synthesis was dramatically reduced. The mechanism of this decreased cellular protein synthesis in vaccinia virus-infected cells is unclear (Moss, 1990). This method of stimulator cell production proved to be simple, rapid and repeatable and was very efficient in terms of lymphocyte numbers. In addition, vFIV/GL14-*gag* infection of lyphoblasts allows for more

specific stimulation than FIV-infection since only FIV-GAG specific clones will be activated. For these reasons, fixed vFIV/GL14-*gag* infected lymphoblasts were used as the source of antigen for restimulation of pCTL.

The use of fixed vFIV/GL14-*gag* infected skin fibroblasts as stimulator cells should be investigated. PBMC could be incubated on fixed monolayers of cells during the in vitro culture period. This would have advantages over the use of vFIV/GL14-*gag* infected lymphoblasts for stimulation since many skin fibroblasts can be harvested from a single biopsy and time could be saved by preparing 6-well plates containg fixed monolayers in advance.

The source of antigen for restimulation may affect the target antigens recognised. This has been demonstrated recently for bovine herpesvirus (BHV) type 1-specific CTL using either fixed vBHV infected fibroblasts or UV inactivated BHV for restimulation (Denis *et al.*, 1993). The authors suggested that this may be due to different processing of viral glycoproteins in the stimulator cells. In our system this problem is unlikely to arise since the same cell type would be used for restimulation and as a target so that identical processing would be expected. In addition, our target antigen does not require glycosylation.

In these experiments, the assumption was made that detection of FIV p24 by ELISA and/or by immunoblotting indicated that lymphoblasts were presenting endogenously processed FIV antigens. This may result in an underestimate of potential stimulating capacity since class I antigen presentation is likely to occur before detectable levels of protein are present. Also, fragments of stimulator cells have been shown to be capable of activating pCTL (Engers *et al.*, 1975; Dunlop and Blanden, 1976; Finberg *et al.*, 1978). Ultimately, the ability of a stimulator cell preparation to present antigen effectively to pCTL can only be assessed with a functioning CTL assay. The use of stimulator cells for generating secondary CTL responses is therefore dealt with in Chapter 5.

CHAPTER 5

DETECTION OF CYTOTOXIC EFFECTOR CELLS FROM CATS EXPERIMENTALLY INFECTED WITH FIV

5.1 INTRODUCTION

The object of this study was to develop a ^{51}Cr release cytotoxicity assay which could be used to measure FIV-specific CTL responses in infected cats. A total of 3 cats were used in this experiment. Two were infected with FIV/GL14 (E3 and F23) and 1 remained uninfected as a control (F22). The assay utilised the autologous target cell system and the antigen presentation system developed in the experiments described in Chapters 3 and 4 respectively.

5.2 MATERIALS AND METHODS

5.2.1 Experimental animals

Three outbred, SPF domestic shorthair cats were used (Table 5.1). Cats E3 and F23 were inoculated with 1ml of a 1:100 dilution of FIV/GL14 (batch number 001) in sterile PBS by intraperitoneal injection. Cat F22 was given PBS alone as a control. Cats were housed in a purpose built facility with FIV/GL14 infected cats separated from the uninfected cat. Clinical examinations were carried out each time blood samples were taken.

5.2.2 Serology

The presence of circulating antibodies to FIV was detected from stored plasma (5.2.4) by ELISA and by indirect immunofluorescence assay (IFA). Plasma samples were also tested for the presence of FIV p24 antigen by ELISA (2.2.7.).

5.2.2.1 Detection of anti-FIV antibodies by ELISA

Capture antigens in the ELISA were either recombinant FIV p17 or FIV p24 (Reid *et al.*, 1991) or synthetic multiple antigenic peptides (MAP) corresponding to V3 and TM2 (Avrameas *et al.*, 1993) regions of FIV ENV (Figures 5.1 and 5.2). These regions of ENV are conserved between FIV isolates GL8, GL14 and PET.

Microtitre plates (Immunlon 1, Dynatech Laboratories, USA) were coated overnight at 4°C with proteins at 10µg/ml in coupling buffer (0.1M $NaHCO^3$, 10mM EGTA, pH 9.6) and 100µl was added to each well. Plates were washed 4 times with Tris-buffered saline (TBS; 0.15M NaCl, 50mM Tris, pH 7.6)/ 0.5% Tween-20, then blocked for 1 hour with 100µl TMT (5% goat serum, 2% skimmed milk powder in TBS/0.5% Tween-20). After washing the plates 3 times, 100µl of serum (diluted 1:20 with TMT) was added for 1 hour. Plates were washed 5 times then 100µl goat anti-cat IgG conjugated to horseradish peroxidase (1:300 in TMT) was added for 1 hour. Plates were washed 5 times and then developed with 10µl

TMB (3,3',5,5'-tetramethylbenzidine, Kirkegaard and Perry Laboratories Inc., Maryland, USA) for 5-10 minutes. The reaction was stopped with 0.1M hydrofluoric acid and the O.D. was read at 650nm using a microplate reader (Model EL312, Biotek Instruments, UK).

5.2.2.2 Detection of anti-FIV antibodies by IFA

Eight-well tissue culture chamber/slides (Lab-Tek, Miles Laboratories Inc., Illinois, USA) containing a 1:1 mixture of uninfected CRFK cells and CRFK cells chronically infected with FIV were provided by Mr M Golder. These slides were fixed in acetone and stored in methanol at -20°C. Prior to use, slides were washed in tap water for 3 minutes and allowed to dry.

Serial ten-fold dilutions of plasma samples from 1:10 to 1: 10,000 were made in PBS/5% FCS then 25μl of sample was added to duplicate wells. Positive and negative control plasma samples were included. The slides were incubated for 2 hours at 37°C in a moisture chamber. At the end of the incubation, slides were washed twice in PBS for 3 minutes, rinsed in tap water and allowed to dry. 25μl of fluorescein-labeled goat anti-cat IgG (Kirkegaard and Perry Laboratories Inc., Maryland, USA), diluted 1:30 with PBS/5% FCS, was added to each well. The slides were incubated for 1 hour at 37°C in a moisture chamber then washed as before and allowed to dry. The slides were examined under UV light using a x25 objective. The highest dilution at which fluorescent activity could be detected was recorded as the end-point titre of the sample.

5.2.3 Lymphocyte subset analysis and haematology

Blood samples were taken from the experimental cats 24 days prior to infection, immediately before infection and 24 and 74 days post-infection for analysis of $CD4^+$, $CD8^+$ and $CD8^{+(low)}$ lymphocytes subsets by flow cytometry (kindly performed by Dr Brian Willett). Details of the method have been described previously (Willett *et al.*, 1993). At the same time points, 1ml of blood was taken into EDTA and submitted to the Haematology Laboratory in this department for routine analysis.

5.2.4 Effector cells

Blood samples of 10ml were taken from each cat (2.2.1) 2 weeks before infection and then at intervals post-infection. One ml was centrifuged at 6500 rpm to separate the plasma which was stored at -20°C for serological analysis (5.2.2). PBMC (responder cells) were separated from the remaining 9ml on a density gradient (2.2.1) and resuspended in complete RPMI at 2 x 10^6 /ml in 1ml cultures in 24-well

tissue culture plates. Autologous stimulator cells (4.2.3) were recovered from liquid nitrogen, washed in PBS, resuspended in complete RPMI and added to the responder cells at a stimulator:responder ratio of 1:10 to 1:20. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in a humidified incubator. On day 2, cultures were supplemented with human rIL2 at 100 U/ml. Cultures were fed with complete RPMI and human rIL2 every 2-3 days. After 6-7 days the cultures were harvested by gentle aspiration and the lymphocytes were recovered by centrifugation at 1000 rpm for 5 minutes. Cell pellets were resuspended in complete RPMI and viable cell counts were performed by trypan blue dye exclusion. These lymphocytes were used as the bulk effector cell populations in the cytotoxicity assays.

On the following occasions this protocol was varied. The stimulator cells for the 9 week post-infection assay were prepared using stored lymphoblasts rather than fresh lymphoblasts as described previously (4.2.3).

In order to assess the contribution of human rIL2 in the restimulation cultures to the observed responses, PBMC isolated at 21 weeks post-infection were cultured in the presence of either 50 or 200 U human rIL2/ml prior to assay.

A fraction of PBMC from the 9 weeks post-infection sample were used directly on isolation as effector cells in the assay.

5.2.5 Target cells

Autologous skin fibroblasts which had been stored at third passage were recovered from liquid nitrogen storage and cultured for a further 1 or 2 passages (3.2.2). Confluent monolayers were trypsinised, washed and seeded into round-bottomed 96-well tissue culture plates at 1 x 10^4 cells/well in complete MEM alpha. ^{51}Cr sodium chromate (Amersham, UK) was added at 1µCi/well in 50µl of complete MEM alpha. Following an overnight incubation, skin fibroblast monolayers were washed 4 times with MEM alpha containing 2% FCS then 50µl of a 1:200 stock dilution of vFIV/GL14-*gag* or vWT in 2% MEM alpha, or 2% MEM alpha alone, was added to each well. In the final assay at 21 weeks post-infection, vFIV/GL14-*gag* and vWT were used at a 1:400 dilution. Plates were centrifuged at 900 rpm for 5 minutes. After 1 hour the virus inoculum was replaced with complete MEM alpha and incubation was continued for a further 2 hours. These cells were then used as target cells in the cytotoxicity assays.

5.2.6 ^{51}Cr release cytotoxicity assays

The culture medium was removed from the target cells which were washed once in complete RPMI then left in 100µl of complete RPMI.

Three two-fold dilutions of the bulk effector lymphocytes were performed in complete RPMI in 96-well plates. 100µl of lymphocytes was added to each experimental well of target cells in triplicate at 4 L:T ratios. On each plate, 100µl of Triton X-100 was added to 6 wells containing target cells alone in complete RPMI at a final concentration of 10%. Similarly, 100µl of complete RPMI was added to a further 6 wells containing target cells. The results from these wells gave the maximum or spontaneous isotope release from the target cells respectively. The assay plates were centrifuged at 900 rpm for 5 minutes to increase the contact between the lymphocytes and target cells. Cultures were incubated for 4 hours at 37°C and then centrifuged at 900 rpm for 5 minutes. A volume of 125µl of culture medium was harvested from each well into a 96-well plate using a multichannel pipette and 100µl of each sample was then transferred individually from the 96-well plate into a plastic tube for counting (LP3 tubes, Denley Instruments Limited, UK). The activity of each sample was counted for 1 minute in a gamma counter (Minaxi gamma, Packard, Berkshire, UK).

A summary of the method for the cytotoxicity assays is shown in Figure 5.3.

5.2.7 Analysis of the results

^{51}Cr release for each sample was measured in counts per minute (cpm). The mean and standard deviation values were calculated for triplicate cultures. The specific lysis occurring in test wells was calculated as a percentage using the formula;

$$\frac{\text{X-spon}}{\text{max-spon}} \times 100 = \%\ \text{specific lysis}$$

Where:

X = activity (mean cpm) from test well triplicates

spon = spontaneous isotope release (mean cpm) from target cells

max = total isotope release (mean cpm) from target cells

Background ^{51}Cr release from target cells was calculated by expressing the spontaneous release (mean cpm) as a percentage of the maximum release from the target cells using the formula;

$$\text{spon/max} \times 100 = \text{background } ^{51}\text{Cr release } (\%)$$

5.3 RESULTS

5.3.1 Experimental cats

The 3 experimental cats remained clinically healthy and maintained their body weights throughout the experiment.

5.3.2 Serology

Prior to experimental FIV infection cats tested seronegative for FIV by ELISA and IFA.

Following experimental infection the development of plasma antibodies to FIV proteins p17, p24, TM2 and V3 was determined by ELISA. The results are shown in Figures 5.4 and 5.5. Plasma antibody profiles against each FIV antigen are similar in FIV infected cats E3 and F23. Cat F23 seroconverted to V3, TM2, p17 and p24 between 2 and 4 weeks post-infection. In cat E3, antibodies to p24 appeared slightly later, between 4 and 7 weeks post-infection. There was a steep rise in antibody levels initially with most reaching a peak at around 9 weeks post-infection. After this, antibody levels plateaued or fell slightly. Uninfected control cat F22 remained seronegative for FIV throughout the experiment.

O.D. values for negative samples on the ELISA were generally very low. Plasma from cat E3 gave higher background readings against recombinant peptides p17 and p24, although this did not affect the ability to detect seroconversion. The onset of seroconversion in FIV-infected cats was confirmed by IFA (Figure 5.6). Non-specific staining was often seen at the lowest plasma dilutions.

All plasma samples were negative for FIV p24 antigen.

5.3.3 Lymphocyte subset analysis and haematology

PBMC were isolated from the cats at intervals during this experiment and $CD4^+$, $CD8^+$ and $CD8^{+(low)}$ lymphocyte subsets were enumerated by flow cytometry. In FIV infected cats E3 and F23, the $CD4^+$:$CD8^+$ ratio was inverted by 74 days post-infection. In the uninfected control cat F22, the $CD4^+$:$CD8^+$ ratio was maintained between 1.2-1.7. Relative and absolute counts of lymphocyte subsets gave similar profiles (Figures 5.7 and 5.8). In FIV infected cat E3, the proportion of $CD8^{+(low)}$ lymphocytes rose sharply as infection progressed (Figure 5.9).

In parallel to flow cytometry, blood samples were submitted for routine haematology. At the point of infection a marked leucocytosis was seen in cat E3 (Figure 5.10). This was due to a mature neutropenia (Figure 5.11, B). A fall in total

WBC count was seen in cat E3 following FIV infection although this did not result in leucopenia. At 3.5 weeks post-infection, cat E3 showed a marked lymphocytosis which had resolved by 10.5 weeks post-infection (Figure 5.11, A).

5.3.4 Effector cells

Yields of PBMC from 10ml heparinised blood samples were between 2-8 x 10^7 cells. At the end of the culture period the number of viable cells was between 80-95% of the original population. This provided maximum L:T ratios from 50:1 to 160:1 in the assays.

5.3.5 Target cells

Spontaneous isotope release was <35% of the maximum except on a single occasion (see later). The spontaneous ^{51}Cr release was found to be consistently higher from vFIV/GL14-*gag* infected targets than from uninfected targets from the same cat, with vWT infected targets giving intermediate values. This pattern was reflected in the background ^{51}Cr release values (spon/max x 100) since the maximum release was unaffected by vaccinia infection (Table 5.2). In the 21 week assay, when vFIV/GL14-*gag* was used at half the previous multiplicity of infection (m.o.i.), background ^{51}Cr release was reduced compared to previous assays.

At 9 weeks post-infection a fraction of directly isolated PBMC was used as effectors in a cytotoxicity assay. This assay was invalid (backgrounds >35%) due to poor uptake of ^{51}Cr by the target cells.

Overspill of detergent from the maximum release wells into the adjacent test wells was observed macroscopically in the cytotoxicity assay at 14 weeks post-infection. This resulted in falsely elevated ^{51}Cr release in wells containing autologous and heterologous vFIV/GL14-*gag* infected target cells and F23 effectors at the 2 lower L:T ratios. In subsequent assays, maximum release wells were separated by at least 1 row from other wells and care was taken not to overfill any wells.

^{51}Cr release from control target cells was found to vary between culture plates in a single assay. The degree of interplate variation in 1 assay (21 weeks post-infection) is demonstrated in Figures 5.12 and 5.13. Figure 5.12 shows the variation in the mean values for spontaneous and maximum ^{51}Cr release from vFIV/GL14-*gag*-infected skin fibroblasts from 3 cats, each on 4 separate plates. The difference between maximum and spontaneous ^{51}Cr release, which comprises the denominator in the calculation of % specific lysis, was not constant between plates (Figure 5.13, A). Similarly the percentage spontaneous ^{51}Cr release varied widely, by as much as 20% in this assay (Figure 5.13, B). Therefore, wherever possible, background

isotope release and % specific lysis were calculated for individual plates although in earlier assays, control wells were not always included for all target cell types on each plate.

5.3.6 ^{51}Cr release cytotoxicity assays

PBMC were isolated from 2 FIV/GL14 infected cats, E3 and F23, and 1 control cat, F22, at 2 weeks prior to infection (E3 and F23 only) and then at 1, 2, 4, 7, 9, 14 and 21 weeks post-infection. Bulk cultures of lymphocytes derived from circulating PBMC, were restimulated in vitro for 6-7 days and then tested for their ability to recognise different target cell types in 4 hour cytotoxicity assays. The results are presented in Figures 5.14 to 5.20.

In cytotoxicity assays carried out before and 1 week after infection, only low levels of lytic activity were detected (Figures 5.14 and 5.15). Antigen-specific cytotoxic effector cells were first detected from FIV-infected cat E3 at 2 weeks post-infection (Figure 5.16). This response was detected in all subsequent assays where results were available for this cat, i.e. at 7, 14 and 21 weeks post-infection. Restimulated lymphocytes from cat E3 killed autologous, GAG-expressing targets with levels of specific lysis ranging from 38%-64% at the highest L:T ratios. At 7 and 14 weeks post-infection heterologous vFIV/GL14-*gag* infected targets were recognised in addition to autologous targets. This heterologous recognition was seen only at the maximum L:T ratio and at a lower level than that of autologous targets.

Results were unavailable for lymphocytes from cat E3 at 4 weeks post-infection on autologous or heterologous GAG-expressing targets since the plate containing these cultures was disturbed during the assay and the culture fluids were lost. No responses were detected from restimulated lymphocytes from any cat taken at 9 weeks post-infection. This finding prompted retrospective immunoblot analysis of the stimulator cells used in this experiment since they were prepared from stored rather than fresh lymphoblasts. p55 antigen could not be demonstrated in these preparations. All previous and subsequent restimulation procedures employed stimulator cells prepared from fresh mitogen-activated lymphoblasts. Such preparations were always positive for FIV p55 on immunoblot.

In contrast to cat E3 which showed consistent GAG-specific cytotoxic responses from 2 weeks post-infection, antigen specific cytotoxic responses from cat F23 were not detected until 14 weeks post-infection (Figure 5.19). In this case, recognition was restricted to autologous cells.

Effector cells from the uninfected control cat F22 usually did not give specific lysis above 10% and this never exceeded 20% on any target cell type.

In order to assess the contribution of human rIL2 added during the restimulation period to the observed responses, PBMC isolated at 21 weeks post-infection were cultured with either 50 or 200 U human rIL2/ml prior to assay. PBMC from cat E3 cultured with 50 U human rIL2/ml recognised autologous vFIV/GL14-*gag* infected target cells, but not heterologous targets. When the same cells were cultured in the presence of 200 U human rIL2, no response was detected. In cat F23, the pattern of recognition was less clear. Higher specific lysis was seen on autologous targets than on heterologous targets at 200 U human rIL2/ml but there was no clear dose-response relationship.

Figure 5.1

FIV ENV V3 loop amino acid sequence

The V3 loop has been sequenced for 17 FIV isolates. Numbers refer to the homology between isolates at each position. The peptide sequence used in the V3 MAP is indicated. In FIV isolates GL8, GL14 and PET, the sequence is as shown. Each MAP unit consists of 8 peptide branches on a polylysine core. (Dr George Reid, unpublished data with permission)

H_1

S_1

L_1 L_3 F_2 V_2 P_1 G_2

H_1 P_2 K_1 H_1 H_1 S_1 T_3 I_5 T_5 G_1 R_7 H_2 K_3

C_{17} Q_{16} R_{15} T_{16} Q_{16} S_{17} Q_{15} P_{16} G_{17} S_{11} W_{17} L_8 [R]$_{17}$ A_{10} I_{17} S_{17} S_{17} W_{16} K_{10} Q_{14} R_{12} N_{17}

MAP start

Disulphide linked

β turn

β sheet

β turn

R_{16} K_1

W_{16} S_1

E_{15} G_2

MAP end

C_{14} Q_{14} L_{17} S_{16} V_{10} K_{16} V_6 K_{14} E_{13} S_6 E_{11} E_{16} [D]$_{17}$ P_{17} R_{17} W_6

G_2 T_2 T_1 I_7 R_1 A_1 R_3 K_1 N_1 K_6 V_1 S_1

S_1 P_1

α helix.

Figure 5.1

Figure 5.2

FIV ENV TM2 peptide amino acid sequence

The TM2 MAP is composed of 4 peptide branches on a polylysine core. This sequence is conserved between FIV isolates GL8, GL14 and PET.

Cys-Asn-Gln-Asn-Asn-Gln-Phe-Phe-Cys-Lys-Gln-Gln

Figure 5.2

Figure 5.3

Summary of the ^{51}Cr release assay protocol

Prior to experimental infection with FIV, skin fibroblasts were harvested and stored from experimental cats for use as target cells in the assays (Chapter 3). Stimulator cells were prepared from vFIV/GL14-gag infected lymphoblasts (Chapter 4). At intervals post-infection PBMC were isolated from the infected and control cats and restimulated with antigen in vitro for 6-7 days. These cells were then used as the bulk effector cell population in the cytotoxicity assays.

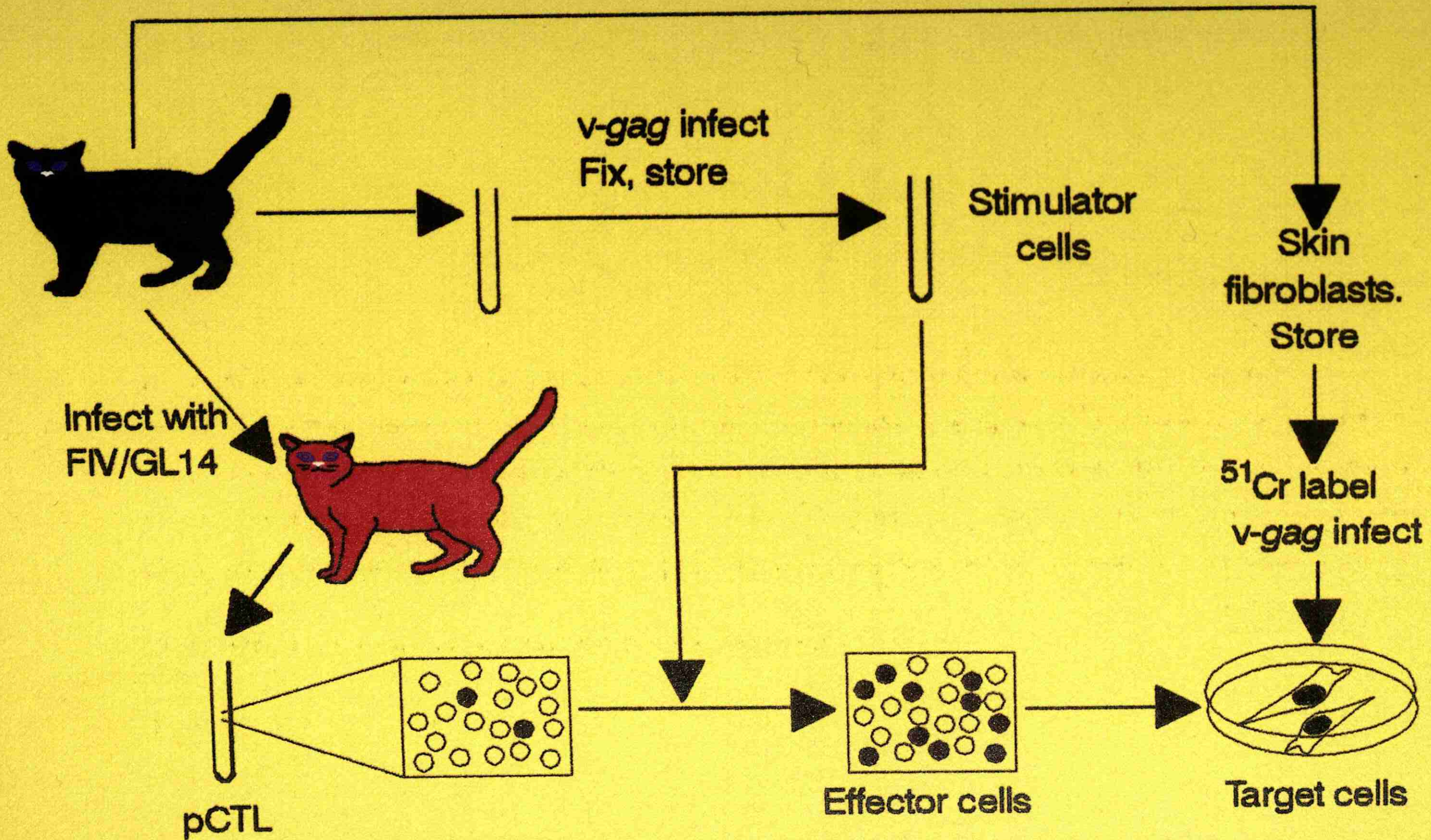


Figure 5.3

Figure 5.4

Development of plasma antibodies to FIV core proteins detected by ELISA following experimental FIV infection

A. Capture antigen was recombinant FIV p17

B. Capture antigen was recombinant FIV p24

O.D. = optical density.

E3
F23
F22 (control)

A. Anti-FIV p17

3.0
2.5
2.0
1.5
1.0
0.5
0.0
O.D. (650nm)
-5
0
5
10
15
20
Weeks post-infection

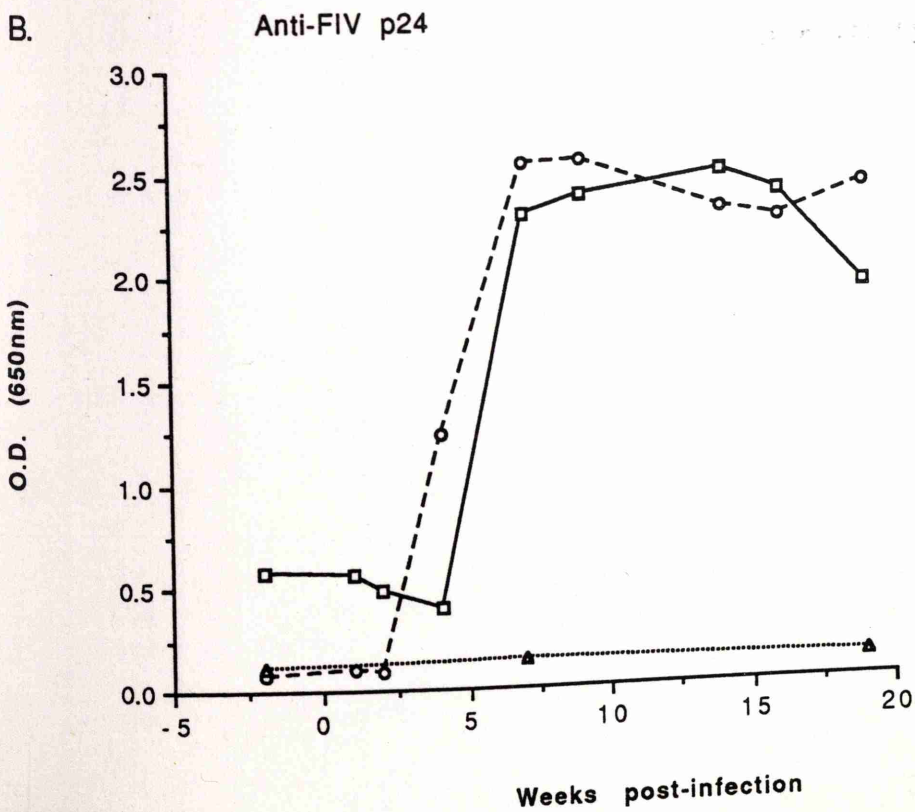


Figure 5.4

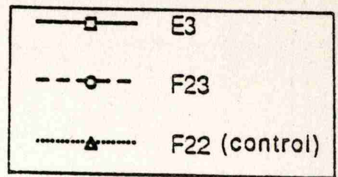
Figure 5.5

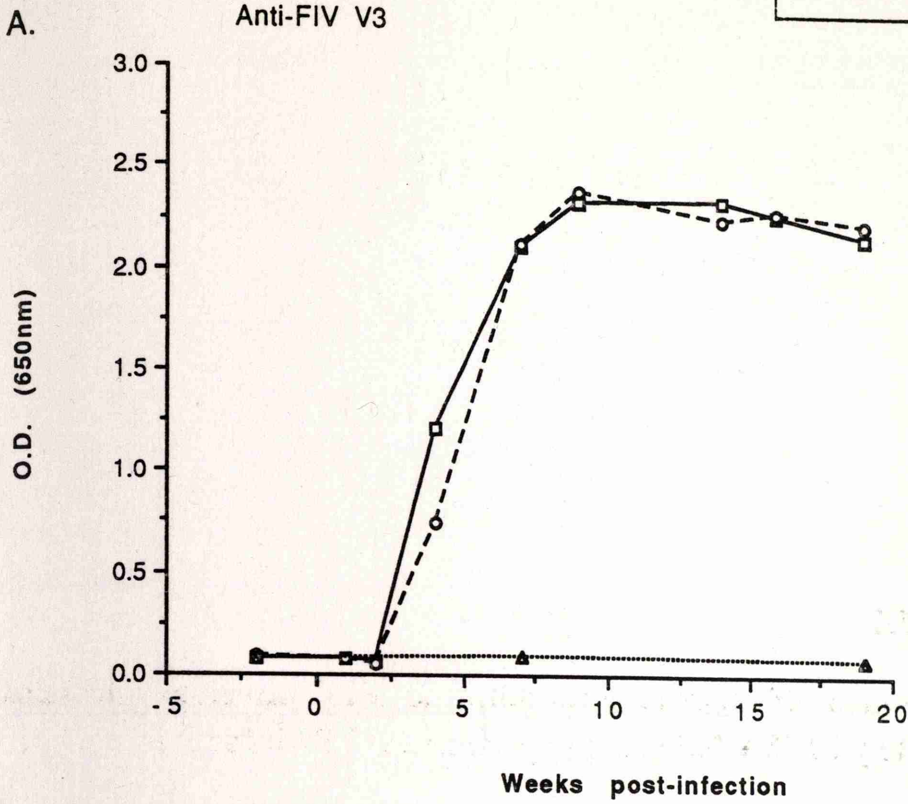
Development of plasma antibodies to FIV envelope proteins detected by ELISA following experimental FIV infection

A. Capture antigen was FIV V3 MAP

B. Capture antigen was FIV TM2 MAP

O.D. = optical density.





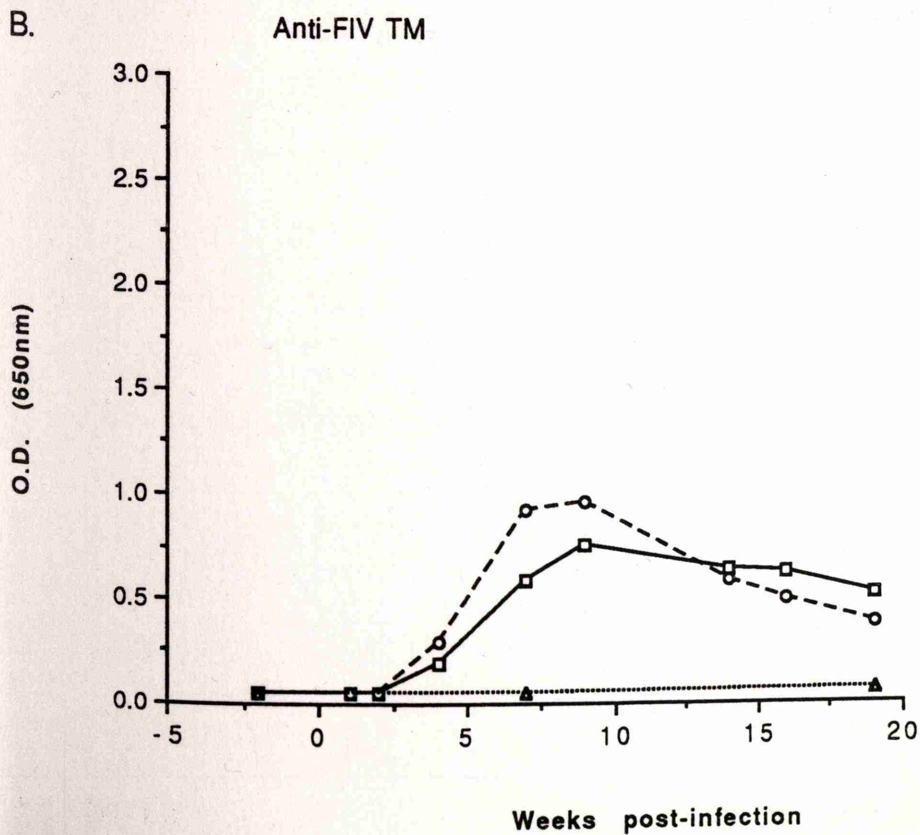


Figure 5.5

Figure 5.6

Development of antibodies to FIV detected by IFA following experimental FIV infection

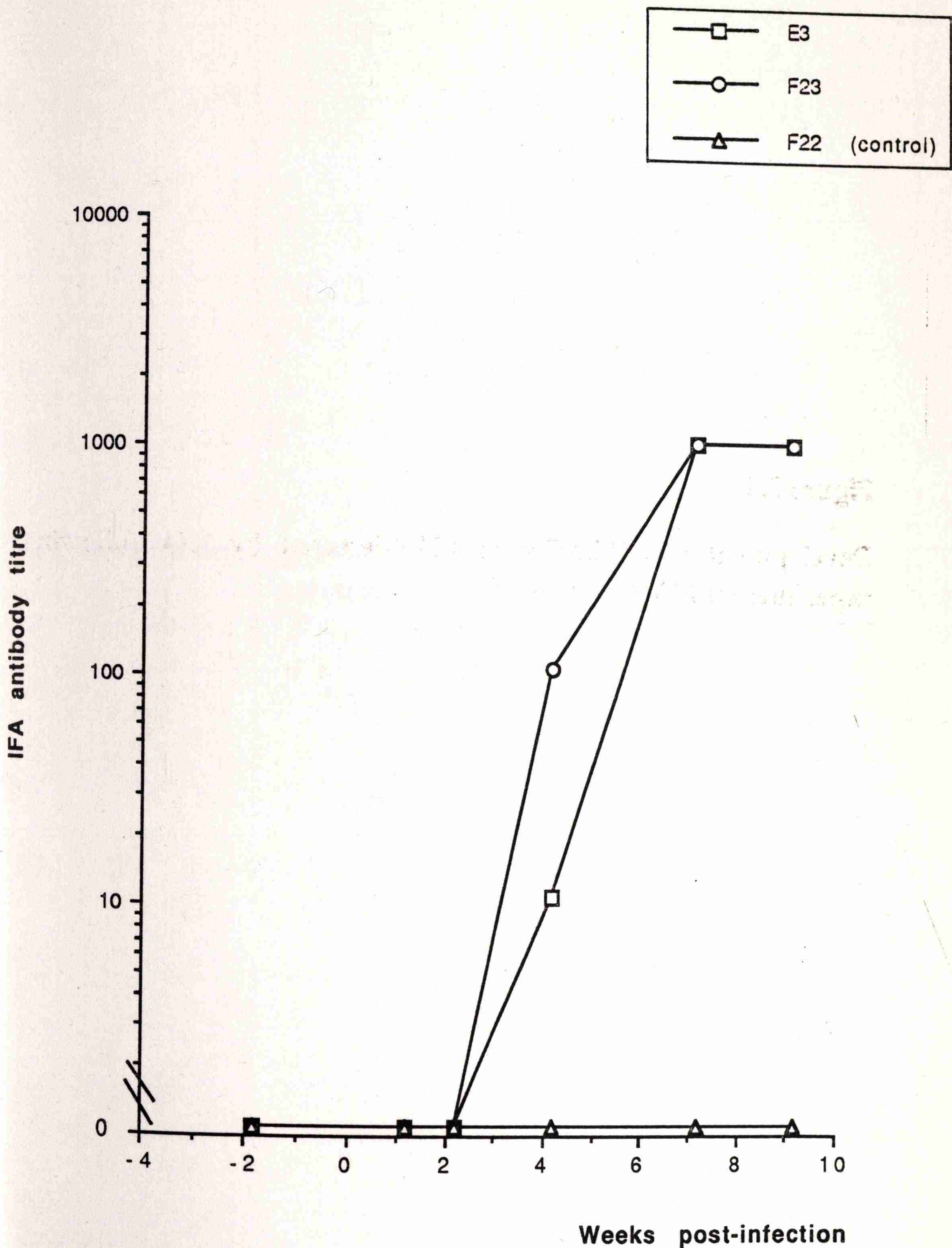
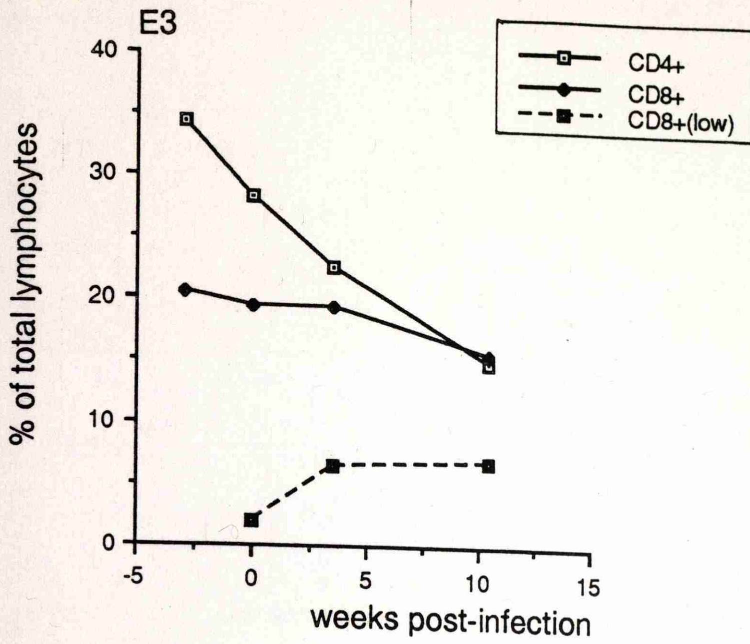
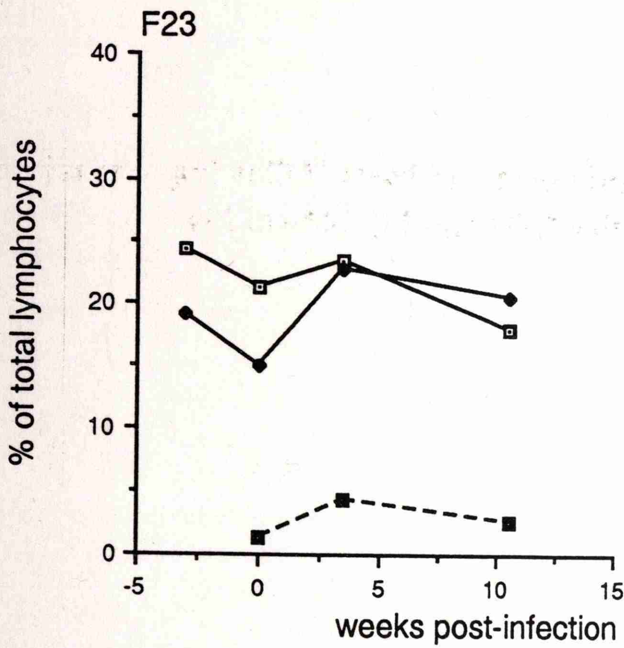


Figure 5.6

Figure 5.7

Changes in lymphocyte subsets following experimental FIV infection. I Relative CD4^{+} and CD8^{+} counts





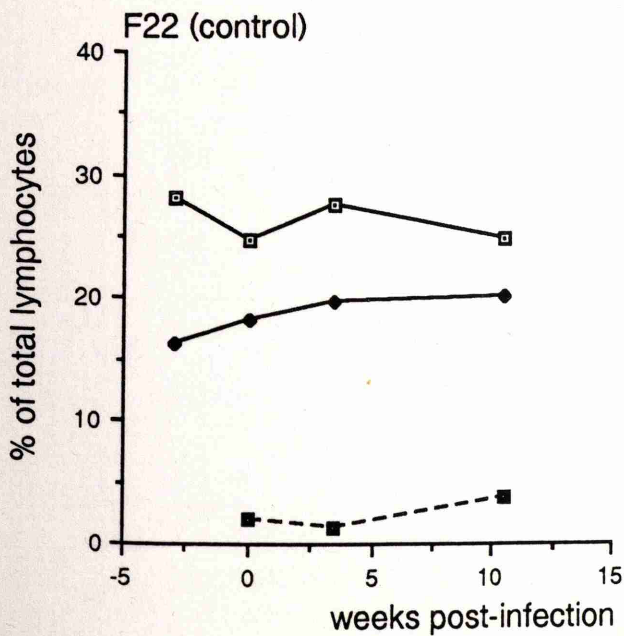
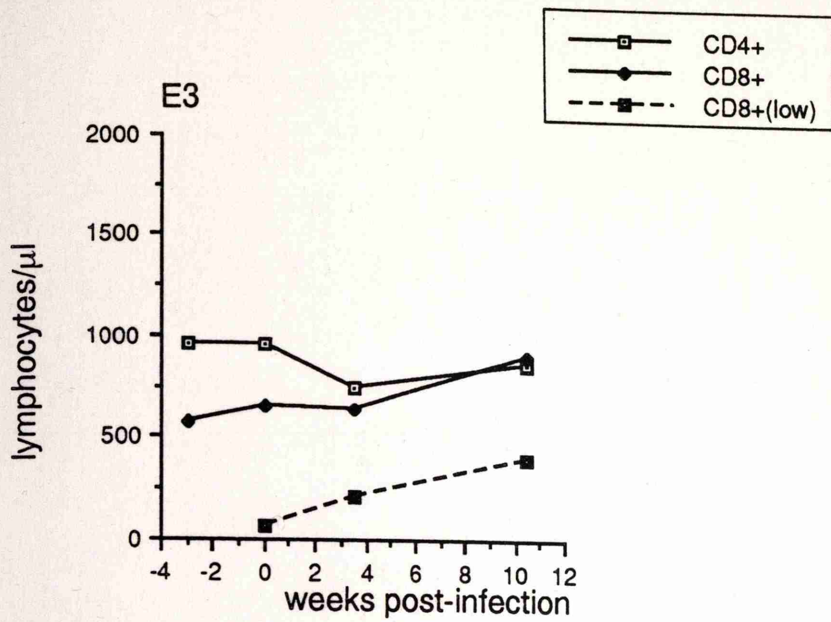
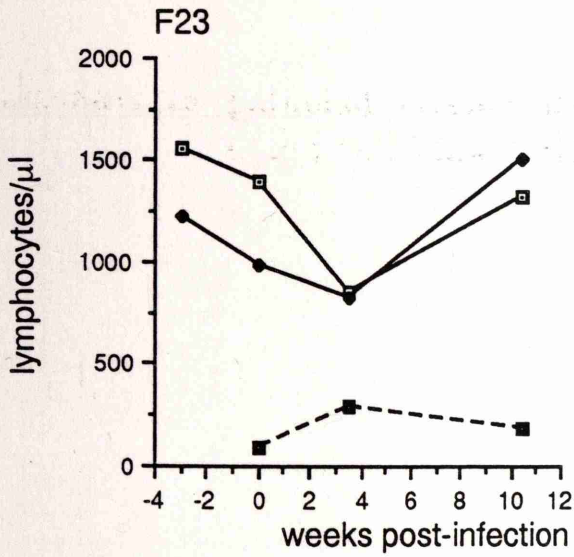


Figure 5.7

Figure 5.8

Changes in lymphocyte subsets following experimental FIV infection. II Absolute $CD4^+$ and $CD8^+$ counts





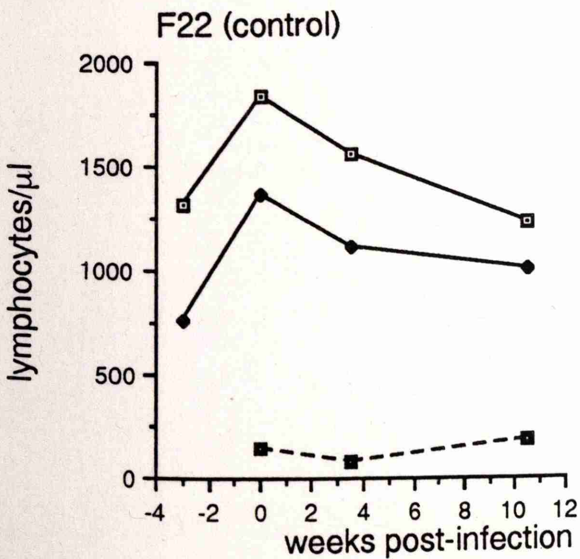


Figure 5.8

Figure 5.9

Changes in lymphocyte subsets following experimental FIV infection. III Proportion of $CD8^+$ lymphocytes expressing $CD8^{+(low)}$

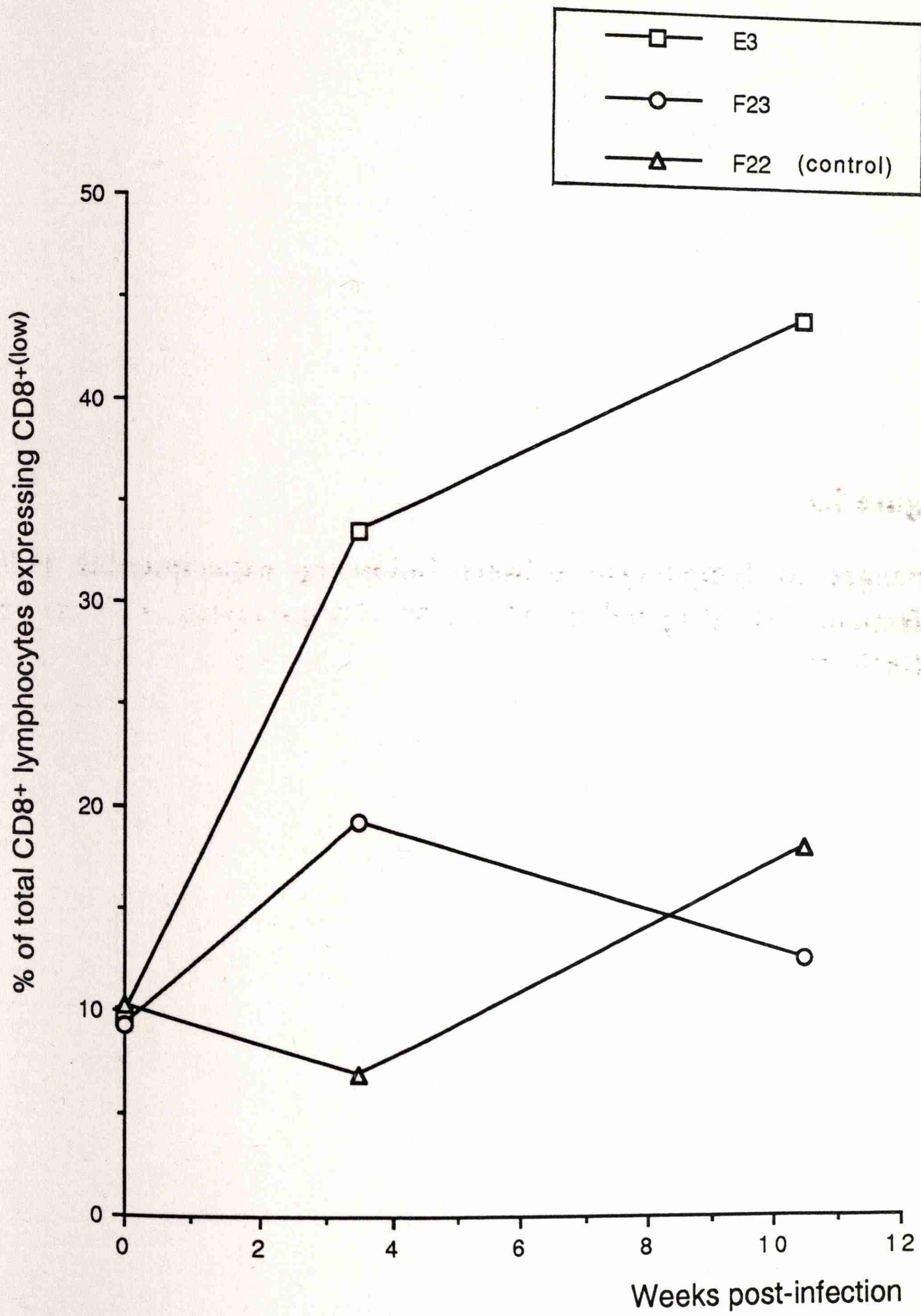


Figure 5.9

Figure 5.10

Changes in total leucocyte counts following experimental FIV infection

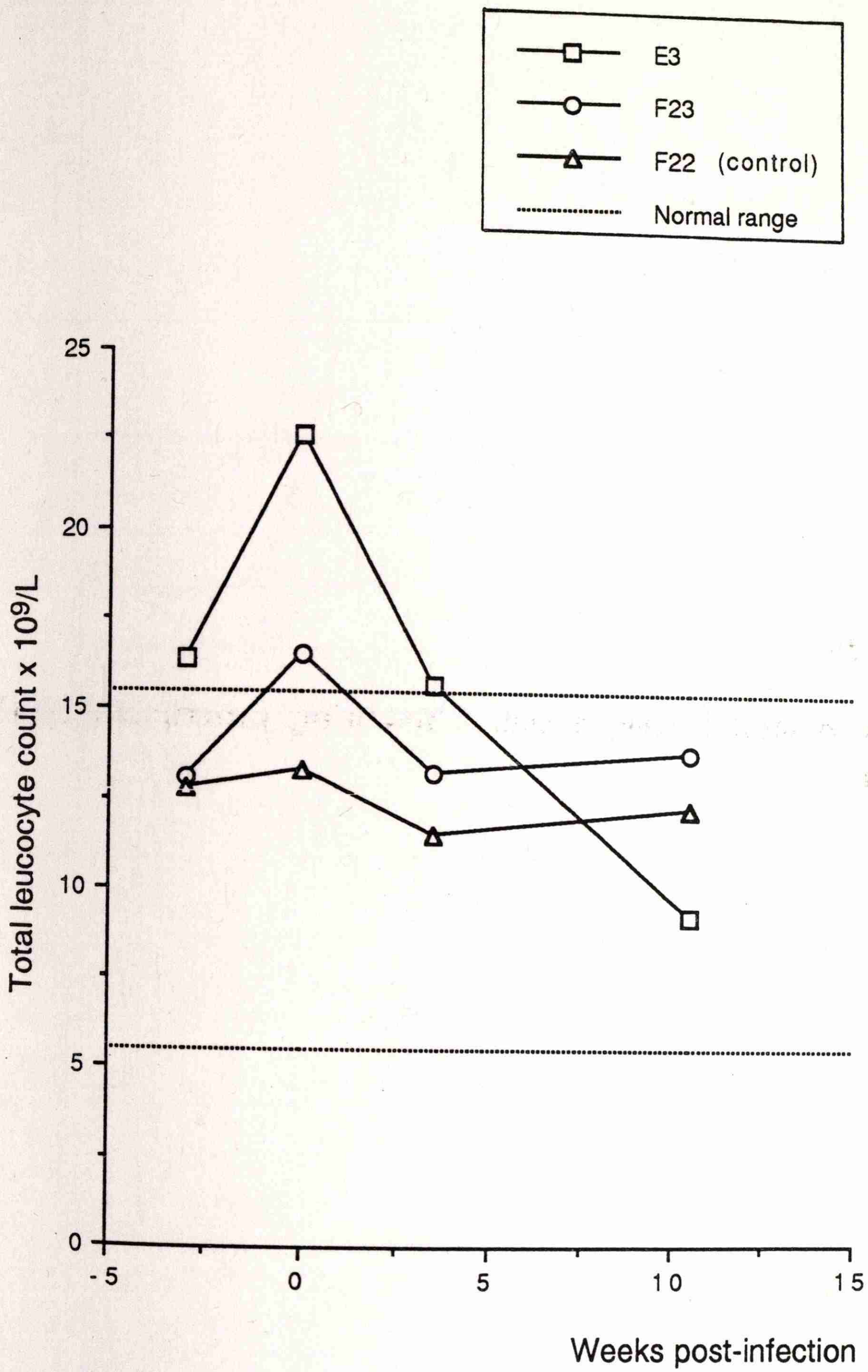


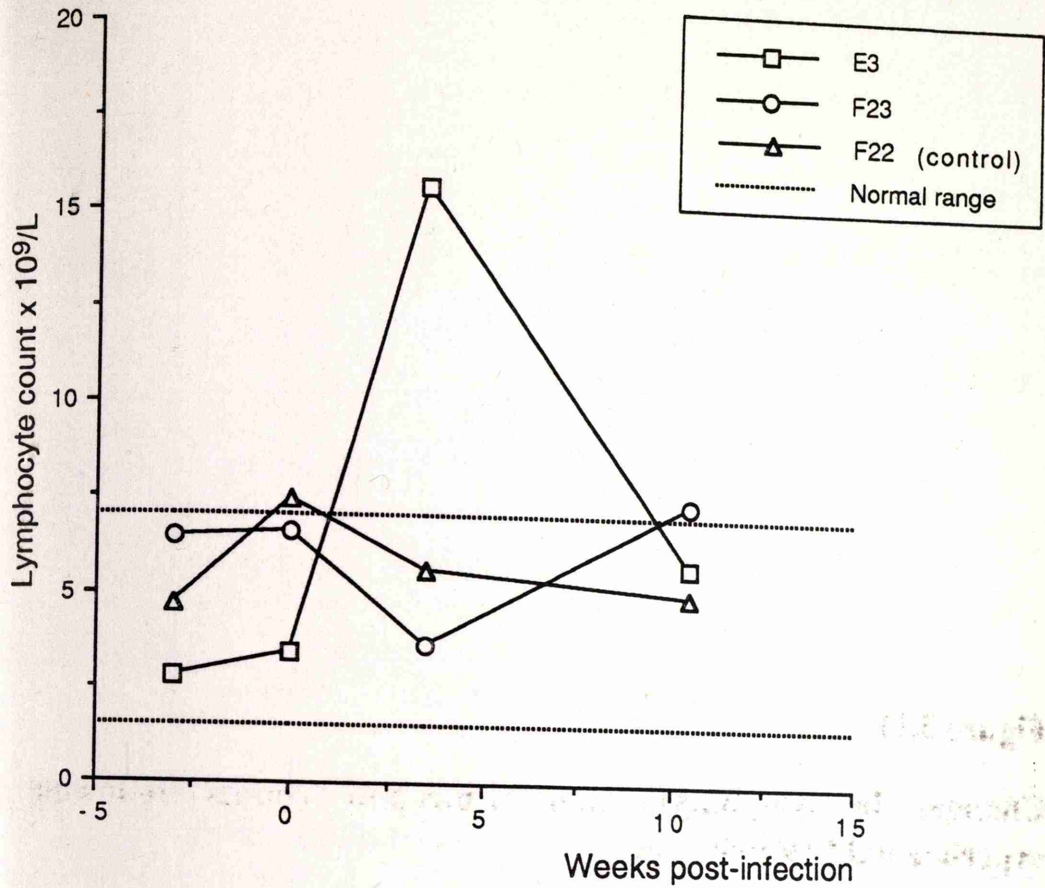
Figure 5.10

Figure 5.11

Changes in lymphocyte and neutrophil counts following experimental FIV infection

A. Lymphocyte counts

B. Neutrophil counts



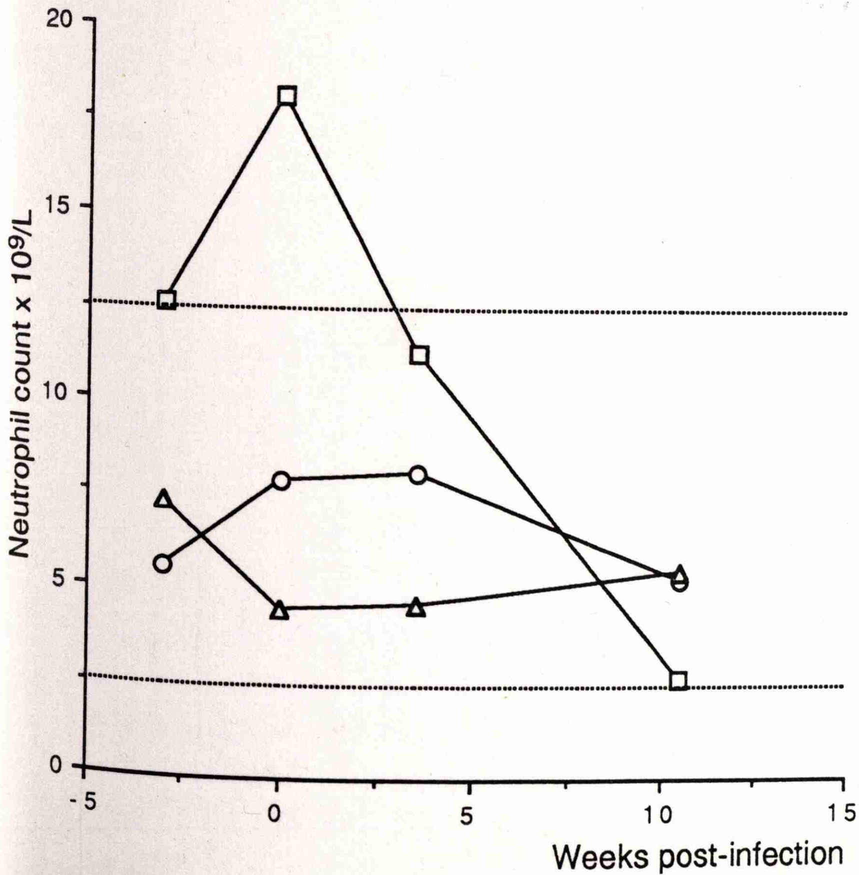
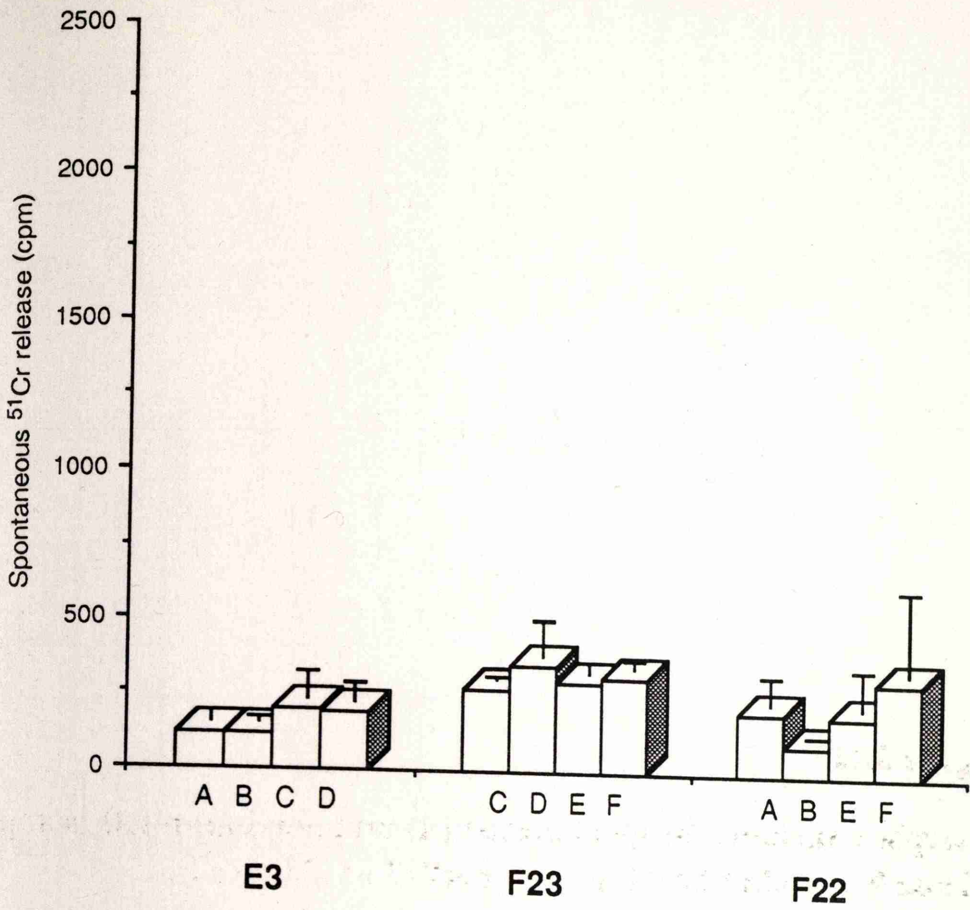


Figure 5.11

Figure 5.12

Interplate variation in spontaneous (A) and maximum (B) isotope release from skin fibroblast target cells in a single assay

Mean values for spontaneous and maximum ^{51}Cr release from vFIV/GL14-*gag*-infected skin fibroblasts from 3 cats E3, F23 and F22, each on 4 separate assay plates A-D. Bars = SD.



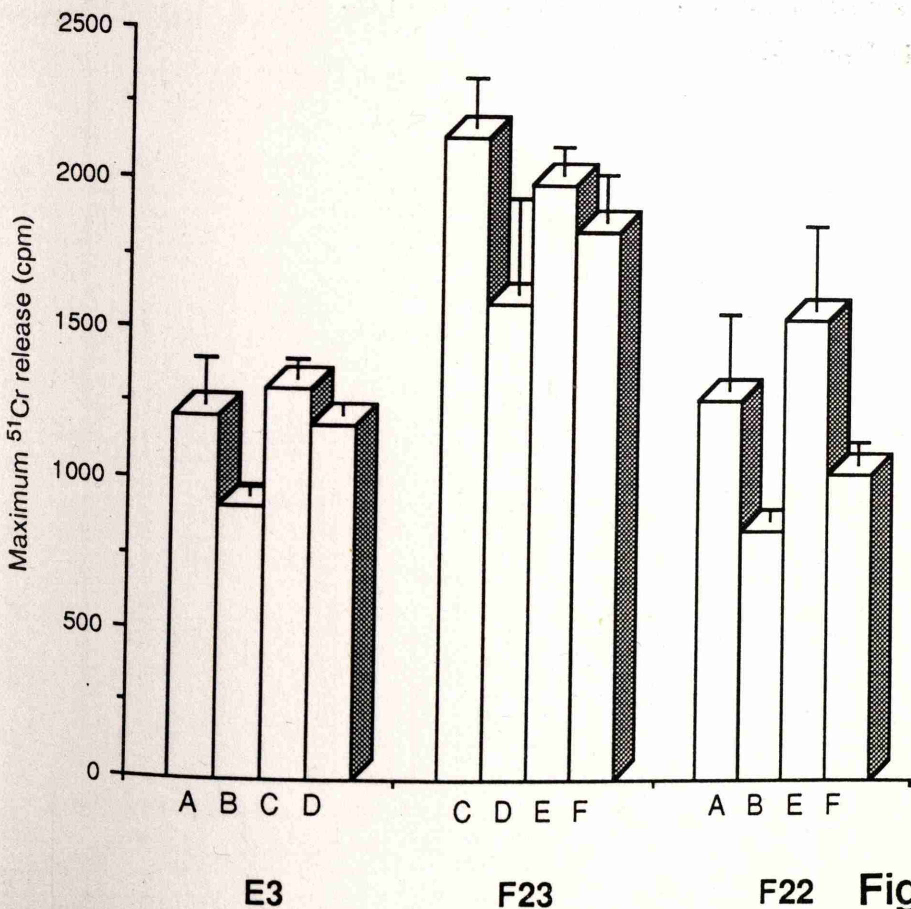
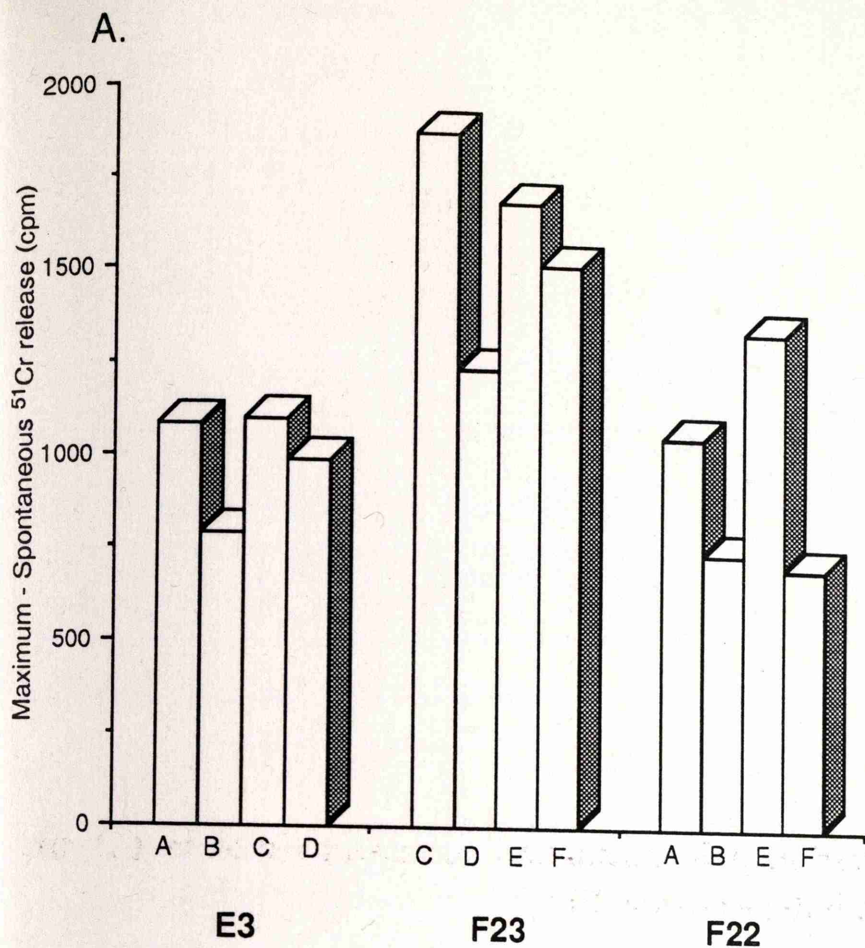


Figure 5.12

Figure 5.13

Interplate variation in maximum-spontaneous release (A) and background isotope release (B)

Mean values for spontaneous and maximum ^{51}Cr release from vFIV/GL14-*gag*-infected skin fibroblasts from 3 cats E3, F23 and F22, each on 4 separate assay plates A-D.Values are derived from the data shown in Figure 5.12.



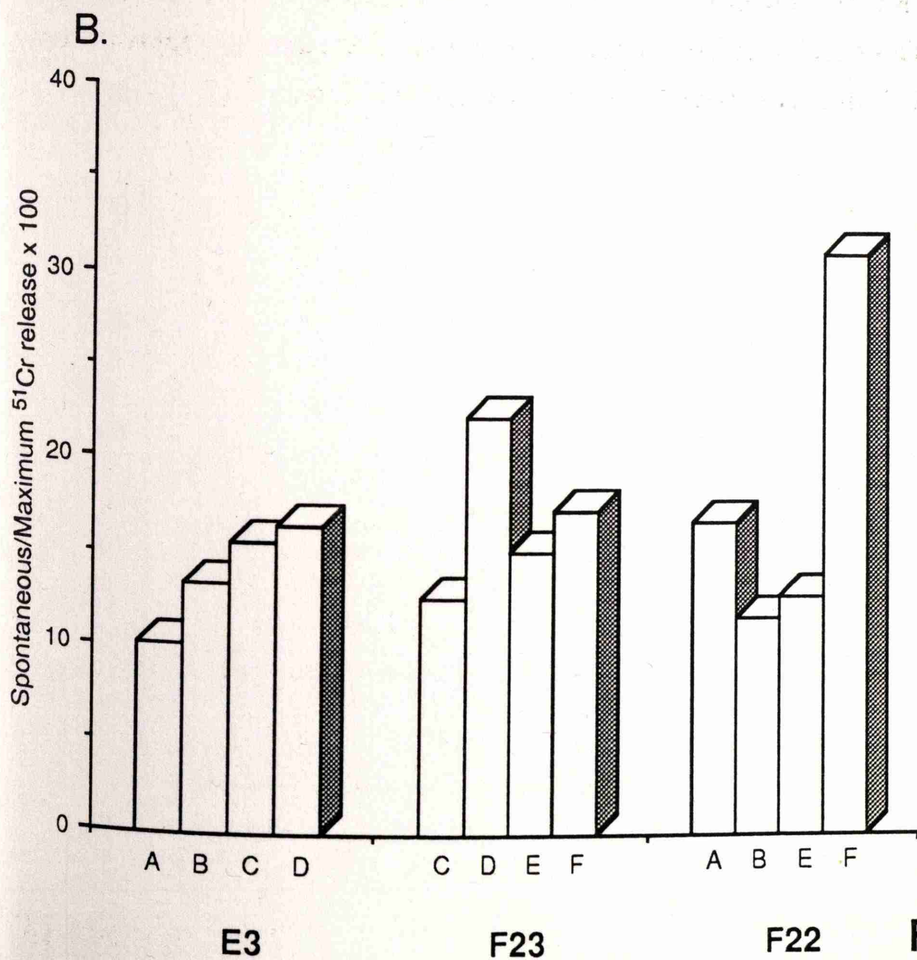


Figure 5.13

Figure 5.14

Cytotoxic activity of bulk restimulated lymphocytes from experimental cats prior to FIV infection/ mock infection

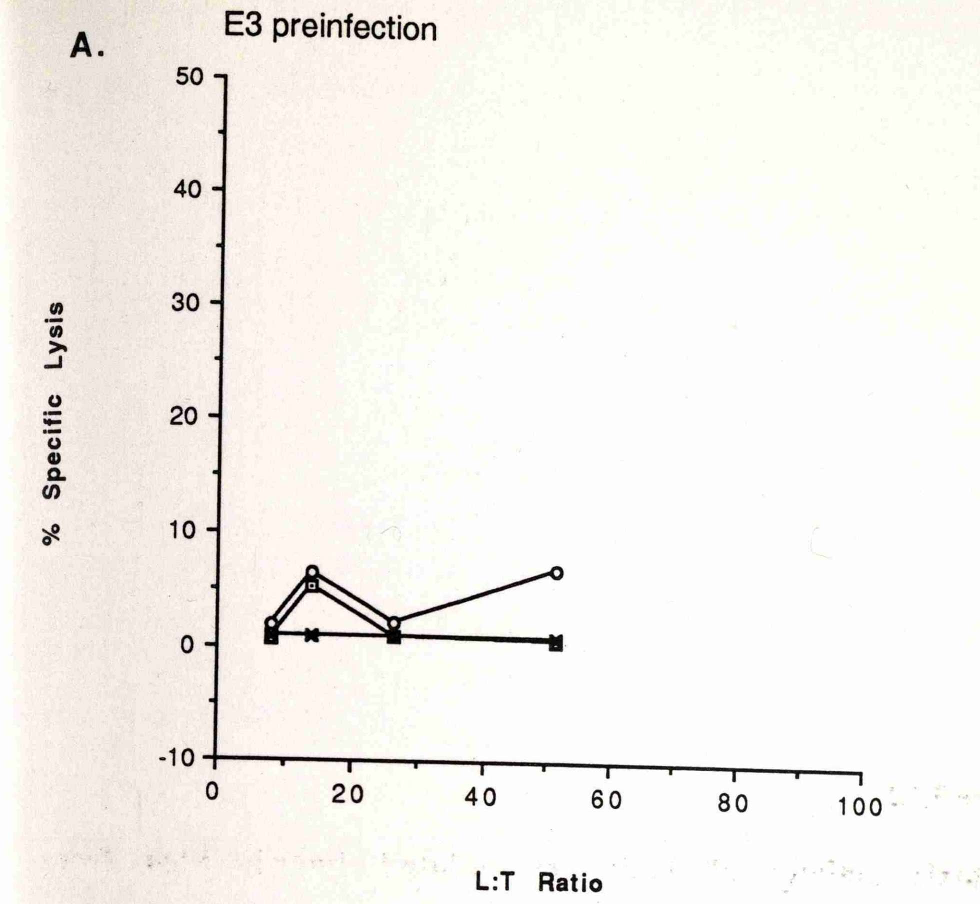
A=E3, B=F23

Aut = Autologous targets

Het = Heterologous targets

vGAG = vFIV/GL14-*gag* infected

L:T = Lymphocyte: target ratio



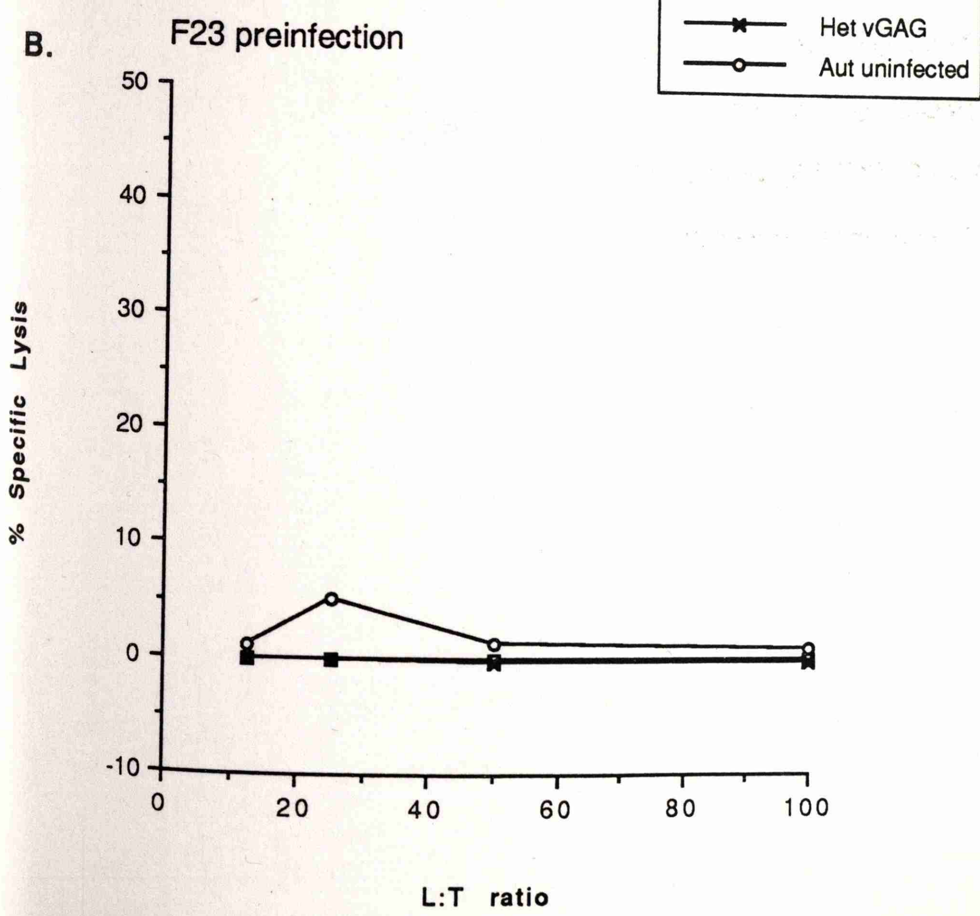


Figure 5.14

Figure 5.15

Cytotoxic activity of bulk restimulated lymphocytes from FIV-infected cats at 1 week post-infection

A=E3, B=F23, C=F22 (control)

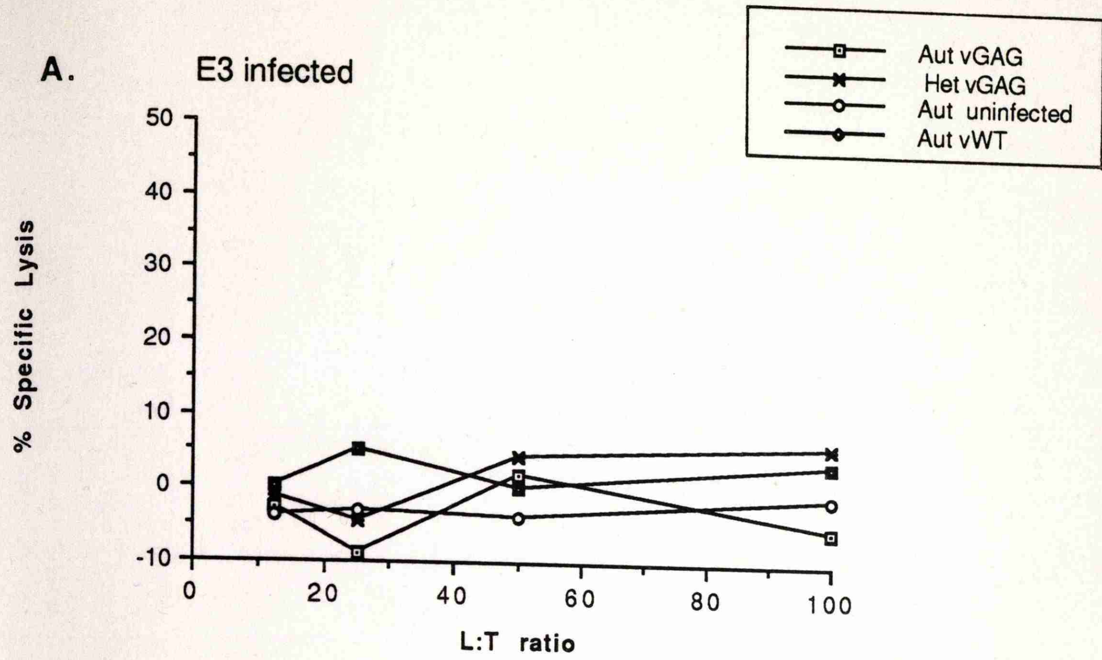
Aut = Autologous targets

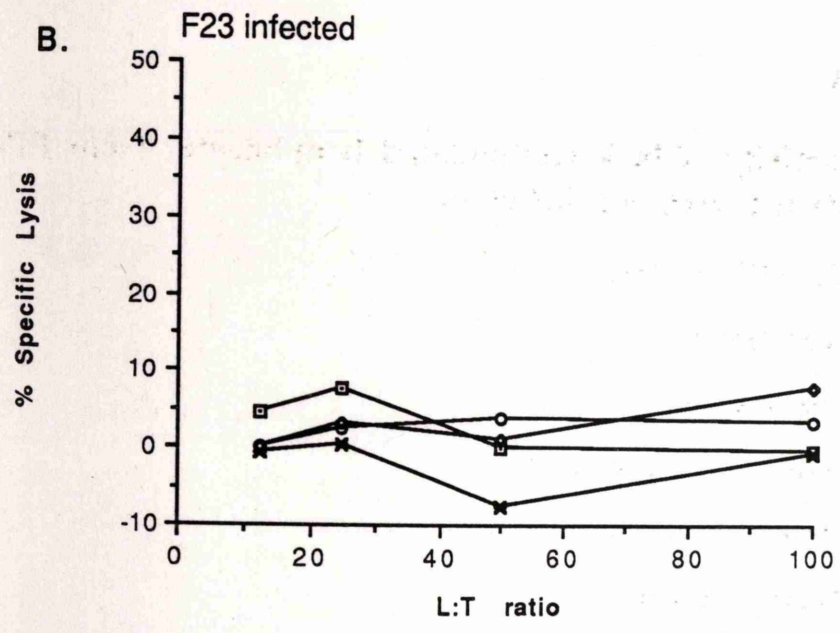
Het = Heterologous targets

vGAG = vFIV/GL14-*gag* infected

vWT = Wild type vaccinia virus infected

L:T = Lymphocyte: target ratio





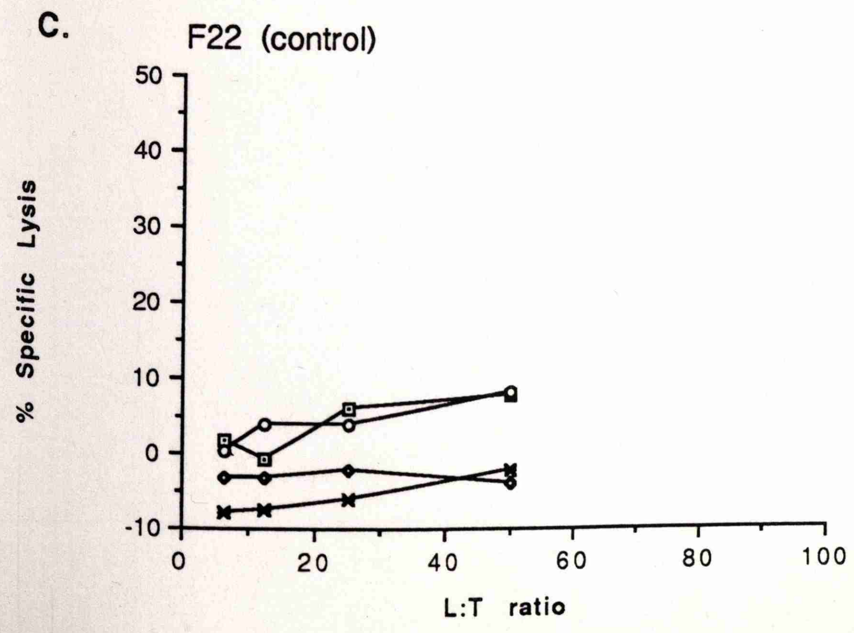


Figure 5.15

Figure 5.16

Cytotoxic activity of bulk restimulated lymphocytes from FIV-infected cats at 2 weeks post-infection

Effectors from E3 recognise autologous, FIV-GAG expressing targets at the highest L:T ratio

A=E3, B=F23, C=F22 (control)

Aut = Autologous targets

Het = Heterologous targets

vGAG = vFIV/GL14-*gag* infected

vWT = Wild type vaccinia virus infected

L:T = Lymphocyte: target ratio

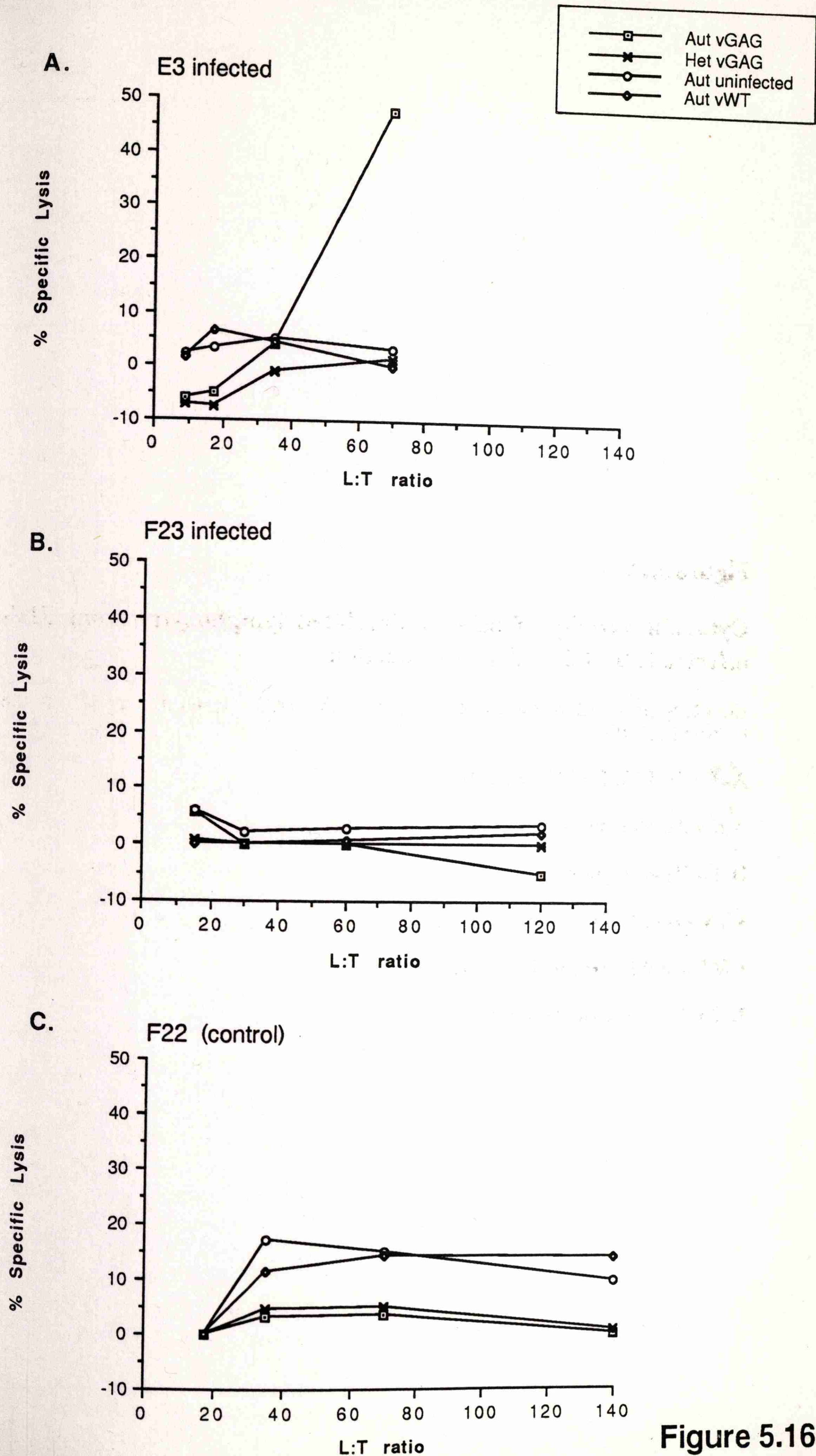


Figure 5.16

Figure 5.17

Cytotoxic activity of bulk restimulated lymphocytes from FIV-infected cats at 4 weeks post-infection

A=E3, B=F23, C=F22 (control)

Aut = Autologous targets

Het = Heterologous targets

vGAG = vFIV/GL14-*gag* infected

vWT = Wild type vaccinia virus infected

L:T = Lymphocyte: target ratio

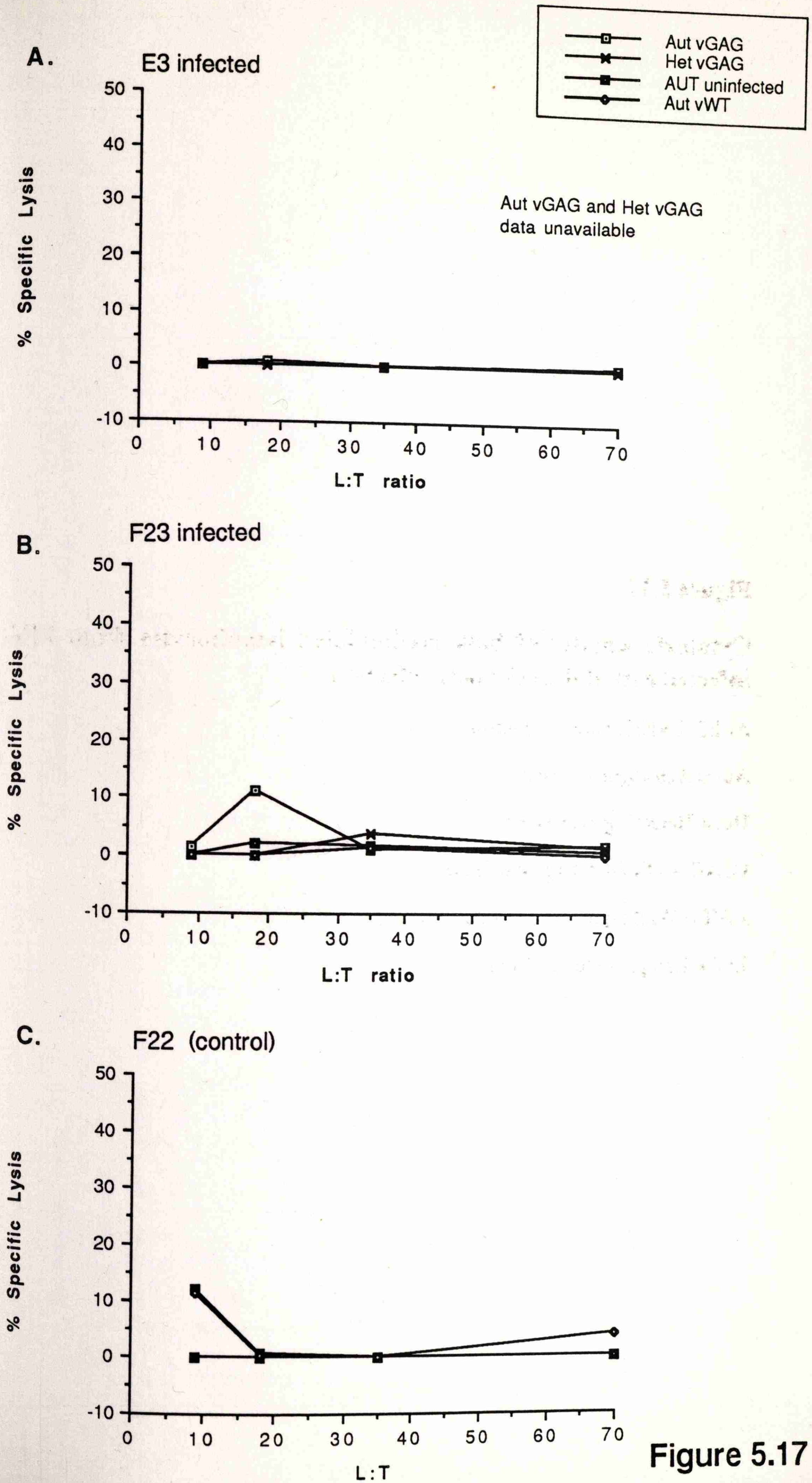


Figure 5.17

Figure 5.18

Cytotoxic activity of bulk restimulated lymphocytes from FIV-infected cats at 7 weeks post-infection

A=E3, B=F23, C=F22 (control)

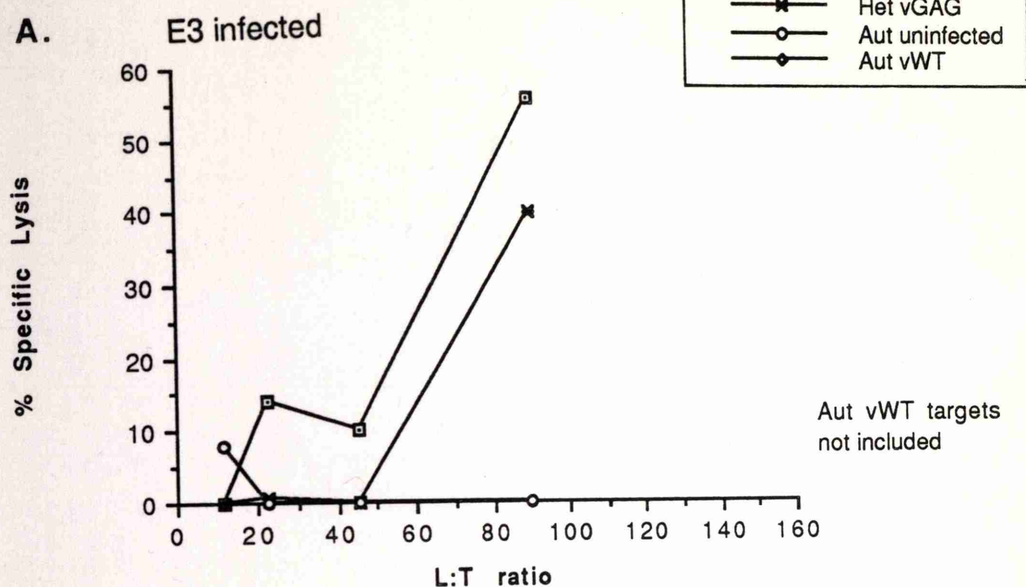
Aut = Autologous targets

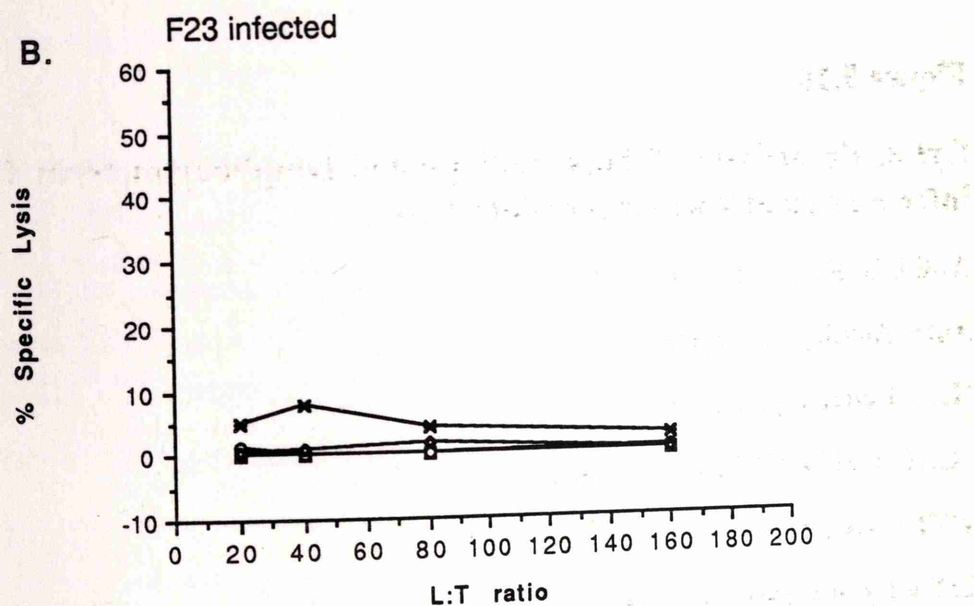
Het = Heterologous targets

vGAG = vFIV/GL14-*gag* infected

vWT = Wild type vaccinia virus infected

L:T = Lymphocyte: target ratio





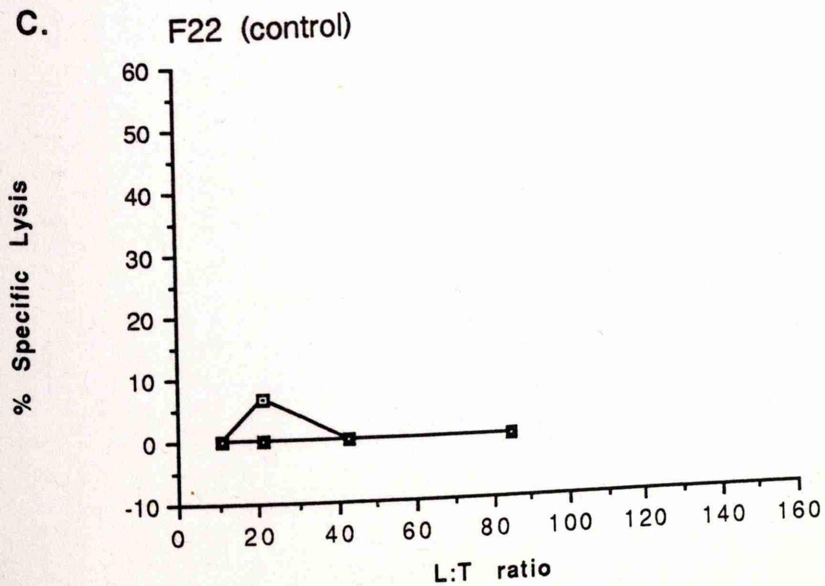


Figure 5.18

Figure 5.19

Cytotoxic activity of bulk restimulated lymphocytes from FIV-infected cats at 14 weeks post-infection

Effectors from E3 recognise autologous, FIV-GAG expressing targets and heterologous (F22) FIV-GAG expressing targets. Effectors from F23 recognise autologous, FIV-GAG expressing targets but not heterologous (E3) FIV-GAG expressing targets.

A=E3, B=F23, C=F22 (control)

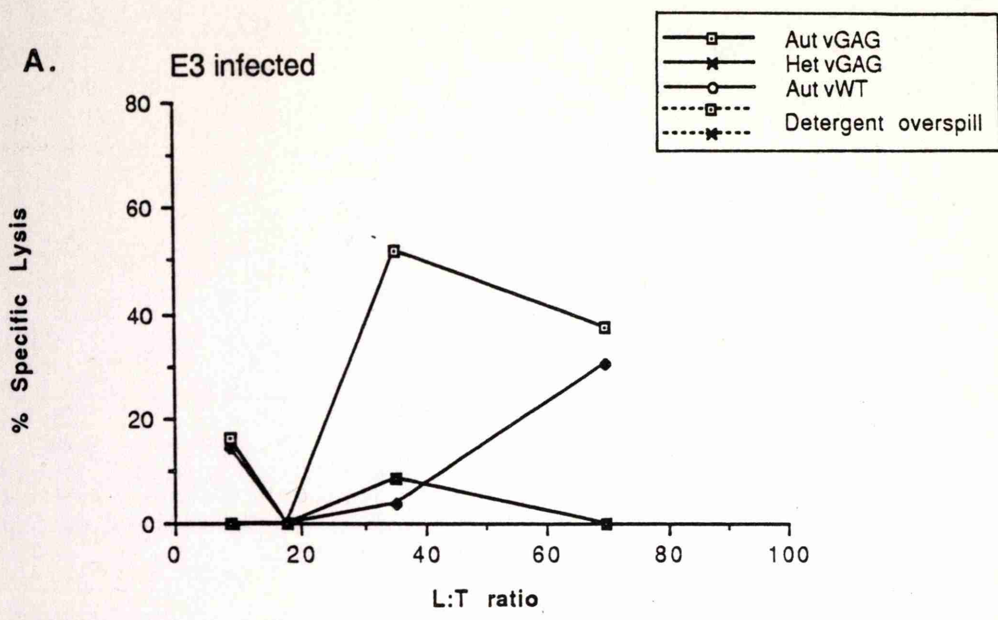
Aut = Autologous targets

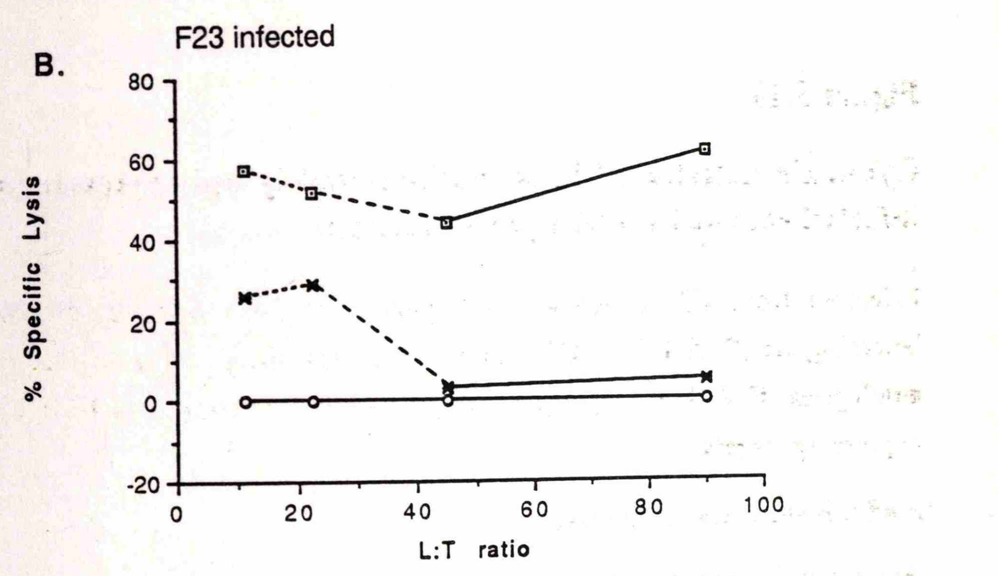
Het = Heterologous targets

vGAG = vFIV/GL14-*gag* infected

vWT = Wild type vaccinia virus infected

L:T = Lymphocyte: target ratio





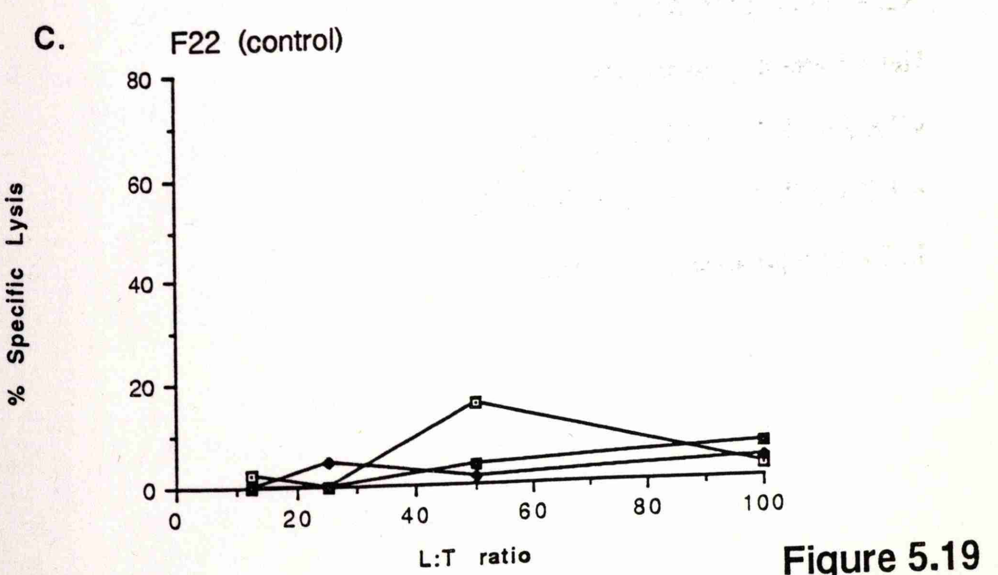


Figure 5.19

Figure 5.20

Cytotoxic activity of bulk restimulated lymphocytes from FIV-infected cats at 21 weeks post-infection

Antigen restimulation in the presence of 50 U IL2/ml was compared with 200 U IL2/ml.

A=E3, B=F23, C=F22 (control)

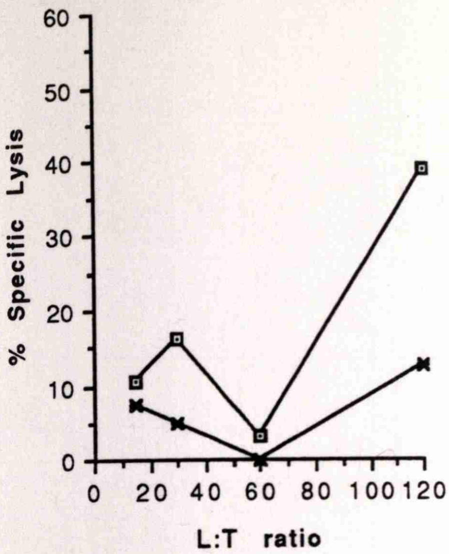
Aut = Autologous targets

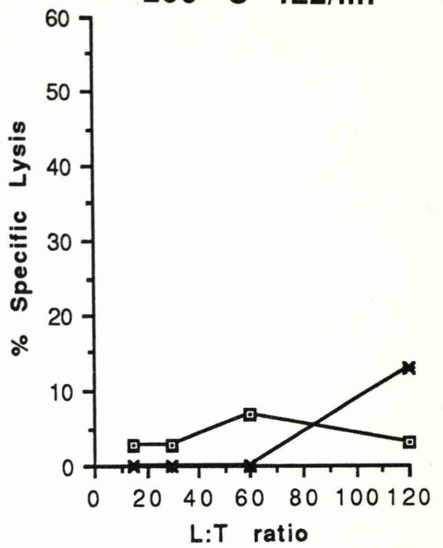
Het = Heterologous targets

vGAG = vFIV/GL14-*gag* infected

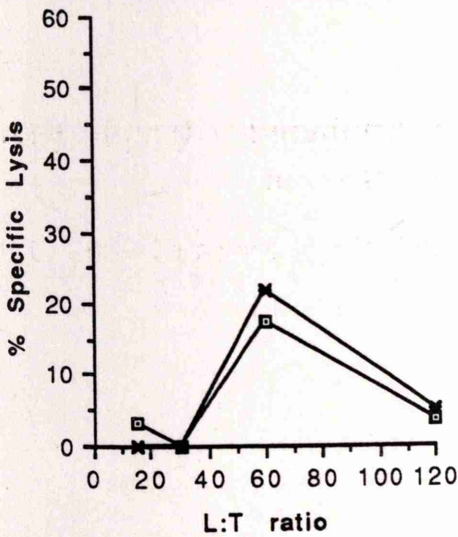
L:T = Lymphocyte: target ratio

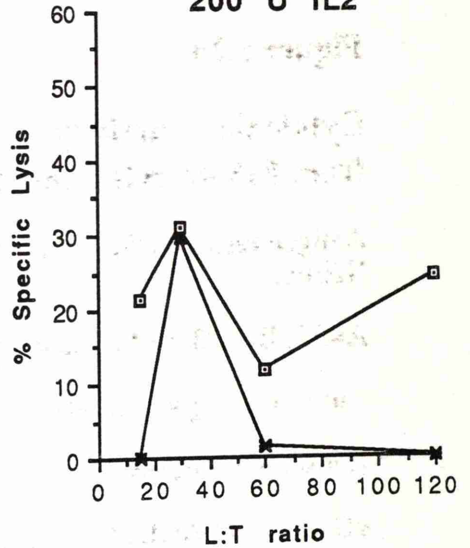
A. E3 infected



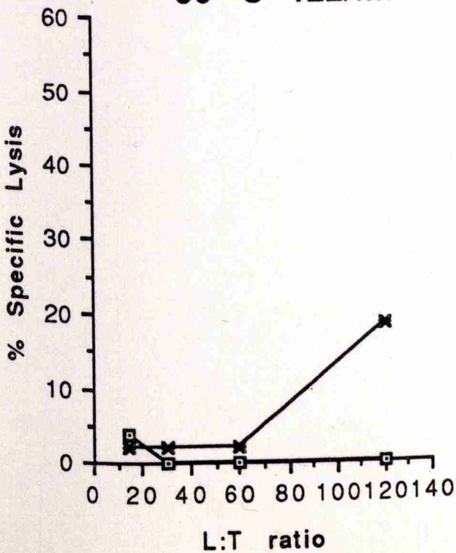


B. F23 infected





C. F22 (control)



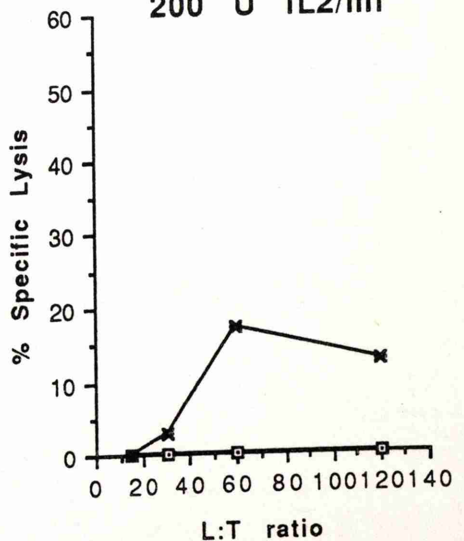
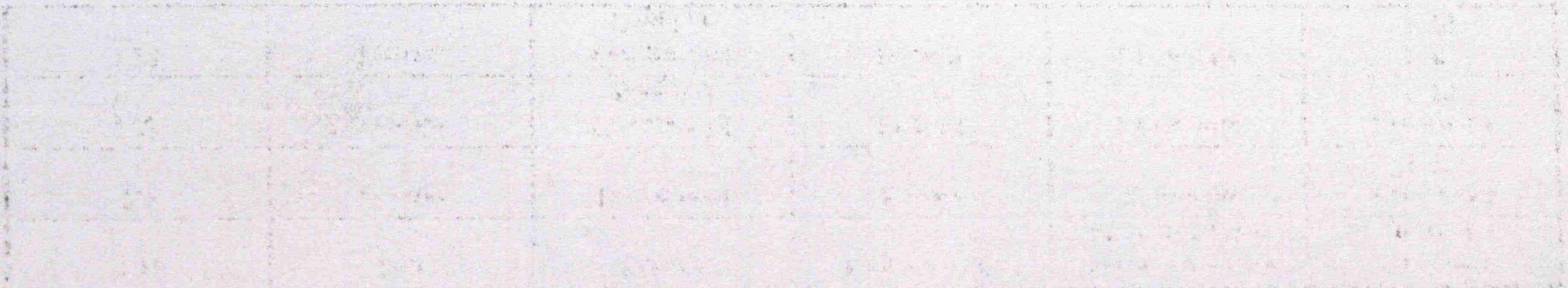


Figure 5.20

Table 5.1 Experimental cat data



Cat	Sex	Source	Date of Birth	Age at Infection/ Mock Infection	Inoculum (route)
E3	Female	Home-bred	8-5-89	49 months	FIV/GL14 (I/P)
F23	Female	Commercial Supplier	14-6-91	24 months	FIV/GL14 (I/P)
F22	Female	Commercial Supplier	14-6-91	24 months	PBS (I/P)

Table 5.1

Table 5.2

Variation in background ^{51}Cr release (%) from target cells from cats E3, F23 and F22 in each assay

Results are from a single plate except where shown. vFIV/GL14-*gag* and vWT were used to infect target cells at a 1:200 stock dilution in all assays except at 21 weeks post-infection where a 1:400 dilution was used.

	E3			F23			F22		
Weeks p.i.	vGAG	vWT	Uninfected	vGAG	vWT	Uninfected	vGAG	vWT	Uninfected
-2	29	-	11	21	-	9	-	-	-
1	34	24	19	23	17.6	14	24	20	12.5
2	23	13	7.2	21.7+/-0.9(n=3)	19.8	15.8	19.8 (n=2)	18.9	16.4
4	-	17.7	8.6	18.6	16.4	9.9	17.3	13.6	8.7
7	34+/-1.0 (n=2)	-	16	34.5+/-0.5(n=2)	15.3	12.4	27.5+/-1.5 (n=2)	20	10.5
14	15.7	22	-	22	21	-	17.8	23.4	-
21	13.4+/-2.4(n=4)	-	-	16.8+/-3.6	-	-	18+/-7.7(n=4)	-	-

Table 5.2

5.4 DISCUSSION

An assay system was developed which was capable of detecting secondary antigen-specific cytotoxic responses from PBMC from FIV-infected cats. FIV-GAG-specific cytotoxicity could be detected from 2 weeks post-infection.

This was the first occasion where cats had been experimentally infected with the GL14 isolate of FIV. Adult cats were used in this study since natural FIV infection affects kittens only infrequently and greater numbers of lymphocytes can be obtained from larger animals. Titration of FIV/GL14 in vivo was not justified since only 2 infected cats were required for this experiment. The virus inoculum used here was sufficient to infect both cats as evidenced by seroconversion. The onset of seroconversion, between 2-4 weeks, is in agreement with observations by others (Yamomoto *et al.*, 1988; Hosie and Jarrett, 1990).

Plasma from cat E3 gave high background readings on ELISA using p17 and p24 as capture antigens. It has been found in this laboratory that some normal cats have antibodies which recognise GST, an immunoaffinity tag attached to the recombinant proteins to facilitate purification (Reid *et al.*, 1991; Chapter 6). Cross-reacting antibodies to GST may be responsible for the higher background readings from cat E3 on ELISA against the recombinant FIV-GAG proteins, particularly since high background readings are not seen where FIV-ENV MAP (which do not contain GST) are used as capture antigens.

Clearance of the initial peak of plasma antigenaemia in HIV and SIV infections is temporally related to the onset of virus-specific immune mechanisms (Tindall and Cooper, 1991; Yasutomi *et al.*, 1993; Reimann *et al.*, 1994). Detection of FIV p24 antigenaemia was attempted in this study to determine if a similar correlation exists in FIV infection. In the absence of a reliable method for detecting FIV antigenaemia a commercial ELISA, designed to detect FIV p24 antigen from tissue culture samples, was used. The detection of p24 in plasma is complicated by the presence of circulating virus-specific antibodies which form complexes with free antigen. However, FIV antigenaemia could not be detected in FIV-infected cats using the method described here, even before seroconversion.

The problem of detecting FIV antigenaemia can be circumvented if viral burden is assessed more directly. Infectious FIV has been quantified from PBMC and plasma of infected cats by end point dilution using tissue culture techniques (Meers *et al.*, 1992). More recently, quantitative PCR techniques have been used to determine FIV proviral burden (Lawson *et al.*, 1993; Margaret Hosie, personal communication).

During the antigen restimulation of PBMC in vitro there was a decrease in cell numbers. This finding was surprising since antigenic stimulation would be expected to induce proliferation of specific precursor cells with non-responding cells being maintained. It is possible that there was active killing of FIV-infected lymphocytes by cytotoxic effectors during this period. Both $CD4^+$ and $CD8^+$ lymphocytes are potential targets for FIV infection (Brown *et al.*, 1991). CTL themselves can be suceptible to cell mediated lysis in certain circumstances (Teh and Yu, 1983). Alternatively, activated cells may be undergoing apoptosis and this has been demonstrated in PBMC from FIV-infected cats. However, these explanations would not account for cell loss in control (F22) cultures. It is more likely that culture conditions were limiting. The addition of feline conditioned medium during the culture period may enhance cell survival. Conditioned media are produced by mitogen activation of lymphocytes and contain factors which support the growth of T cells. In addition to feline IL2, conditioned medium would be expected to contain other feline T cell growth factors which may be limiting in the restimulation cultures.

The method devised for ^{51}Cr labelling of target cells was found to be convenient and effective. Skin fibroblasts were used at fourth or fifth passage which was the earliest possible given that fibroblasts were stored prior to use. Labelling of target cells was unsuccessful on 1 occasion because of poor isotope uptake, with target cells on all assay plates being equally affected. The reason for this failure is not known.

When designing the assay it was anticipated that a major factor limiting the ability to detect cytotoxic activity would be the small and variable number of PBMC which could be isolated from cats since this would restrict the maximum L:T ratio. This problem would potentially be exacerbated in FIV-infected cats since a leucopenia of variable duration is often seen within a few weeks of infection (Shelton *et al.*, 1990(b); Sparkes *et al.*, 1993). Steps were taken to ensure that the available cells were used as economically as possible. Triplicate rather than quadruplicate cultures were used since it was considered that the potential increase in variance would be offset by the increase in L:T ratio allowed. It is likely that the target cell number could be reduced further in these assays given the level of maximum ^{51}Cr release achieved in these experiments.

In general ^{51}Cr release assays are considered invalid when the spontaneous release from target cells is >30-35% (Walker *et al.*, 1987; Hoffenbach *et al.*, 1989; Koup *et al.*, 1989; Riviere *et al.*, 1989(b); Oldstone, 1990; Buseyne *et al.*, 1993(a)). In these

experiments, spontaneous ^{51}Cr release never exceeded 35% of the maximum release (Table 5.2).

vFIV/GL14-*gag* infection of target cells was accompanied by higher background ^{51}Cr release than that seen in uninfected target cells (Table 5.2). Ideally, background ^{51}Cr release should be similar in all target cell types. Vaccinia viruses should therefore be used at the lowest m.o.i. which allows expression of recombinant protein in the target cells. For reasons described previously (3.4), the infectivity of vFIV/GL14-*gag* for feline fibroblasts could not be determined. Consequently the amount of vFIV/GL14-*gag* used to infect target cells is expressed as a dilution of virus stock (shown to give expression of GAG in target cells by immunoblot) rather than a m.o.i.. In the 21 week post-infection assay, the virus was diluted a further two-fold compared to previous experiments (1:400 rather than 1:200) and background values from vFIV/GL14-*gag* infected target cells were consequently reduced. No vWT or uninfected targets were used in this experiment so an intra-assay comparison was not possible. It has previously been shown that FIV-GAG is detectable in the target cells by immunoblot at a 1:500 dilution of the stock (Figure 3.9).

Specific recognition of target cells by effector lymphocytes was assessed qualitatively. The values for specific lysis of different target cell types by each effector population were presented graphically. Firstly, the extent of specific killing by lymphocytes from FIV-infected cats was compared with that for the uninfected control cat at each time point. Where higher values were found from the FIV-infected cats, the relative extent of specific lysis of different target cells was compared for each FIV-infected animal to gain information on the nature of the recognition in terms of antigen specificity and MHC-restriction. In limiting dilution analysis, where the identification of individual assay wells as positive or negative is crucial, the threshold for positive wells has been defined by Carmichael *et al.* (1993) as 10% specific lysis above spontaneous release, although the exact value will vary between systems.

Uninfected autologous target cells were used as a control for antigen specificity and were useful in revealing the increase in spontaneous ^{51}Cr release from target cells accompanying vaccinia virus infection. vWT infected targets were used to control for recognition of vaccinia antigens. vWT was used to infect control target cells at a dose of virus equivalent to that of vFIV/GL14-*gag* based on the infectivities of these stocks for $TK^{-}143$ cells. A recombinant vaccinia virus expressing an irrelevant antigen may also be useful for this purpose.

The inability to MHC type these cats means that it is only possible to speculate on the contribution of MHC-restriction to these results. Interpretation of the observed responses is further complicated by the heterogeneous nature of bulk effector populations. In these experiments heterologous targets were included in each assay. At 2 weeks post infection, lymphocytes from E3 killed autologous but not heterologous (F23) targets (Figure 5.16). At 7 weeks post-infection, heterologous targets (F23) were also recognised although to a lesser extent than autologous targets (Figure 5.18). At 14 weeks post-infection lymphocytes from E3 again recognised both autologous and heterologous targets but in this case the latter were from a different cat (F22) (Figure 5.19). One explanation for these results is that the 3 experimental cats may share class I antigens so that MHC-restricted killing is being detected. The likelihood of this increases with the degree of relatedness of the animals. Cat E3 was home-bred and was therefore unrelated to the other 2 cats which were obtained from a commercial supplier. The birth dates of cats F23 and F22 (Table 5.1) show that they cannot be more closely related than half-siblings. Although this does not rule out class I homologies, it is unlikely since reciprocal recognition was not seen, i.e. E3 GAG-expressing targets were not recognised by F23 effectors at 14 weeks post-infection. MHC-restricted killing in this system is likely to be restricted by class I products since skin fibroblasts cell would not be expected to express class II products, although target cell MHC-antigen expression requires investigation.

A system for rapid DNA typing in the cat is being developed (Dr S. O'Brien, personal communication). Until such a system becomes available two-dimensional electrophoresis could be used to determine differences in class I products between cats so that truly MHC-mismatched targets could be identified (Vasilov *et al.*, 1983).

There are several possiblities for the effector cell type mediating heterologous recognition and more than one phenotype may be involved. In these experiments, the effectors which recognise heterologous targets may show specificity for alloantigens or they may recognise FIV-GAG in a non-MHC restricted manner. The inclusion of uninfected heterologous targets would have made it possible to distinguish between these possibilities. Specificities for vaccinia virus antigens and common antigens (such as FCS) can be ruled out since vWT infected autologous targets and uninfected autologous targets were not recognised.

Heterologous killing may be mediated by the same population which mediates killing of vFIV/GL14-*gag* infected targets but in a different differentiation state. Prolonged in vitro exposure of murine CTL to IL2 can result in a broader

specificity of lytic activity (Brooks, 1983; Teh and Yu, 1983; Shortman *et al.*, 1984; Kane and Clark, 1986) although this was never seen before 8-9 days (Shortman *et al.*, 1984) whereas, in the present experiments, lymphocytes were cultured for a maximum of 7 days.

Other potential cytotoxic effectors in these bulk cultures are natural killer cells (NK cells) and lymphokine-activated killer cells (LAK cells) also called NK-like cells. NK and LAK cells are characterised by a broad range of killing (Young and Cohn, 1987). NK cells are active directly ex vivo whereas mitogen-activated NK-like cells are generated on incubation with high concentrations of IL2 and lyse a broader range of targets (Abo *et al.*, 1982). It is not clear if LAK cells represent a further differentiation state of NK cells or whether they are distinct populations (Grimm *et al.,* 1982; Vujanovic *et al.*, 1988), although there is strong evidence for the latter in humans (Abo *et al.*, 1982). Previous workers have been unable to demonstrate NK activity in feline PBMC (Tompkins and Tompkins, 1985) but broadly specific NK-like LAK cells have been observed in this species following long term culture of lymphocytes in the presence of human rIL2 (Tompkins *et al.*, 1987).

IL2 was included in the restimulation cultures because it is essential for expansion and differentiation of activated lymphocytes (Rouse *et al.*, 1988; Figure 4.1). Human rIL2 was used because the feline molecule is not yet available. Feline IL2 has recently been cloned (Cozzi *et al.*, 1993) and found to have 81% identity to human IL2 at the aa level. Work by Tompkins *et al.* (1989) showed that human rIL2 had equivalent biological activity on human and feline lymphocytes. The contribution of human rIL2 to observed responses in the ^{51}Cr release assay in the present study should be ascertained. A preliminary attempt to titrate the human rIL2 concentration in the restimulation cultures gave conflicting results (Figure 5.20) and requires further investigation. It would be useful to compare the activities of lymphocytes cultured with antigen alone or with human rIL2 alone to those cultured with both. In the human system, the concentration of rIL2 used has varied between experiments from 5 U/ml (Bourgalt *et al.*, 1993; Carmichael *et al.*, 1993) to 100 U/ml (Walker *et al.*, 1989; Littua *et al.*, 1992; Johnson *et al.*, 1993) although this variation may reflect the fact that the rIL2 was from different sources. Others use conditioned medium as a source of human IL2 (Chenciner *et al.*, 1989; Hoffenbach *et al.*, 1989). It may be useful to include feline conditioned medium in the restimulation cultures since, although it would be necessary to titrate each batch, it may provide a more appropriate environment than human rIL2 and may enhance feline lymphocyte survival, as mentioned earlier.

Whatever the cell type responsible for killing heterologous cells it is unlikely to be an artefact of in vitro culture but rather generated in response to FIV infection since only low levels of heterologous recognition are seen by lymphocytes from the control cat F22 or cats E3 and F23 prior to infection.

The results presented here are similar to those of Hannant and Mumford (1989) who were investigating cytotoxic effector cells induced in response to equine influenza virus infection. These workers demonstrated cytotoxic activity against autologous and heterologous targets but only if the targets expressed viral antigens. Furthermore, killing of heterologous targets, but not of autologous targets could be abrogated by reducing the concentration of equine conditioned medium in the restimulation cultures.

Monoclonal antibodies which recognise feline cell surface molecules CD4, CD8, a pan-T marker, Class I and Class II are available so that characterisation of the effectors and the role of MHC antigens in the observed responses by positive and negative selection of lymphocyte subsets and antibody blocking experiments should be relatively straightforward. The addition of antibodies which recognise conserved regions of feline class I molecules in the restimulation cultures would enable its role in activation to be assessed.

Cytotoxic activity was first detected from cat E3 at 2 weeks post-infection but not until 14 weeks post-infection in F23. Whether these findings reflect the onset of cytotoxic activity in vivo cannot be determined since the assay has not been fully optimised. Both cats serconverted between 2-4 weeks post-infection. If these results reflect a genuine earlier cytotoxic response in E3 this may be a consequence of the larger virus dose/kg which this cat received or an age-related effect.

FIV/GL14 infection was followed by inversion of the $CD4^+/CD8^+$ ratio in both cats in agreement with observations by others (Ackley *et al.*, 1990; Novotney *et al.*, 1990; Barlough *et al.*, 1991; Tompkins *et al.*, 1991; Torten *et al.*, 1991; Bishop *et al.*, 1992(a); Hoffmann-Fezer *et al.*, 1992) (Figures 5.7 and 5.8). In cat E3, where cytotoxic responses were detected from 2 weeks post-infection, a rapid increase in the $fCD8^{+(low)}$ population was seen (Figure 5.9). This is a distinct population of $fCD8^+$ cells which express low levels of the marker (Willett *et al.*, 1993). This pattern was not seen in cat F23, where responses were not detected until 14 weeks post-infection, or in the uninfected control cat. Unfortunately, temporal constraints preventéd lymphocyte subset analysis beyond 10.5 weeks post-infection so it is not known if a similar rise in $CD8^{+(low)}$ cells accompanied detectable cytotoxic activity in cat F23. This observation should be investigated further in studies using more

cats and also by isolating fCD8$^{+(low)}$ cells by fluorescence-activated cell sorting and assaying this subpopulation directly for effector activity.

The haematological data were difficult to interpret because of the small numbers of cats involved and the limited duration of monitoring (Figures 5.10 and 5.11). The reason for the marked leucocytosis in cat E3 at the point of infection is unclear. Physiological leucocytosis can occur in cats during blood sampling when the release of catecholamines causes contraction of the splenic capsule resulting in a rapid increase in the numbers of circulating lymphocytes and neutrophils (Jain, 1993). However, the observed leucocytosis is accounted for by a mature neutrophilia. This haemogram resembles a typical "stress" haemogram in cats which involves mature neutrophilia, eosinopenia and lymphopenia. The source of any stress may be environmental although in cat F23, which was housed with E3, neutrophils were in the normal range.

Similarly, the rapid and marked lymphocytosis in cat E3 at 3.5 weeks post-infection was unexpected since haematological changes in acute FIV-infection usually involve cytopenias (Pedersen *et al.*, 1987; Yamamoto *et al.*, 1988; Callanan *et al.*, 1992 (b)).

The emphasis in this study was to devised a system capable of measuring cytotoxic effector cells. Now that this goal has been achieved the next step is to further optimise the assay. There are many variable parameters in the system and the number of lymphocyte available imposes limits on the number of conditions which can be compared in parallel. Priorities would be to maximise lymphocyte activation and survival during the pre-assay culture period, minimise the target cell number, titrate m.o.i. and optimise the assay length. Statistically significant data on the pattern of the CTL response to FIV could then be obtained from sequential post-infection assays involving a larger group of animals including age and sex-matched controls.

CHAPTER 6

IDENTIFICATION OF A RECOMBINANT FIV ENVELOPE FUSION PROTEIN WHICH STIMULATES PBMC FROM NAIVE CATS TO PROLIFERATE IN VITRO

6.1 INTRODUCTION

A major concern of research in this department is the use of FIV as a model for the development of strategies to produce a vaccine against HIV. FIV vaccines produced from inactivated whole virus and inactivated virus-infected cells protect cats from experimental challenge (Yamamoto *et al.*, 1993). Subunit vaccines have potential advantages over preparations containing whole virus arising from more specific immune stimulation which may increase efficacy and cross-protection, and greater safety. Recombinant FIV proteins have been prepared in this laboratory for use in prototype subunit vaccines and as diagnostic reagents (Reid *et al.*, 1991). As part of this programme of research, cats were inoculated with recombinant FIV p24 in immunostimulating complexes (ISCOMs) (Hosie *et al.*, 1992). ISCOMs are cage-like particles with a mean diameter of 35nm composed of antigen and Quil A which are capable of inducing cell-mediated, as well as humoral immune responses (Morein *et al.*, 1987; Morein, 1988). It was of interest to determine the host cellular immune response stimulated by this immunogen. In the absence of a working CTL assay at this stage of the project, in vitro proliferative responses to p24 were measured from PBMC of vaccinates and unimmunised cats. A recombinant protein encompassing the V3 and V4 domains (Rigby *et al.*, 1993) of FIV/GL8 ENV was included as a negative control antigen in the proliferation assays. The V3/V4 region of FIV *env* had been cloned for use as a potential immunogen since this region encodes a major neutralising domain (Lombardi *et al.*, 1993; De Ronde *et al.*, 1994).

Proliferative responses to p24 were not detected from PBMC of cats immunised with p24 ISCOMs in this preliminary study (data not presented). However, an incidental finding that recombinant V3/V4 stimulated fresh PBMC from naive cats to proliferate in vitro was investigated further and is the subject of this Chapter. The response to V3/V4 was dose-dependent and, under optimal conditions, was comparable to that seen with lectin mitogens. The ability of the V3/V4 region of FIV ENV to stimulate lymphocytes to proliferate may have important implications in the immunopathogenesis of FIV infection. HIV infection of man is characterised by a progressive immune dysfunction which eventually becomes clinically apparent as ARC and AIDS. The most consistent immunological derangement in HIV infected humans is a decline in numbers of circulating CD4+ cells (Fahey *et al.*, 1990). Many potential mechanisms have been proposed to account for the loss of CD4+ cells (1.2.6) but the crucial events which result in CD4+ cell depletion in vivo are not known. Infection of cats with FIV resembles human infection with HIV in its clinical course and pathological parameters, including progressive CD4+ cell depletion (Sparger *et al.*, 1989; Siebelink *et al.*, 1990; Ackley *et al.*, 1990).

FIV infection therefore represents an excellent experimental model in which to study this phenomenon (Jarrett *et al.*, 1990; Gardner, 1991; Kindt *et al.*, 1992).

6.2 MATERIALS AND METHODS

6.2.1 Production and immunoaffinity purification of recombinant polypeptide V3/V4GST

The V3/V4 region of FIV/GL8 ENV coding sequence, bases 961-1473 inclusive, was amplified by PCR. The product was cloned directly into pGEX-2T (Figure 6.1) as a *Bam*HI/*Eco*RI restriction fragment and used to transform *E.coli* (strain JM83) as described by Reid and colleagues (1991). This cloning was carried out by Dr G Reid. The pGEX system directs the expression of recombinant proteins as fusions with the schistosome protein GST, facilitating affinity purification of the product with glutathione, the substrate for GST. Clones containing the insert were selected and grown in 1L cultures. A sample of the culture was retained as a control for SDS-PAGE analysis, then expression of the recombinant protein was induced by addition of isopropyl beta-D-thiogalactoside (IPTG), as described by Smith and Johnson (1988). The product was released from the cells by sonicating on ice for 2 x 30 second periods using a 100w ultrasonic disintegrator (Fisons, Crawley, England, UK).

After centrifugation, the crude lysate was incubated with glutathione-agarose beads (Pharmacia, Milton Keynes, Bekshire, UK) and the slurry was used to pack a column 1cm in diameter. The column was washed in PBS and the fusion protein was eluted by adding reduced glutathione. This fraction was analysed by SDS-PAGE and its protein concentration was estimated by Bradford's assay (Bradford, 1976). The antigenic integrity of the product was checked by reactivity in ELISA with FIV-seropositive and normal cat sera. Comparative aa sequences of V3/V4 from FIV/GL8 and 5 other FIV isolates are shown in Figure 6.2.

6.2.2 Purification of polypeptide by conventional methods

Ammonium sulphate precipitation of V3/V4GST was carried out. The crude lysate was centrifuged and the supernatant retained. Solid ammonium sulphate was added to the lysate in 1.5ml polypropylene reaction tubes to give 20-70% saturation in 5% increases to screen for the cut containing the product. Precipitation was carried out for 15 minutes on ice. Precipitates were collected by centrifugation at 10,000 rpm for 10 minutes.

Precipitates were resuspended in a solution containing 8% (v/v) glycerol, 1.6% (w/v) SDS, 570mM 2-mercaptoethanol, 33mM Tris pH 6.7 and 0.016% (w/v)

bromophenol blue. Samples were denatured by boiling for 2 minutes and then analysed by SDS-PAGE using a 12% resolving gel and a 6% stacking gel. Gels were cast between glass plates using 1.5mm spacers and sealed with Teflon tape. Gels were run at 10mA overnight at room temperature. Gels were stained by soaking in 45:45:10 methanol: water: glacial acetic acid (fix) containing 0.2% (w/v) Coomassie Brilliant Blue R-250 for 1 hour. Excess stain was removed by soaking in fix without dye.

6.2.3 Lymphocyte proliferation assays

The cats used were bred and housed at the Glasgow Veterinary School SPF colony. Two of the cats had received routine vaccinations for feline herpesvirus, feline calicivirus and feline panleucopenia virus, and 2 others had been inoculated with recombinant FIV p24 in ISCOMs (Hosie *et al.*, 1992). All cats were seronegative for FIV. The mean age of the cats was 15 months (range 8-24 months). Samples of peripheral blood (5ml) were collected from 15 cats and 2 humans into pfh and PBMC were separated by density gradient separation as described in Chapter 2. PBMC were washed in 50ml of PBS. The cells were resuspended in 5ml of complete RPMI and maintained in a 25cm^2 tissue culture flask at 37°C for 1 hour. The non-adherent cell population was collected, counted and resuspended at 1 x 10^6/ml. Cells were seeded into 96-well, round-bottomed tissue culture plates at 100μl/well. V3/V4GST was added to these cultures at a final concentration of 0.01 to 20μg/ml. Control cultures were incubated with V5GST, p24GST, GST (all produced under identical conditions to those described for V3/V4GST and provided by Dr G Reid), ovalbumin or with ConA (7.5μg/ml) or culture medium alone. All antigens and ConA were diluted in culture medium and added to cultures at 100μl/well. Triplicate cultures were set-up for each assay condition. Assay plates were incubated at 37°C in a humid environment containing 5% CO_2 for 4 days. At the end of the incubation, each well was pulsed with 0.5μCi of ^{3}H-thymidine and incubated overnight, the time course having been optimised in preliminary experiments. Cells were harvested using a semi-automatic system (Skatron Combi Cell Harvester, Norway) and the activity from each cultures was read on a liquid scintillation counter (Beckman LS 1801).

Mean values were calculated for each assay condition and the stimulation index (S.I.) calculated as follows;

$$S.I. = \frac{\text{mean cpm of test wells}}{\text{mean cpm of background wells}}$$

where backgound wells contain medium alone.

6.2.4 Monoclonal antibody blocking

Monoclonal antibody vpg15 binds a 24K protein expressed on the surface of cells permissive for FIV replication, and blocks FIV infection of T cells and fibroblasts in vitro (Hosie *et al.*, 1993). The ligand of vpg15 has been identified as the feline homologue of CD9 and is believed to act as a cellular receptor for FIV (Willett *et al.*, 1994). vpg15 was purified from hybridoma culture supernatant (kindly provided by Mr T Dunsford) by protein A affinity chromatography. The antibody was added at 50µl/well at a range of concentrations to cultures of PBMC stimulated with V3/V4GST at a final concentration which was found to stimulate optimum proliferation.

6.3 RESULTS

6.3.1 Expression of V3/V4GST recombinant fusion protein

The V3/V4 region of FIV/GL8 ENV was cloned into pGEX-2T and used to transform *E.coli*. Following the addition of IPTG, the recombinant protein was expressed as a fusion protein with GST, V3/V4GST, which was released by sonication. The product was purified over glutathione-agarose beads and examined by SDS-PAGE and ELISA. SDS-PAGE analysis revealed a major band at the expected molecular weight of 45K. This band was not present prior to the addition of IPTG. The final yield of purified fusion protein was approximately 5 mg/L. The fusion protein reacted with plasma from FIV seropositive cats, but not with normal cat plasma, by ELISA (Figure 6.3).

Further attempts to use immunoaffinity purification to isolate V3/V4 GST were unsuccessful. The reason for this is not known.

6.3.2 Purification of polypeptide by conventional methods

As an alternative to immunoaffinity purification, ammonium sulphate precipitation of the crude lysates was carried out. Precipitates were examined by SDS-PAGE and the results are shown in Figure 6.4. V3/V4GST was first present in the precipitate at 35% ammonium sulphate, but at low yield compared to the crude lysate. At 40% ammonium sulphate, contaminating proteins were present in the precipitate. Ammonium sulphate precipitation was not a suitable method for purifying V3/V4GST from the crude lysate.

6.3.3 Lymphocyte proliferation assays

The ability of V3/V4GST fusion protein to stimulate PBMC was determined by lymphocyte proliferation assays. The S.I. for V3/V4GST and ConA for the 15 FIV seronegative cats and 2 humans tested are listed in Table 6.1. In all 15 cats there was a proliferative response to the recombinant fusion protein V3/V4GST. This response was dependent on the concentration of V3/V4GST over the range tested (Figure 6.5). Optimum proliferative responses in feline PBMC were achieved using a concentration of 5-10 μg/ml V3/V4GST. The mean S.I. using V3/V4GST to stimulate PBMC was 6.3 (S.E. = 0.7). With ConA stimulation, the mean S.I. was 15.4 (S.E. = 5.5). The S.I. with control antigens V5GST, p24GST, GST alone and ovalbumin, was never in excess of 1.5. Human PBMC showed no response to V3/V4GST (Table 6.1, Figure 6.6).

6.3.4 Monoclonal antibody blocking

In order to investigate whether the lymphoproliferative effect of V3/V4GST fusion protein was mediated though an interaction with the putative host cell receptor for FIV, monoclonal antibody blocking experiments were carried out using vpg15. At concentrations of 0.01-10μg/ml, vpg15 did not inhibit proliferation of feline PBMC stimulated with either 5μg/ml or 10μg/ml V3/V4GST (Figure 6.7).

Figure 6.1

pGEX

The pGEX expression system directs the synthesis of fusion proteins with GST, encoded by Sj26, under the control of the IPTG inducible tac promoter (Smith and Johnson, 1988). Arrows indicate the direction of transcription.

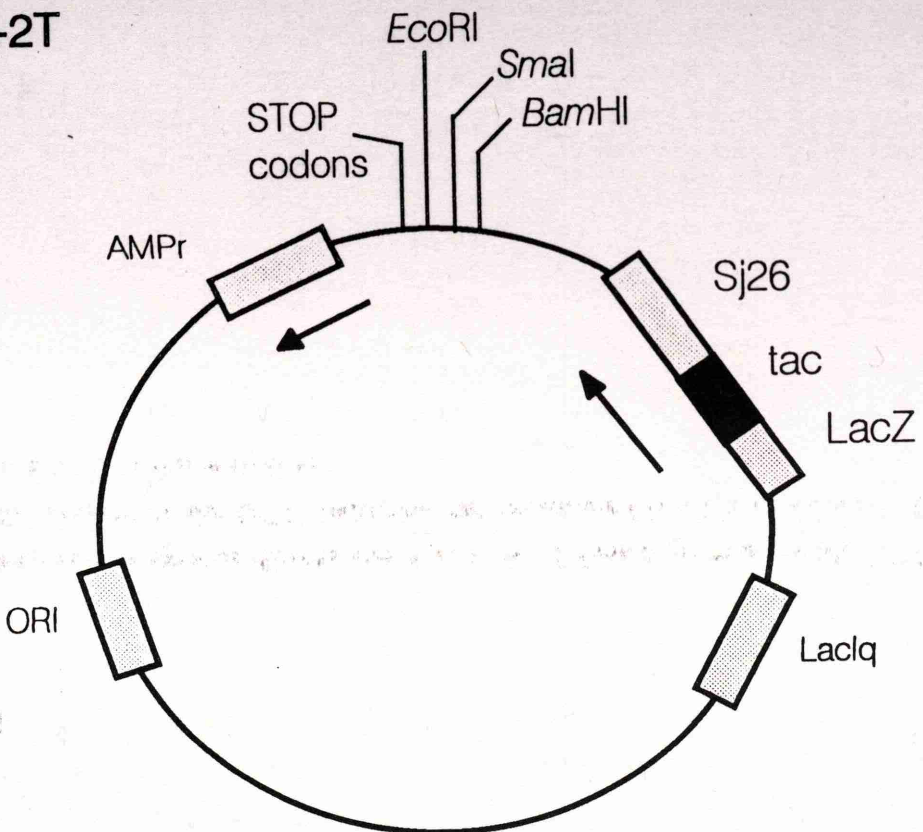


Figure 6.1

Figure 6.2

Comparative amino acid sequences of FIV *env* corresponding to the V3/V4 fragment

G8, G2, and G14 are UK isolates (Rigby *et al.*, 1993); PPR (Phillips *et al.*, 1990), PET (Talbott *et al.*, 1989) and 14 (Olmstead *et al.*, 1989) are US isolates. Bars represent glycosylation sites.

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G8    DPLQIPLINYTFGPNQTCMWNTSQIQDPEIPKCGWWNQIAYYNSCRWESTDVKFHCQRTQSQPGLWLRAISSWKQRNRWEWRPDF
G2    ............................SD...................Q...............T...T...............
G14   .............................D.........R...........D.......K.....S.S.................
PPR   ...................................................N...Y.........T.I.T.....R.K.......
PET   .......................................M.......K.EAK.............S.F.................
14    .......................................M.......K.EAK.............S.F.................
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V3

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G8    ESEKMKISLQCNSTQNLTFAMRSSGDYGEVTGAWIEFGCHRNKSKLHTEARFRIRCRWNVGDNCSLIDTCGETQNVSGANPVDCT
G2    ....VRV......T.K................................SD..............T......NDP...........
G14   ....V.V........K................................S...............T............D.H.....
PPR   ....V..........H...............M.............RF............A....T......KNL...........
PET   K.K.V.....P.N..K.............................N................SDT......N.P...........
14    K.K.E.....P...QK.............................N...K...A........SDT..N...N.P...QK......
```

V4

Figure 6.2

Figure 6.3

Detection of antibodies recognising V3/V4 from FIV-infected cats

Plasma samples from 4 FIV seropositive cats recognised the fusion protein on ELISA (triangles, large circles, diamonds and squares), whereas plasma from a seronegative cat (small circles) did not react.

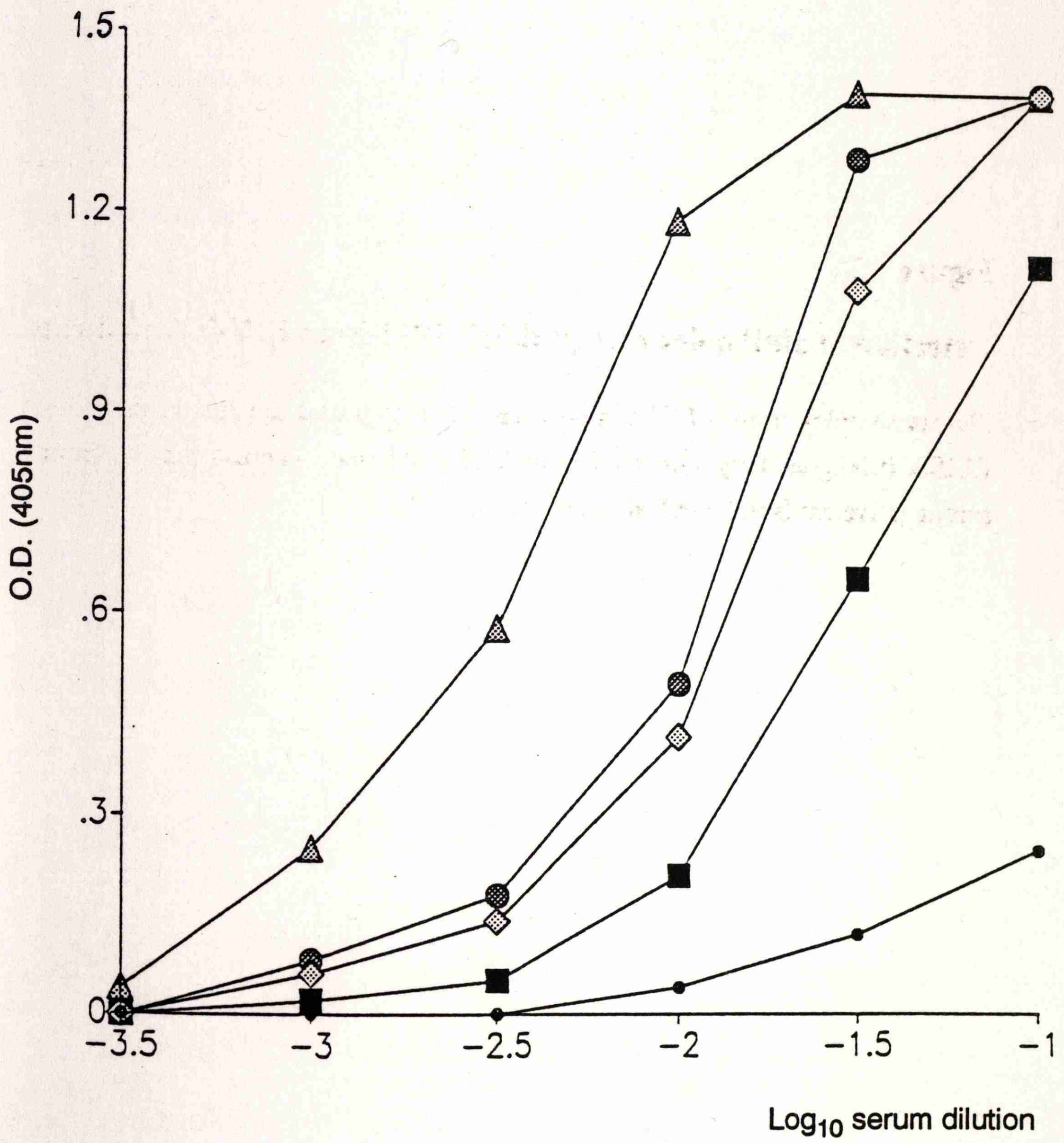

Figure 6.3

Figure 6.4

Ammonium sulphate precipitation of V3/V4GST

Lane a contains molecular weight markers, lane b is empty, lane c contains crude lysate, lanes d-n contain 30%-80% ammonium sulphate precipitates increasing in 5% steps.

The product V3/V4GST (45K) was present at 35% ammonium sulphate but at a low yield compared to the crude lysate. At 40% ammonium sulphate other contaminating proteins were present. Therefore, this was not a suitable method for purification of V3/V4GST from the crude lysate.

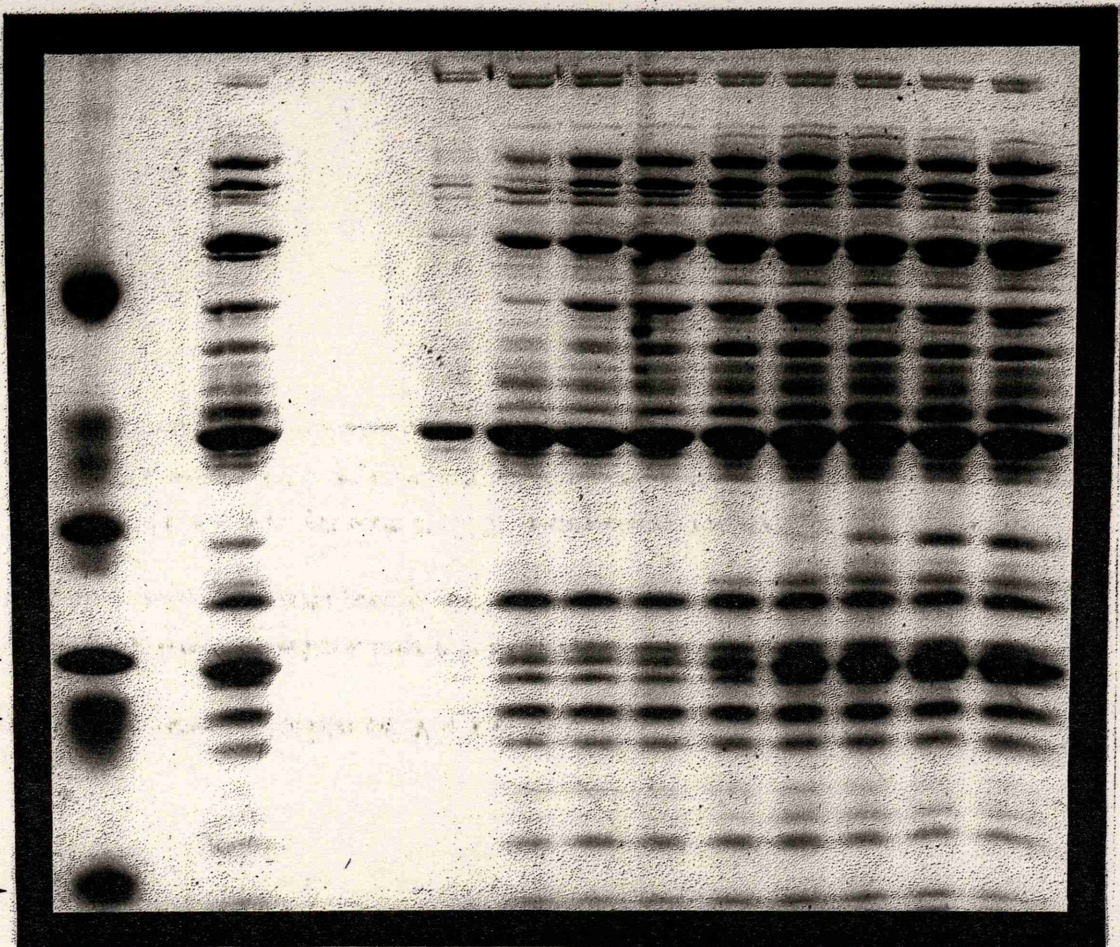

Figure 6.4

Figure 6.5

Proliferation of PBMC from a naive cat, in response to V3/V4GST

Proliferation of PBMC from cat G4 incubated with V3/V4GST was measured by ^{3}H-thymidine uptake. There was no response to control antigens.

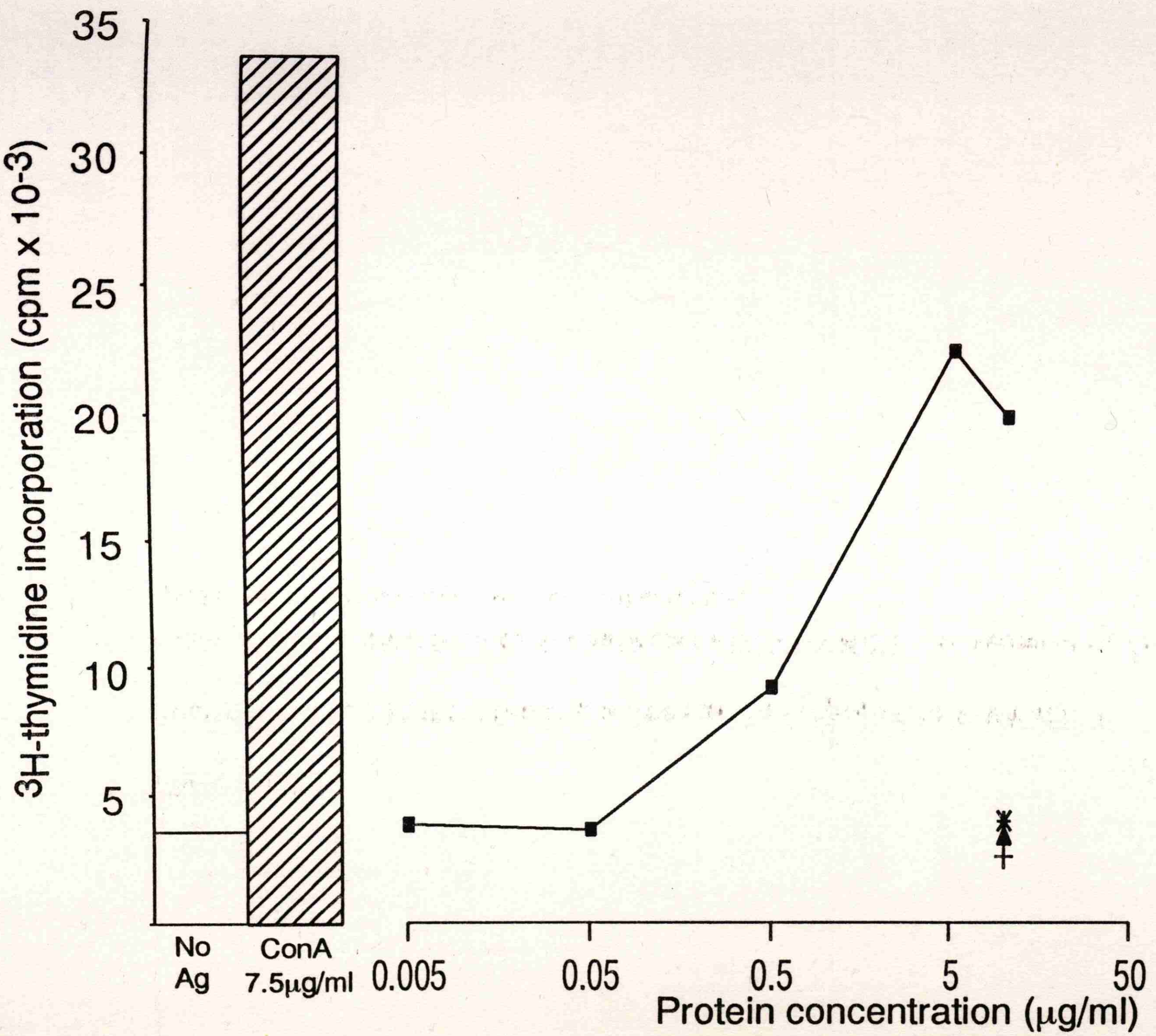

Figure 6.5

Figure 6.6

Comparison between responses of feline (A3,G4,A9) and human (JAB) PBMC to ConA and V3/V4GST

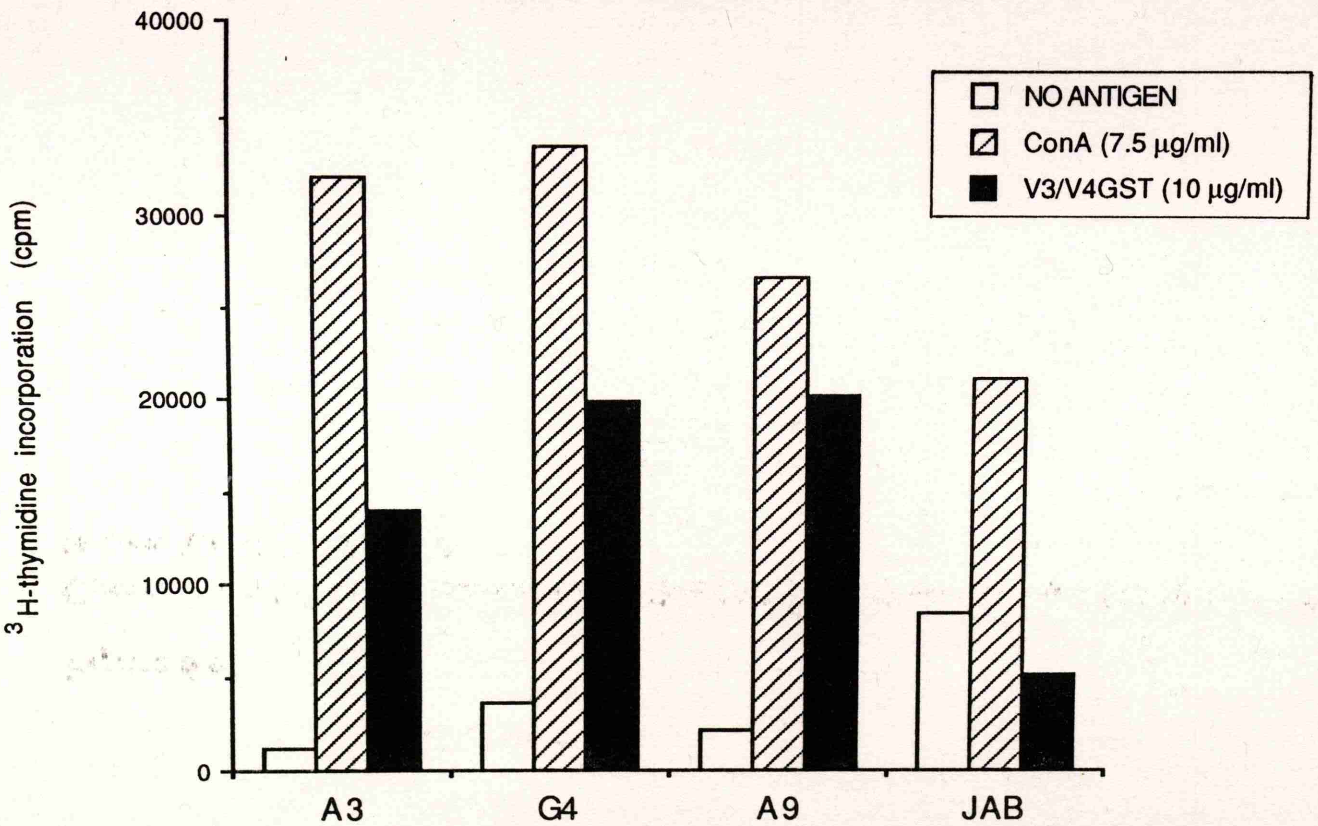

Figure 6.6

Figure 6.7
Titration of monoclonal antibody vpg15 on naive feline PBMC stimulated with V3/V4GST

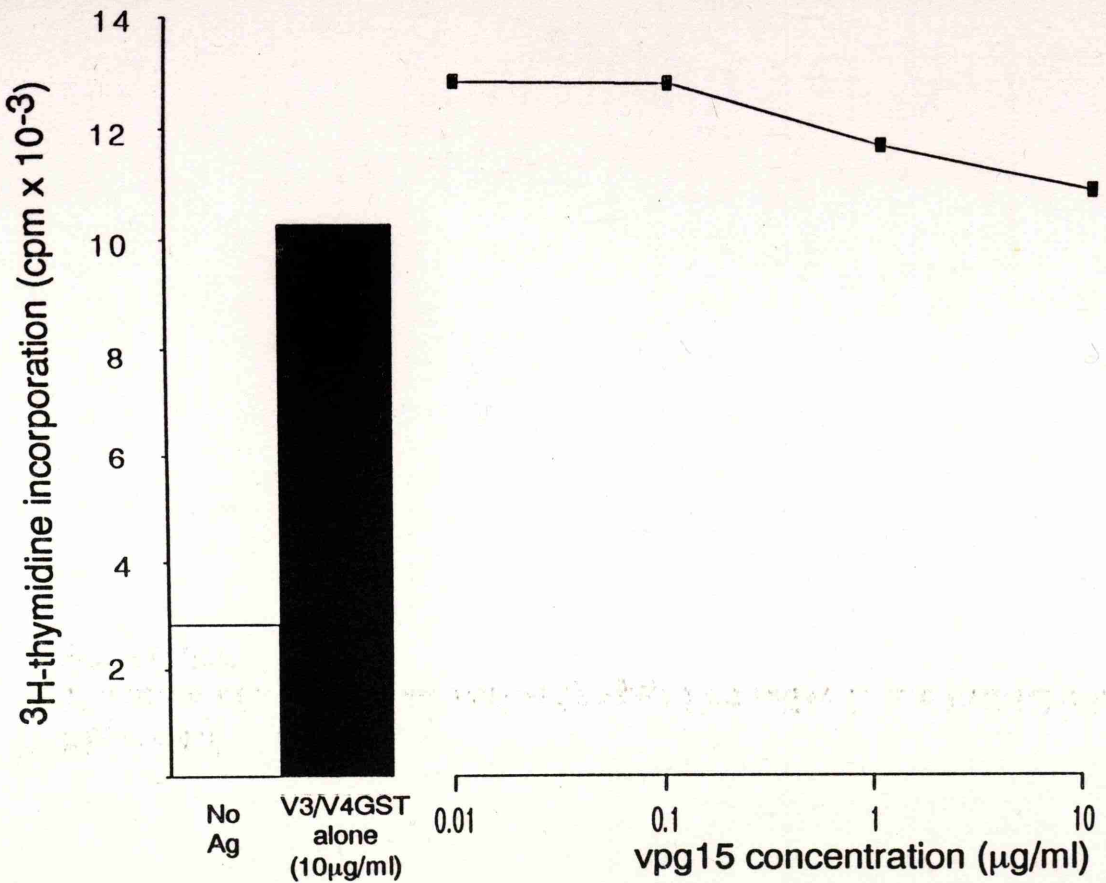

Figure 6.7

Table 6.1

Responses of feline and human PBMC to V3/V4GST

V3/V4GST was used to stimulate PBMC from 15 naive cats and 2 humans and proliferation was measured by ^{3}H-thymidine incorporation. Stimulation indexes obtained with ConA are included for comparison.

SAMPLE	V3/V4GST (μg/ml)	V3/V4GST S.I.	ConA S.I. (7.5μg/ml)
Feline			
G1	10.0	2.7	7.0
C1	10.0	2.9	3.0
G4	5.0	6.3	9.4
Z2	5.0	4.8	6.0
L2	5.0	7.4	5.5
A8	10.0	3.6	8.5
A3	10.0	11.9	27.2
A9	10.0	9.4	12.3
A1	10.0	5.7	5.2
G4	10.0	7.7	9.0
E3	5.0	5.0	24.2
285	5.0	6.0	7.1
321	10.0	8.0	4.4
Sa	10.0	3.6	11.9
E1	10.0	10.4	91.0
Human			
MJB	10.0	0.7	2.3
JAB	10.0	0.6	2.5

Table 6.1

6.4 DISCUSSION

It was shown that the recombinant fusion protein V3/V4GST stimulated a high level, dose-dependent proliferative response in unprimed feline lymphocytes. This effect was specific for the V3/V4 gene product, as shown by the absence of a response to GST alone or to another FIV ENV fusion protein (V5GST) or to an FIV GAG fusion protein (p24GST) all produced under identical conditions, or to ovalbumin.

The demonstration that a viral polypeptide stimulates lymphocytes from animals which have not previously encountered that virus to proliferate is unusual. Classic antigen-induced proliferative responses require immunological priming with the antigen before proliferation in vitro is detectable. Although hyper-responsive individuals may exist whose lymphocytes will proliferate on first exposure to an antigen such individuals would be expected to be rare and could not account for the results presented here as the response was seen consistently in 15 cats and was specific for V3/V4GST.

There are several possible explanations for these observations. Firstly, V3/V4GST may mimic an antigen to which these cats have been immunologically primed and the observed proliferation would therefore represent a cross-reactive, anamnestic response. If this were the case, all the cats would have to have been exposed to the priming antigen. Since these cats were chosen at random from several different pens there are few potential sources for such an antigen, although dietary exposure cannot be ruled out. In addition, the priming antigen would require to stimulate an unusually high level of precursor lymphocytes to account for the magnitude of the response. As stated earlier (5.4), it has been noted in this laboratory that some cats have antibodies which recognise GST on ELISA without previous exposure to the antigen. These cats might therefore be expected to show T cell proliferation to GST in vitro. However, the absence of a proliferative reponse to other GST fusion proteins or to GST alone suggests that this response in PBMC from naive cats is V3/V4 specific.

Secondly, the fusion protein may directly activate cells by cross-linking a cell surface molecule. There are several precedents for this effect, notably the cross-linking of the T cell receptor/CD3 complex (Weiss A *et al.*, 1986). If V3/V4GST-induced proliferation were to operate through a surface receptor cross-linking effect, one of the main candidates for its target would be the cellular receptor for the virus. Whilst we cannot rule out this possibility, we were unable to inhibit the proliferative response to V3/V4GST with monoclonal antibody vpg15, which has been shown to bind a cellular receptor for FIV and to prevent FIV infection in vitro

over a similar dose range to that tested here (Hosie *et al.,* 1993). A third possibility is that V3/V4GST is mitogenic for feline lymphocytes. This hypothesis would account for both the magnitude and the apparently unprimed nature of this response. Conventional mitogens stimulate PBMC from a wide range of species. As V3/V4GST did not stimulate human PBMC, its mitogenic activity would have to have a species restricted effect. It will be interesting to determine the species specificity of the response to V3/V4GST.

Finally, the stimulation of unprimed PBMC by V3/V4GST might represent a superantigen response. Superantigens bind to conserved regions of class II MHC molecules to form ligands capable of inducing proliferative responses in T_h cells which express specific Vβ products in their T cell receptor. Since there is a limited number of families of Vβ genes (14 in the mouse) compared to the vast diversity of antigen specific clones, superantigens have the potential to stimulate a much higher proportion of T cells. Known superantigens are a diverse group of molecules derived from infectious agents including bacteria (White *et al.,* 1989), mycoplasms (Cole and Atkin, 1991) and retroviruses (Choi *et al.,* 1991). The possibility that HIV may encode a superantigen has been raised previously (Janeway, 1991), but evidence remains uncertain (discussed in 1.2.6 *i)*). It is suggested that excessive stimulation by a superantigen may account for the depletion of this of $CD4^+$ T cells, which is a feature of both HIV and FIV infections (Polk *et al.,* 1987; Ackley *et al.,* 1990), by exhaustion or provision of further infectable targets. In this model, polyclonal B-cell activation might be due to either a direct effect of the superantigen, or may occur secondary to increased T_h activity.

It was not possible carry out further investigations into the mechanism whereby FIV/GL8 ENV V3/V4 stimulates proliferation of feline PBMC in this study due to the limited availability of recombinant protein. This was due to failure of immunoaffinity purification after production of the initial batch despite repeated attempts using this method. Conventional purification techniques were therefore attempted. V3/V4GST has a high isoelectric point relative to most other proteins (8.26 cf 5-6) and it was anticipated that this would facilitate purification by ammonium sulphate precipitation followed by ion exchange chromatography. However, ammonium sulphate precipitation was not a suitable method. The use of alternative vector systems may be required to obtain more V3/V4 so that its action can be studied further (Chapter 7).

When more V3/V4 becomes available the mechanism of its action can be investigated. In most species, this problem could be approached by assessing the response to V3/V4GST in isolated populations of T and B cells. T or B cell

mitogens would be expected to induce proliferation in purified lymphocytes, whereas conventional or superantigens require the presence of both populations for a response. Adding V3/V4GST to T cells in the presence of fixed or unfixed B cells could then be used to differentiate between classical antigen-induced proliferation and a superantigen response since the latter does not require intracellular processing. However, class II MHC antigens are constitutively expressed on feline T cells (Rideout *et al.*, 1990) and therefore a superantigen response may be seen in in isolated T cells. When monoclonal antibodies which recognise feline Vβ chains become available, flow cytometric analysis of PBMC pre- and post-stimulation could be used to detect changes in T cell Vβ subsets. The response to V3/V4GST in cultures of PBMC which had been depleted or enriched for the proliferating subset could be used to confirm the responding population. The effect of adding antibodies to cell surface molecules on the response to V3/V4GST could be investigated e.g. using antibodies to class II, Vβ chains, CD4 and CD8. Another approach would be to use quantitative PCR for Vβ chains following stimulation with V3/V4GST. A feline Vβ chain has been cloned (Dr Rojko, personal communication) which might be used as a source of degenerate primers.

CHAPTER 7

GENERAL DISCUSSION

Persistent infection of cats with FIV results in a progressive immune dysfunction which eventually becomes manifest as a clinical immunodeficiency syndrome. The immune mechanisms responsible for the maintenance of the asymptomatic state are of particular interest since these represent a natural, though incomplete, immunity to FIV. An understanding of these mechanisms will provide us with a rational basis for eventual therapeutic and prophylactic immunological interventions.

The humoral response to FIV was characterised soon after the first isolation of the virus (Hosie and Jarrett, 1990) but there has been little published on the role of cellular immunity to FIV. In particular, information on the CTL response to FIV would be useful for several reasons. Firstly, despite the fact that many infected cats have high levels of antibodies to FIV, the infection persists and recovery from infection has not been documented. Ironically, since the detection of circulating antibodies to FIV is the basis of the diagnostic test for infection, their presence is associated with a guarded prognosis. Secondly, the role of virus-specific CTL in containing other persistent virus infections is well established (reviewed in Chapter 1). Thirdly, there is compelling circumstantial evidence that CTL play a role in containing virus in HIV-1 and SIV infections (McMichael and Gotch, 1993). The CTL response to HIV-1 is of much greater magnitude than that seen in other virus infections; high levels of primary CTL activity can be detected in PBMC from asymptomatic patients, pCTL frequencies are high and many CTL epitopes have been identified within the virus. HIV-1 infection in man shares many of the pathogenic features of FIV infection in the cat (Jarrett *et al.*, 1990; Siebelink *et al.*, 1990). Hence, FIV infection in the cat provides a unique experimental system in which to study the contribution of CTL in the immune response to an immunosuppressive lentivirus in its natural host.

The studies carried out during this project were principally concerned with the development of an assay system for detecting feline CTL. The problems experienced by previous workers in performing cytotoxicity assays in the cat have been reflected in the absence of a consistent and reliable method to this point. Adaptation of the ^{51}Cr release cytotoxicity assay (Brunner *et al.*, 1968), the standard method for detecting murine and human CTL, for use in the cat is not straightforward. In particular, a suitable feline target cell system has been elusive. In the absence of a system for MHC-typing in the cat, autologous targets must be used. No reliable transforming factor for feline cells in vitro has been identified so that target cells must be able to grow well in culture and survive storage between assays.

In Chapter 3 the development of a target cell system for the assay is described. Others have detected FIV-specific cytotoxic cells using stored FIV-infected or

uninfected autologous T lymphoblastoid cells as targets (Song *et al.*, 1992). It was found (Chapter 4) that the regular addition of fresh lymphocytes was necessary to maintain FIV-infected T lymphoblastoid cell lines and viability after storage was variable. For these reasons the use of lymphocytes as targets was not attempted here. Renal, bone marrow and skin fibroblasts were assessed for suitability as target cells. Fibroblast cells are not normally infectable by FIV so an alternative method of expressing endogenously processed FIV antigens in these cells was sought. vFIV/GL14-*gag* infection of fibroblasts was found to be suitable for this purpose. The in vitro growth characteristics and onset of GAG antigen expression following vFIV/GL14-*gag* infection were found to be similar in all fibroblast types studied. Feline renal fibroblasts have been used previously in ^{51}Cr release assays to detect cytotoxic cells raised against inactivated feline herpesvirus I and feline panleucopenia virus vaccines (Tham and Studdert, 1987(a); Tham and Studdert, 1987(b)). However, the highly invasive procedure necessary to harvest renal fibroblasts could not be justified here as their use offered no specific advantages. In addition, as assay based on the use of renal fibroblasts would not be suitable for use in naturally infected animals for ethical reasons.

Skin fibroblasts were selected over bone marrow fibroblasts as target cells for the assay based on the more reliable recovery from storage of these cells. Other workers have used skin fibroblasts as target cells to detect FeLV-specific cytotoxicity (Zeidner *et al.*, 1993). A colorimetric detection system was used by these workers as an alternative to ^{51}Cr release. The use of skin fibroblasts targets coupled with a colorimetric detection system has been used by other workers for detecting CAEV-specific CTL (Lichtensteiger *et al.*, 1993). Zeidner and colleagues found that the use of ^{51}Cr release to detect target cell lysis was not possible due to high levels of spontaneous isotope release from skin fibroblasts (Dr NS Zeidner, personal communication). In our hands background ^{51}Cr release from skin fibroblast target cells was acceptable (Chapter 5). ^{51}Cr labelled human skin fibroblast cells have been used successfully by others as target cells for detection of, for example, CMV-specific and HIV-1-specific CTL (Quinnan *et al.*, 1982; Shepp *et al.*, 1988).

The system of ^{51}Cr labelling developed here is different from those described previously in that skin fibroblasts are incubated with the isotope in 96-well plates overnight. Targets can therefore be handled as monolayers which has several advantages over using suspension cells; target cells only need to be counted once, washing to remove excess ^{51}Cr and vFIV/GL14-*gag* can be completed more quickly as plates do not require centrifugation between washes, ^{51}Cr labelling and vFIV/GL14-*gag* infection are carried out in the assay plate so that less manipulations of the target cells are required and finally the workload is spread over 2 days.

Since it was first shown that greater and more rapid cytotoxic activity could be measured from lymphocytes primed in vivo by restimulating in vitro prior to assay (Häyry and Defendi, 1970), an in vitro restimulation step has become standard in the measurement of CTL function. Many studies have been carried out to determine the requirements for the generation of secondary CTL responses from pCTL in vitro (Dunlop and Blanden 1976; Gardner and Blanden, 1976; Zinkernagel and Doherty, 1979). In most cases there is a requirement for specific antigen presented by autologous class I MHC products to activate pCTL (Figure 4.1). Expansion and maturation of this activated population requires the participation of various cytokines.

Different types of antigen presenting systems were prepared for the activation of FIV-specific pCTL in vitro (Chapter 4). Virus-infected cells are often used by others as a convenient source of appropriately processed antigen for activation. This is achieved either by adding live virus (Kulkarni *et al.*, 1993) or virus-infected cells (McMichael and Askonas, 1978; Offit *et al.*, 1991) to cultures. Inactivated viruses have been used successfully for restimulation but only where they retain a fusogenic capacity, e.g. Sendai virus (Koszinowski and Simon, 1979) and influenza virus inactivated by UV light (McMichael and Askonas, 1978), suggesting that the insertion of viral proteins into APC for processing is required. Viral peptides have also been used for activation of pCTL. They are believed to act by associating with class I molecules at the cell surface (Townsend *et al.*, 1986(b)). The use of synthetic viral peptides for restimulation thus depends on the selection of a viral CTL epitope of 8-10 aa in length. Addition of appropriate synthetic peptides to PBMC cultures results in a much more powerful restimulation than the use of virus-infected cells (Dr A Carmichael, personal communication).

Mitogen activated autologous PBMC are often used for restimulation of HIV-1-specific pCTL (Nixon *et al.*, 1988). The rationale behind this procedure is that mitogen activation increases HIV-1 antigen expression so that these cells can present antigen to pCTL. This method of stimulator cell production was attempted here for FIV. However, due to the length of time taken to achieve expression of antigens detectable on ELISA and the individual variation between cats this method was not pursued. It is possible that mitogen activated PBMC from FIV-infected cats may be capable of presenting viral antigens to pCTL before FIV antigens can be detected on ELISA. Given the many variables in the CTL assay system it was decided to use a stimulator cell preparation which could be shown to be expressing FIV antigens to give the best chance of achieving a working assay. vFIV/GL14-*gag* infected autologous lymphoblasts were found to express GAG proteins repeatably and to high levels and preparation of these cells was rapid and efficient compared to FIV-

infected lymphoblasts. Fixation of vFIV/GL14-gag infected stimulator cells was carried out to protect pCTL from vaccinia virus cpe. A low concentration of paraformaldehyde (0.1%) was used for fixation of the stimulator cells as others have shown that stimulation capacity can be lost as the concentration of fixative increases (Bubbers and Henney, 1975; Lightbody and Kong, 1978). Inactivation of vaccinia virus in the final preparations of stimulator cells was not formally demonstrated here although vaccinia virus cpe could not be detected in restimulation cultures. Stimulator cells could be used in infectious centre assays (3.3.4) to detect any residual infectious vaccinia virus particles. Recently, fixed, recombinant vaccinia virus-infected lymphoblasts stimulator cells have been shown to stimulate secondary HIV-1-specific CTL responses (Van Baalen *et al.*, 1993).

The cytokine requirements for the expansion of activated T cells in vitro have not been fully determined (Rouse *et al.*, 1988). The participation of IL2 in this process is now undisputed (Kern *et al.*, 1981) and exogenous IL2 was added to restimulation cultures here (Chapter 5). The role of other cytokines is less well defined. A plethora of cytokines have been shown to have various and often conflicting effects on this process in vitro. The pleiotropic nature of these substances makes interpretation of the data difficult in all but the simplest of systems. The addition of IL1 (Gery *et al.*, 1972), previously called lymphocyte activating factor, to restimulation cultures, was considered here. The role of IL1 in T cell activation is controversial. Some APC (monocytes and macrophages) produce IL1 following PHA or LPS stimulation whereas dendritic cells, which have a powerful antigen presenting capability, do not produce IL1 although its presence can enhance their action. IL1 enhances production of lymphokines, including IL2 and IL3, and IL2 receptor expression by T cells (Mizel, 1982). Under certain conditions IL1 causes T cell proliferation and this phenomenon is utilised in a bioassay to measure IL1 activity. Overall it seems that IL1 has at least an enhancing effect on the process of T cell activation, although it is no longer thought to be essential. The existence of a feline homologue of IL1 has been shown (Goitsuka *et al.*, 1987) but the recombinant molecule is not yet available. The extent of cross-species reactivity is variable (Bird and Sakltavala., 1986; Thieme *et al.*, 1987). Feline IL1 could have been prepared by overnight stimulation of adherent PBMC with LPS and the culture supernatant assayed for IL1 activity on a murine bioassay (Dr C Lawrence, personal communication). Given the problem of removing LPS from the preparation and the facilitatory rather then essential role of IL1 in other systems it was decided not to include IL1 in the initial attempts to generate secondary CTL.

In the experiments described in Chapter 5 the prototype assay system was used to detect cytotoxic effector cells from cats experimentally infected with FIV.

Secondary, GAG-specific cytotoxic effector cells could be generated from PBMC from 2 weeks post-infection in 1 FIV-infected cat and from 14 weeks post-infection in the other. Now that this assay has been established the next step is to fully optimise the system as discussed in Chapter 5. The phenotype of cytotoxic effector cells in FIV infection can also be determined. Previous authors have described FIV-specific cytotoxicity as being mediated by CD8+ effector cells although the evidence presented did not support this assertion (Song *et al.*, 1992). The assumption was based on the preferential expansion of CD8+ cells during in vitro restimulation. A common approach to determine the phenotype of effector cells is to use positively and negatively selected lymphocyte subsets as effectors and compare the lysis due to each with that of the unfractionated population. This approach has been used by us to show that immunisation of cats with a synthetic FIV peptide induces antigen-specific CD8+ cytotoxic cells which recognise autologous targets (Flynn *et al.*, in press). Supporting evidence for the identity of the effector population could be provided by adding monoclonal antibodies to class I and class II MHC products and CD4 and CD8 molecules and observing the effects on specific lysis.

The identification of effector cells as CTL requires the demonstration of MHC-restriction of the response. In addition CTL would be expected to express the fCD8 cell surface marker and consequently be restricted by class I MHC products since the only situation described to date where CD4+ CTL have potential in vivo significance is in measles virus infection (Jacobson *et al.*, 1984). Care must be taken when describing feline cytotoxic responses as MHC-restricted. Previous authors have interpreted the lysis of autologous but not heterologous targets as an MHC-restricted response (Song *et al.*, 1992) without evidence of genuine MHC mismatching. Others have described "MHC-restricted feline CTL" where controls for antigen-specificity and heterologous target recognition were not included in the data (Tham and Studdert, 1987(a); Tham and Studdert, 1987(b)). Until MHC-typing of cats becomes routinely available, immunoprecipitation of radiolabelled class I MHC molecules followed by two-dimensional electrophoresis would allow the selection of genuinely class I MHC mismatched targets. Preliminary attempts to use this technique in these studies were unsuccessful (data not presented). The murine monoclonal antibody W6/32 (monomorphic anti-human class I heavy chain, Parham *et al.*, 1979) was used to immunoprecipitate class I molecules. Although this antibody has been used by others for this purpose (Pollack *et al.*, 1988), W6/32 does not consistently recognise feline class I MHC products (Dr B Willett, personal communication). However, an anti-sheep class I monoclonal antibody has been identified which consistently cross-reacts with feline class I MHC products (VPM 19, Serotec). The availability of this reagent might therefore allow mismatched

targets to be identified. In addition, the expression of class I MHC molecules on feline skin fibroblasts cells could be investigated.

A consequence of the use of bulk effector cell populations is that there is likely to be more than 1 effector cell type (discussed in Chapter 5). The activity of NK cells in particular may mask CTL responses. A monoclonal antibody which identifies feline NK-cells has recently been identified (Dr M Tompkins and Dr B Willett, personal communications). The antibody recognises the human CD57 molecule (American Tissue Culture Collection, TIB200) and cross-reacts with the feline molecule. The availability of this antibody will allow the removal of NK-cells prior to in vitro culture allowing easier interpretation of the results of bulk culture assays.

So far we have detected GAG-specific cytotoxic responses in cats infected with FIV/GL14. The extent of recognition of vFIV/GL14-*gag* infected targets by CTL raised against heterologous FIV isolates should be determined. The 225 aa construct in vFIV/G14-*gag* contains only 7 non-synonymous changes from a consensus sequence determined for 4 European, 1 Japanese and 2 American FIV isolates (Dr M Rigby, personal communication). Therefore, it is expected that it will be possible to use vFIV/G14-*gag* infected targets to detect anti-GAG CTL from cats infected with different isolates. In addition to vFIV/GL14-gag, we now have available a vFIV/PET-*env* recombinant (kindly supplied by Dr EB Stephens, University of Florida, USA). A further 2 vFIV(G8)-*env* recombinants, 1 encoding the full length envelope protein and the other encoding the surface region only, are in the final stages of preparation. We are collaborating with Dr M Mackett (Paterson Institute for Cancer Research, Manchester, UK) in the construction of these recombinant viruses.

In Chapter 6, the chance finding that a recombinant FIV ENV protein, V3/V4, caused proliferation of feline lymphocytes was investigated further. The implications of this finding for the pathogenesis of FIV have been discussed. Further investigation into the effects of V3/V4 were prevented by the failure to purify further batches of protein. Recently, full length recombinant ENV from the SIV isolate $SIV_{mmPBj14}$, a virus which has been shown to encode a superantigen, has been successfully expressed using a recombinant vaccinia virus vector (Gonzàlez *et al.*, 1994). Recombinants expressing full length and SU FIV/GL8 ENV are in the final stages of preparation (Dr M Mackett, personal communication) and cells infected with these recombinants may provide a suitable source of protein for the investigations outlined at the end of Chapter 6.

The assay system designed in this study provides the first step in the determination of the role of antiviral CTL in the immune response to FIV infection. Importantly,

the availability of this assay will allow us to generate quantitative data on the CTL response to individual FIV proteins at different stages of infection by LDA. LDA is an in vitro technique which allows the retrospective calculation of antigen-specific CTL precursor frequencies, based on measurement of the number of cytolytic clones generated in short-term culture from a given population of cells (Waldmann *et al.*, 1987). Briefly, PBMC are restimulated in vitro and then titrated onto a fixed number of target cells in the cytotoxicity assay. Some wells will contain no precursors and these wells will be negative. Positive wells contain 1 or more clones each derived from single precursor in the initial population. Estimation of precursor frequency is made from a semilog plot of lymphocyte input against the fraction of non-responding cultures. Calculation of the results depends on the generation of a single-hit curve and this is achieved when only a single cell type, the CTL, is limiting. Thus, a prerequisite for LDA is optimisation of the in vitro activation of pCTL and the cytotoxicity assay so that the effects of a single precursor cell can be detected.

The cat/FIV model may allow us to address some of the unanswered questions regarding CTL response to HIV-1 infection. Monitoring the onset of FIV-specific CTL activity and its relationship to viraemia and antibody response will be important. These early post-infection events, which are not amenable to study in HIV-1 infection of humans, are likely to be important as CTL are believed to contribute to the control of viraemia and potentially may participate in the prevention of the establishment of infection (see later). This experimental model provides the opportunity to carry out controlled prospective studies so that the frequency of circulating FIV gene-specific pCTL in the acute, asymptomatic and terminal stages of the infection can be measured and related to viral load and markers of disease progression at each stage. This will allow determination of whether CTL activity to *gag* or *env* gene products is correlated with the clinical state of FIV infected cats and has prognostic significance. PCR can be used to quantitate FIV proviral load in cells from infected cats by end-point dilution (Momoi *et al.*, 1993; Matteucci *et al.*, 1993; Dr MJ Hosie, personal communication).

The HIV-1-specific CTL response declines towards the end of the asymptomatic stage. It is crucial to determine the reason for the decline in CTL activity. It is not known if this is a marker of immune failure or whether a causal relationship exists between the fall in CTL activity and the onset of secondary illness. Infection of CTL or their precursors, active suppression of the response secondary to either $CD4^+$ T cell immunodeficiency or defective antigen presentation and exhaustion of the HIV-1-specific CTL response have all been proposed to account for the fall in CTL activity (reviewed in Chapter 1). If the latter were the case then precursor frequencies of CTL specific for other viruses should be unaffected. There is

conflicting data on whether the deficit is HIV-1-specific or generalised (Rook *et al.*, 1985; Shearer *et al.*, 1985; Pantaleo *et al.*, 1990; Carmichael *et al.*, 1993). The FIV model gives us the opportunity to carry out controlled longitudinal studies in FIV-infected cats to address this question. Precursor frequencies could be monitored for CTL specific for FIV and contrasted with those for, for example, FeSFV, a persistent, apparently apathogenic retrovirus.

An experimental model also gives us the opportunity to compare the frequencies of FIV-specific CTL precursors in the circulation with those in secondary lymphoid organs. This will be important since only 2% of the total lymphocytes are found in the peripheral blood at any given time and lymphocytes migrate between lymphoid and non-lymphoid organs (Westermann and Pabst, 1990).

The relatively non-invasive nature of our CTL assay makes it suitable for use in naturally infected cats. The pattern of FIV-specific CTL activity can be studied in FIV seropositive cats presenting with clinical signs relating to immunodeficiency. This will allow us to determine if a relationship exists between clinical state and virus-specific CTL activity which would provide a basis for immunological staging in naturally infected cats.

The role of CTL in vivo can be assessed by adoptive transfer of effector cells from immune to non-immune donors. Although others have adoptively transferred activated lymphocytes between feline siblings which were compatible in mixed lymphocyte reaction (MLR) (Zeidner *et al.*, 1993), the MLR would only be expected to detect differences in class II MHC products (Tizard, 1992). When it becomes possible to detect class I MHC-matched cats then meaningful adoptive transfer experiments can be carried out. Adoptive transfer also has potential therapeutic uses. The feasibility of adoptive transfer of autologous $CD8^+$ lymphocytes as a therapeutic measure for HIV-1-infected humans is currently under investigation (Ho M *et al.*, 1993; Torpey *et al.*, 1993; Whiteside *et al.*, 1993; Klimas *et al.*, 1994).

Further detailed analysis of feline CTL responses, in particular the interpretation of data on the recognition of peptide sequences by CTL, will require the ability to determine the MHC haplotype of individual cats. The identification of CTL epitopes in FIV using synthetic peptides will allow us to define the role of CTL more directly. In particular, FIV CTL epitope mapping will enable us to determine whether immune escape from virus-specific CTL, proposed as a mechanism of disease progression in HIV-1 infection (Philips *et al.*, 1991), is involved in the immunopathogenesis of FIV infection. Experimental infection of cats with cloned FIV followed by reisolation of virus and sequencing of CTL epitopes, in parallel with measurement of the CTL response to these regions will enable us to answer this important question. In

addition, FIV epitope data will provide a basis for the induction of individual CTL clones by immunisation so that the effect of specific clones on virus replication in vivo can be assessed. CTL responses can be induced to individual viral epitopes using recombinant infectious vectors or by inoculation of peptides with various adjuvants including Freund's adjuvant, lipid tails, ISCOMs or simply subcutaneous immunisation with free peptide (Lanzavecchia, 1993). We have recently shown in this laboratory that the immunisation of cats with a carrier-free synthetic FIV peptide induces $CD8^+$ cytotoxic cells specific for autologous target cells labelled with constituent peptides or with vFIV/GL14-*gag* (Flynn *et al.*, in press).

Vaccines which protect from experimental FIV challenge have been developed (reviewed in Hosie, 1994). It is believed that virus neutralising antibodies are important in protection although a correlation between the high neutralising antibody titres and protection is incomplete. It will be interesting to determine the contribution of FIV-specific CTL to this protection. For CTL to be capable of eliminating virus they should be able to lyse virally infected cells during the eclipse phase before infectious progeny are released and this has been demonstrated (Zinkernagel and Althage, 1977). Vaccine induced CTL have been shown to protect mice against RSV (Kulkarni *et al.*, 1993). Protection mediated by CTL presumably depends on early viral proteins acting as targets for CTL activity. Interestingly in this regard, an early HIV-1 protein NEF induces a high level CTL response (Culmann *et al.*, 1989; Culmann *et al.*, 1991; Hadida *et al.*, 1992). Anti-NEF CTL may be therefore important in protection from HIV.

It has been suggested that reduced viral load following infection may be a more realistic goal for HIV vaccination than sterilising immunity (Brinchmann, 1993) and CTL have a potential role here. Macaques immunised with vSIV-*env* or recombinant ENV produced in a baculovirus vector showed significantly decreased viral load after challenge compared to control animals. As this effect occurred in the absence of a neutralising antibody response it is assumed that cell mediated immunity is responsible (Ahmad *et al.*, 1994).

As discussed in Chapter 1, antiviral CTL responses can sometimes be harmful to the host making it important to identify protective specificities for CTL induction. Whether this can be achieved by examining the FIV-specific CTL response at the levels of individual viral proteins or whether epitope data, and thus a knowledge of feline class I MHC products, will be necessary remains to be seen. If CTL-mediated immunopathology is a feature of FIV infection then it may eventually be possible to suppress CTL of the appropriate specificities. Gairin and Oldstone (1992), have protected LCMV infected cells from CTL in vitro using synthetic antagonist

peptides. Recently, naturally occurring antagonist peptides have been described in HIV-1 and may provide a mechanism for immune escape from CTL in vivo (Klenerman *et al.*, 1994).

The development of a fully autologous assay system for feline cytotoxic cells provides a fundamental basis for elucidating the role of FIV-specific CTL in the immune response of cats to FIV infection. Perhaps most importantly the ability to detect FIV-specific CTL provides us with an experimental system for studying the contribution of HIV-specific CTL to the immune response in HIV-infected people.

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