STUDIES ON HAEMOPOIESIS IN EARLY FELINE IMMUNODEFICIENCY VIRUS INFECTION

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To Mum and Dad

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abbreviations

ACS	anaemic cat serum
ADCC	antibody-dependent cell-mediated cytotoxic response
AIDS	acquired immune deficiency syndrome
AMS	anaemic mouse serum
ANOVA	analysis of variance
APAAP	alkaline phosphatase, anti-alkaline phosphatase
APS	ammonium persulphate
ARC	AIDS-related complex
ATP	adenosine triphosphate
AZT	3'-azido-3'deoxythymidine
BFU-E	burst-forming unit erythroid
BLV	bovine leukaemia virus
BOE	Boehringer Ingelheim bovine serum albumin
BSA	bovine serum albumin
^{o}C	degrees Centigrade
CAEV	caprine arthritis and encephalitis virus
CFC	colony forming cell
CFU	colony forming unit
CFU-E	colony-forming unit erythroid
CFU-G	colony-forming unit granulocyte
CFU-GEMM	colony-forming unit granulocyte/ erythroid/ macrophage/ megakaryocyte
CFU-GM	colony-forming unit granulocyte/macrophage
CFU-M	colony-forming unit macrophage
CFU-Meg	colony-forming unit megakaryocyte

CM	conditioned medium
CRFK	Crandell feline kidney
CSF	colony stimulating factor
CTP	cytidine triphoshate
DNA	deoxyribonucleic acid
ECM	extra-cellular matrix
EDTA	ethylenediaminetetraacetic acid
EIAV	equine infectious anaemia virus
ELISA	enzyme-linked immunosorbent assay
env	envelope protein-encoding gene
EPO	erythropoietin
FCS	foetal calf serum
FeLV	feline leukaemia virus
FeSFV	feline syncytium-forming virus
FCV	feline calicivirus
FHV	feline herpes virus
FIV	feline immunodeficiency virus
g	gram(s)
gag	group-specific antigen-encoding gene
G-CSF	granulocyte colony-stimulating factor
GL-8	Glasgow 8
GM-CFU	granulocyte-macrophage colony forming unit
GM-CSF	granulocyte-macrophage colony-stimulating factor
gp	glycoprotein
GTP	guanosine triphosphate
Hb	haemoglobin
Hct	haematocrit
HIV	human immunodeficiency virus
HSC	haemopoietic stem cell

HTLV	human T-cell leukaemia virus
ID50	50% infectious dose
IL-1	interleukin 1
IL-2	interleukin 2
IL-3	interleukin 3
IMDM	Iscove's modified Dulbecco's medium
i.u.	international units
LTR	long terminal repeat
M	molar
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
mg	milligram(s)
MHC	major histocompatibility complex
ml	millilitre(s)
mm	millimetre(s)
mM	micromolar
MV	maedi-visna
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
ng	nanogram(s)
nm	nanometre(s)
ORF	open reading frame
p	protein
PCR	polymerase chain reaction
PCV	packed cell volume
PGL	persistent generalised lymphadenopathy
pi	post-infection

PLV	puma lentivirus
PMEA	9-(2-phosphonyl methoxyethyl)adenine
pol	polymerase-encoding gene
PRCA	pure red cell aplasia
RBC	red blood cell(s)
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase or room temperature
RT-PCR	reverse transcriptase PCR
SCF	stem cell factor
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SPF	specific pathogen free
SSC	sodium chloride/sodium citrate buffer
STWS	Scott's tap water substitute
TBE	tris-borate buffer
TBS	tris-buffered saline
TE	tris-EDTA buffer
TEA	tris-acetate buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF	transforming growth factor
Th1	T-helper 1
Th2	T-helper 2
TNF	tumour necrosis factor
TTP	thymidine triphosphate
UTPase	uridine 5'-triphosphatease
WBC	white blood cell(s)
WK(S)	week(s)

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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 December 1991 and March 1995. The author was responsible for all results except as follows: the percentile analysis in chapter 3 was carried out by Katie Knox in the Department of Veterinary Medicine using the Minitab statistical software; FIV antibody tests and virus isolation were carried out by the staff of the Feline Virus Unit in the Department of Veterinary Pathology and haematology differential counts were performed by Ronnie Barron and Kenny Williamson in the Department of Veterinary Pathology.

No part of this thesis has been presented to any university.

Susan E. Grant, September 1995.

SUMMARY

This thesis is a study of the haematological events occurring in early experimental FIV infection in cats. In the first few weeks of infection with FIV, there is intense viral activity and viraemia. This is accompanied by lymphadenopathy, acute clinical illness and a profound neutropaenia of unknown pathogenesis. Cytopaenias of any or all of the blood cell lines are known to occur in late infection in both FIV and HIV. These are important in terms of their own morbidity and in that they limit the type and intensity of therapy which can be given to combat the viral and secondary infections. Their aetiology is likely to be multifactorial. The predictable neutropaenia seen in early FIV infection presented an ideal opportunity to elucidate the role of FIV in bone marrow.

Feline haematological parameters were re-evaluated using percentile analysis of database results. Although the number of results on the database was relatively small, the advantages of the percentile system were demonstrated over the traditional "normal" ranges. A comparison between a small distinct population of research cats and cats from the general population illustrated the danger of using small groups of animals from closed populations to construct "normal" ranges, as there were distinct differences between the two groups regarding total white cell numbers, lymphocytes and eosinophils.

After experimental infection with the Glasgow-8 strain of FIV, cats were sequentially blood sampled and full haematological profiles were obtained over a six month period. All infected animals

developed a neutropaenia 5-6 weeks after infection. This was of variable duration and severity. Those cats with the most profound and prolonged neutropaenia were also clinically ill for a few days. The infected cats also had significantly fewer erythrocytes than control cats, although they were never anaemic. A significant difference in lymphocyte numbers was also recorded between the two groups of control and infected animals, infected animals again having lower numbers but not becoming lymphopaenic. Relative eosinopaenia was noted in infected animals.

In order to establish the efficacy of neutrophil production at this time, serial bone marrow aspirates were taken from the cats and subjected to quantitative assay techniques. These assays enabled the numbers of committed neutrophil, monocyte-macrophage and erythroid precursors to be ascertained. The results showed that there were significantly fewer neutrophil and monocyte-macrophage precursors in infected cats than in control animals. This difference was most pronounced at 5-6 weeks post-infection, when the infected cats were also neutropaenic.

Having demonstrated that neutrophil production in bone marrow was compromised in early FIV infection, it was then important to determine the mechanism of the progenitor cell suppression. Inhibition of neutrophil production could have been due to direct infection and killing of precursors by virus; direct infection and compromise of function of progenitor cells; or by indirect means whereby feeder, stromal or accessory cells were virally infected and growth factor regulation disrupted. Clonally-derived colonies of bone marrow cells grown in the assay system were individually

screened by PCR for evidence of direct viral infection. All colony types (CFU-M, CFU-G, CFU-GM and BFU-E) were found to be positive for the FIV LTR fragment. The proportion of infected colonies varied according to time post-infection, suggesting that there was a dynamic relationship between virus, progenitors and progeny cells. Maximum numbers of infected colonies were found at 12 weeks post-infection.

Immunohistochemistry was performed to investigate whether colony cells were expressing FIV proteins. Colonies from the assay plates were spun onto slides and stained with anti-p24 antibody. The vast majority of cells staining positive were macrophages. Maximum numbers of positive cells were detected at weeks 5-6 post-infection. Very few mononuclear cells stained positively, but this did not exclude the possibility of their being infected. The varying nature of the proportion of cells expressing p24 followed a similar pattern to that of the numbers being positive by PCR, reinforcing the theory that the numbers of infected cells depended on the quantity of infectious virus being produced at any one time.

Peripheral neutrophils from asymptomatic long-term infected cats were screened by PCR for the presence of FIV. No virus was found in these cells. The committed precursor cells from which these cells were derived must also have been free of virus. This was to be expected of long-term infected animals in the asymptomatic stage as viral production would be at low levels, and therefore few precursors would be infected. Circulating mature cells from cats in early infection were not tested.

Findings from the studies outlined above are comparable with the known facts on HIV. Further investigation into the marrow events in FIV would be useful in elucidating the pathogenesis of cytopaenias in both FIV itself and in HIV.

CHAPTER 1
GENERAL INTRODUCTION

1.1. HAEMOPOIESIS

Mammalian leucocytes have a short life span. Haemopoiesis is the process by which mammals replace circulating blood cells by a combination of replication and differentiation of precursor cells to enable a constant supply of mature replacement cells. Haemopoietic tissue can be divided into three categories:- the multipotential progenitor cells; the committed progenitor cells and the maturing cell population. The majority of this tissue is found in bone marrow, the cells all being derived from a common population of stem cells established during embryogenesis (Metcalf & Moore 1971). Although the bone marrow is the major site of haemopoiesis in the post-natal mammal, a potential for extra-medullary haemopoiesis exists in organs such as spleen and liver in times of emergency. A population of stem cells remains in these organs as perhaps a remnant of their pre-natal haemopoietic function. The haemopoietic cells are supported by an extensive network of support cells, forming the bone marrow stroma. The stroma is essential for the maintenance and development of progenitor cells. A simplified diagrammatic view of haemopoiesis is represented in fig 1.1.

1.1.1. HAEMOPOIETIC STEM CELLS

Wright & Lord (1992) reviewed the characteristics of haemopoietic stem cells (HSC). Stem cells are defined as cells with extensive self-renewal capacity, extending throughout the lifespan of the

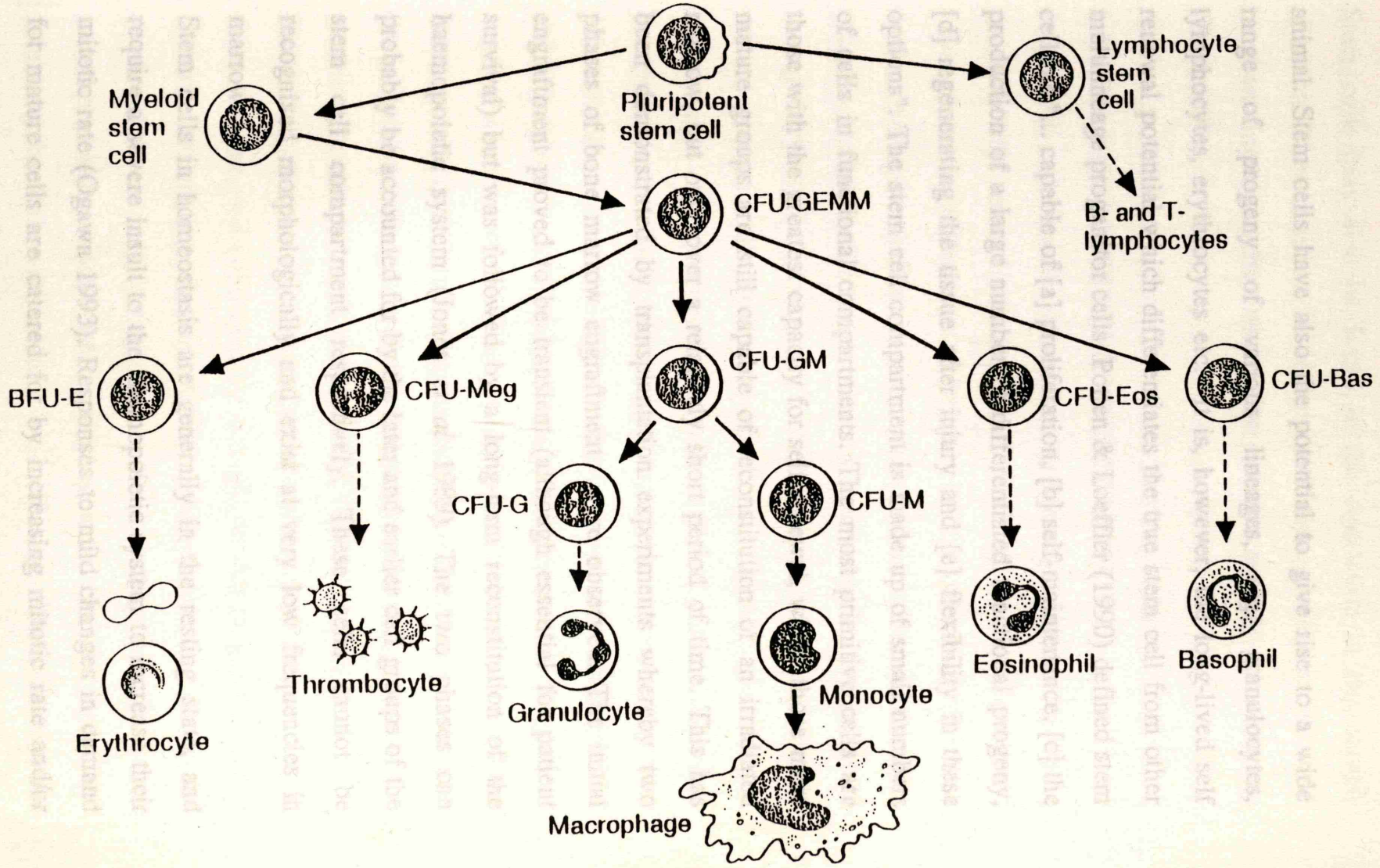


Fig. 1.1. A simplified model of haematopoiesis

animal. Stem cells have also the potential to give rise to a wide range of progeny of various lineages, e.g. granulocytes, lymphocytes, erythrocytes etc. It is, however, the long-lived self renewal potential which differentiates the true stem cell from other multilineage progenitor cells. Potten & Loeffler (1990) defined stem cells as "... capable of [a] proliferation, [b] self-maintenance, [c] the production of a large number of differentiated functional progeny, [d] regenerating the tissue after injury and [e] flexibility in these options". The stem cell compartment is made up of small numbers of cells in functional compartments. The most primitive cells are those with the greatest capacity for self-renewal, whilst those more mature groups are still capable of reconstitution of an irradiated marrow, but only over a relatively short period of time. This has been demonstrated by transplantation experiments whereby two phases of bone marrow engraftment were observed. The initial engraftment proved to be transient (although essential for patient survival) but was followed by a long-term reconstitution of the haemopoietic system (Jones *et al* 1989). The two phases can probably be accounted for by the later and earlier cell groups of the stem cell compartment respectively. These cells cannot be recognised morphologically and exist at very low frequencies in marrow.

Stem cells in homeostasis are generally in the resting state, and require a severe insult to the haemopoietic system to increase their mitotic rate (Ogawa 1993). Responses to mild changes in demand for mature cells are catered for by increasing mitotic rate and/or decreasing maturation time of progenitor cells and their progeny.

Stem cells may also be found in the circulation in the normal situation. These tend to be of the more mature compartment, and numbers increase after stimulation of the marrow compartment (Beck 1991).

1.1.2. HAEMOPOIETIC PROGENITOR CELLS

Progenitor cells are the immediate progeny of stem cells, being progressively more limited in their capacity to self-renew and having a limited differentiation potential (Wright & Lord 1992). Any self-renewal potential serves only to amplify the numbers of mature progeny produced. Most progenitors are unipotential, giving rise to a single type of progeny, but common progenitor cells of closely related mature cell types can be found e.g. the granulocyte-macrophage progenitor cell. Progenitor cells are not morphologically identifiable and any single type is found at less than 1% frequency in marrow. Instead, these cells are identified by their ability to give rise to clonal colonies of their respective progeny in *in vitro* assay systems. Thus, progenitor cells are named colony-forming cells (CFC) or colony-forming units (CFU). For example, the progenitor cells giving rise to mature granulocytes are known as granulocyte colony forming cells, or G-CFC.

The activity and survival of progenitor cells *in vivo* and *in vitro* is dependent on, and controlled by, a large number of growth factors (colony stimulating factors, CSFs) and cytokines. These are produced by a range of stromal cells, including fibroblasts and endothelial cells, monocytes, macrophages and lymphocytes. Their role may be either stimulatory or inhibitory. These factors are interdependent, their role *in vivo* often being unclear due to the large numbers involved and their many interactions. Additive, synergistic

and antagonistic effects of various CSF and cytokine combinations have been demonstrated *in vitro*. It is thought that steady state haemopoiesis is established by a balance of positive and negative regulators. Some factors are involved at the stem cell level. Insults causing changes in peripheral blood cell demands cause changes in cytokine and growth factor production, which in turn lead to a response from the progenitor and maturing cell populations.

1.1.3. MATURING AND MATURE CELLS

Ninety-five percent of haemopoietic cells are maturing and mature cells. These are the progeny of the progenitor cells, committed in lineage and having a progressively limited ability to proliferate. End-stage mature cells are generally incapable of division. These cells may be recognised morphologically in their various stages of maturation.

1.1.4. THE HAEMOPOIETIC MICROENVIRONMENT

Haemopoiesis occurs only in a few selected organs in the body. It is restricted to those sites in which an appropriate "microenvironment" is available. The haemopoietic microenvironment is composed of stromal cells, other connective tissue cells and the extra-cellular matrix (ECM). This tissue is highly organised and composed of endothelial cells, fibroblasts, reticular cells, adipocytes and macrophages (Lichtman 1984). The ECM consists of a large number of components, including collagen of various types, fibronectin, laminin, heparan sulphate, and hyaluronic acid (Dexter *et al* 1984). These components form an important intercellular network and may be restricted to certain sites within the marrow cavity. The proteins involved may be responsible for a large number

of interactions and in the binding of cells to their respective growth factors.

Survival and differentiation of haemopoietic cells apparently depends on intimate interaction between stromal cells and developing haemopoietic cells (Lichtman 1984). Separating stromal and progenitor cells in *in vitro* systems by diffusion chamber or an agar layer results in a decline in haemopoietic activity (Verfaillie 1992). It may be the case that membrane bound forms of growth factors are essential for the regulation of progenitor cell development, hence necessitating direct contact between cells for effect.

It has been recognised for many years for example, that erythrocytes develop in association with marrow macrophages (Lichtman 1984). Clusters of maturing erythroblasts are found in association with macrophages. These are known as erythroblastic islets. As well as providing a prolific source of growth factors, the macrophage is involved in the phagocytosis of the erythrocyte nuclei in the latter stages of maturation. Other similar types of relationship are likely to exist between stromal cells and developing haemopoietic cells.

1.1.5. TRANSFER OF MATURE CELLS TO THE CIRCULATION

The process by which mature cells of the bone marrow are transferred to the circulation is far from random. Cell entry is selective and closely regulated. The endothelial lining of the marrow sinusoids is thought to play an integral part in controlling the release of cells into circulation. Factors such as integrity of the wall, the

state of the pores in the wall and blood flow characteristics within the sinusoids are thought to be important. The endothelium may also produce important signalling factors (Dorshkind 1990). The endothelial basement membrane is in close contact with fixed cells in the sinusoidal spaces known as reticular cells. It has been suggested that the degree of association between these cells and the basement membrane regulates the passage of cells into the sinusoids. Several types of stress e.g. toxaemia have been shown to reduce the area of endothelial membrane covered by reticular cell processes. This may well favour cell egress into the circulation. This, so called, blood-bone barrier may also be responsible for influencing the influx of soluble mediators into the marrow cavity. The physical location of the mature cells and their accompanying cells are important in transfer (Jain 1993). The deformability of the cell is also relevant, immature cells being less deformable than their mature equivalents, and having a higher surface negative charge. The entry of leukocytes and erythrocytes into the vascular space is believed to be transcellular, but selective numbers of these may also be able to cross via apertures in the endothelial wall. Megakaryocytes extend cytoplasmic processes into the sinusoidal lumen from their position in apposition to the sinusoidal wall, from whence platelets can fragment into general circulation.

1.2. RETROVIRIDAE

The retroviridae are a large family of viruses, found primarily in vertebrates. They cause a wide spectrum of disease, including malignancies, wasting diseases, neurological disorders, immunodeficiencies and lifelong viraemias with no apparent ill-effect on the host (Coffin *et al* 1990). Retroviruses are unique in a number of ways, one of which is their ability to integrate the viral genome into that of the host cell in a stable manner, such that it may be transmitted to the progeny. Virus particles can then continue to be replicated in the next generation of host cells. Integration of viral genomic sequences into that of the host cell may alter the host genome at a significant site so that oncogenesis results. Replication of retroviruses is more prone to error than that of other viruses, due to the reduced fidelity of the reverse transcriptase enzyme. Thus, more aberrant viral forms arise, resulting in a genetically diverse population of viral mutants potentially capable of evading host immune responses.

1.2.1. CLASSIFICATION

The retroviridae are classified primarily by pathogenicity into three main groups: the oncoviruses, the lentiviruses and the spumaviruses (Coffin *et al* 1990). The oncoviruses may then be divided into five main subdivisions according to their nucleotide sequences and genomic structure as follows: avian leukosis-sarcoma; mammalian type C; B-type viruses; D-type viruses and the HTLV-BLV group. The lentiviruses subfamily consists of exogenous viruses causing a number of neurological and immunological diseases. These include, as well as feline immunodeficiency virus (FIV), human immunodeficiency virus (HIV) and simian immunodeficiency virus

(SIV), the so-called slow viruses maedi-visna of sheep (MV), equine infectious anaemia (EIA) and caprine arthritis-encephalitis virus (CAEV). Lentivirus infections characteristically involve a prolonged asymptomatic phase of latent infection, followed by a terminal period of clinical disease.

Little is known about the subfamily spumaviridae, and persistent infection with these viruses is not associated with any disease.

1.3. FELINE IMMUNODEFICIENCY VIRUS

Feline immunodeficiency virus (FIV) is a lentiviral infection of cats first isolated in California in 1986 (Pedersen *et al* 1987). Infected animals develop an immunodeficiency syndrome characterised by the late-stage onset of a variety of secondary and opportunistic infections.

1.3.1. VIRAL CLASSIFICATION AND LIFE CYCLE

The FIV virion is typical of the lentivirus family, being 105 to 125nm in diameter, enveloped and spherical to ellipsoid in shape. Bennett & Smyth (1992) reviewed the physical properties of the virus: two main envelope glycoproteins are expressed, a major spike glycoprotein (gp120) responsible for attachment to target cells, and a smaller transmembrane glycoprotein (gp40). The core is bar or cone shaped and contains the major proteins p24, p15 and p10. As with all retroviruses, FIV has a single-stranded RNA genome (sRNA) and replicates by means of the viral encoded reverse transcriptase (RT) enzyme.

FIV infects cells by attachment to receptors and internalisation by endocytosis or membrane fusion. A double-stranded DNA replica of the original RNA genome containing long terminal repeats (LTR)

at each end is manufactured using the reverse transcriptase enzyme. This viral DNA duplex is then inserted into the host cell genome by the viral integrase. This first phase of the replication cycle proceeds using existing virion products contained in the viral core and in the absence of further viral gene expression. Integrated proviral DNA can then code for viral proteins and RNA using the cellular mechanisms. The protein cores are assembled during the process of budding through the plasma membrane of the host cell, where they acquire their lipid membranes, complete with the viral envelope glycoproteins. New virions are then free to infect cells. Replication is represented diagrammatically in fig 1.2.

Integrated provirus contains the three main open reading frames (ORF) common to the lentivirus subfamily, that is *gag, pol* and *env*, as well as the flanking long terminal repeats (LTR) and several smaller ORFs which may encode regulatory factors. The *gag* gene encodes for the main capsid proteins p24, p15 and p10. The *pol* gene encodes for reverse transcriptase, a protease, a dUTPase and an integrase. *env* contains sequences encoding the envelope glycoproteins.

Most differences between viral isolates have been in the *env* area (Bennet & Smyth 1992), although there has been less variation recorded between FIV isolates than for other lentiviruses e.g. HIV.

1.3.2. EPIDEMIOLOGY

FIV was first isolated in 1986 from domestic cats which were showing signs of an immunodeficiency syndrome (Pedersen *et al* 1987). It is widespread, although its prevalence varies across the

globe, the incidence generally being higher in "sick" cat populations than in healthy ones. Retrospective serological surveys indicate that the virus has been present in the feline population as long as samples have been available to test, i.e. certainly since 1968 (Gruffydd-Jones *et al* 1988, Shelton *et al* 1990). Given the endemic nature and widespread distribution of the virus, it is likely it has been present for considerably longer. Hosie *et al* (1989) noted a prevalence of 19% in sick cats and 6% in healthy cats in the UK. Surveys in the USA and Canada revealed respective infection rates of 14% in the sick population and 1.2% in the control population (Yamamoto *et al* 1989) and 15% versus 3.6% (Grindem *et al* 1989), whilst in Japan higher rates of 43.9% and 12.4% respectively, have been recorded (Ishida *et al* 1989). A serological survey conducted in northern Italy suggested a prevalence of 9% in healthy cats and 12.5% in unhealthy animals (Peri *et al* 1994). These different rates of infection may be attributable to different population samples and a larger, more densely populated, free-roaming domestic cat population in Japan.

A related virus, infectious puma lentivirus (PLV), has been isolated from several Florida panthers (Olmsted *et al* 1992). This virus elicits the production of cross-reactive antibodies to FIV. Cross reactive antibodies have also been found in several of the free-ranging African big cat species (Olmsted *et al* 1992, Brown *et al* 1994). The extent of exposure to virus varies widely between cat populations in different parts of Africa, being up to 91% positive in Kruger lions, and as low as 0% in Namibian lions. Brown *et al* (1994) reported the isolation of a genetically distinct lion lentivirus from wild east African lions. Several subtypes appear to exist, each more distinct from each other than FIV is from PLV, suggesting

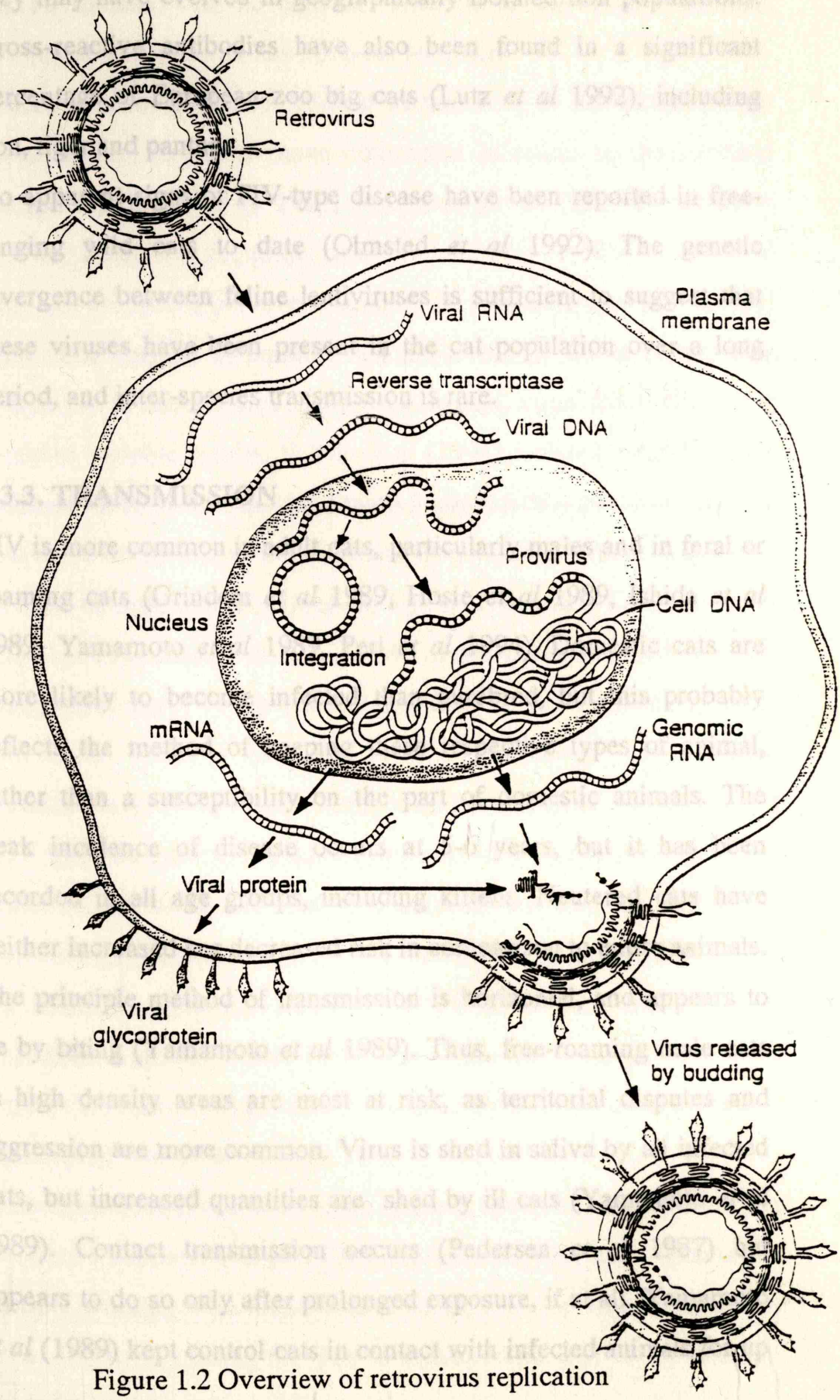


Figure 1.2 Overview of retrovirus replication

they may have evolved in geographically isolated lion populations. Cross-reactive antibodies have also been found in a significant percentage of European zoo big cats (Lutz *et al* 1992), including lion, tiger and panther.

No apparent signs of FIV-type disease have been reported in free-ranging wild cats to date (Olmsted *et al* 1992). The genetic divergence between feline lentiviruses is sufficient to suggest that these viruses have been present in the cat population over a long period, and inter-species transmission is rare.

1.3.3. TRANSMISSION

FIV is more common in adult cats, particularly males and in feral or roaming cats (Grindem *et al* 1989, Hosie *et al* 1989, Ishida *et al* 1989, Yamamoto *et al* 1989, Peri *et al* 1994). Domestic cats are more likely to become infected than purebred, but this probably reflects the method of keeping these respective types of animal, rather than a susceptibility on the part of domestic animals. The peak incidence of disease occurs at 5-6 years, but it has been recorded in all age groups, including kittens. Neutered cats have neither increased nor decreased risk in comparison to entire animals. The principle method of transmission is horizontal, and appears to be by biting (Yamamoto *et al* 1989). Thus, free-roaming male cats in high density areas are most at risk, as territorial disputes and aggression are more common. Virus is shed in saliva by all infected cats, but increased quantities are shed by ill cats (Yamamoto *et al* 1989). Contact transmission occurs (Pedersen *et al* 1987) but appears to do so only after prolonged exposure, if at all. Yamamoto *et al* (1989) kept control cats in contact with infected animals for up

to 14 months with no sign of infection in the control animals. Venereal transmission has not been recorded.

There is no evidence for vertical transmission *in utero*, but kittens born to infected dams have contracted infection in the neonatal period (Callanan *et al* 1991, Wasmoen *et al* 1992, Sellon *et al* 1994). Passively acquired maternal antibody to FIV is acquired through colostrum but levels wane by 10 weeks of age. It is thought that virus may be transmitted in milk or via the saliva of the dam. Attempts to isolate free or cell-associated virus from milk have yielded variable results. Sellon *et al* (1994) isolated virus from the milk of three of four queens infected immediately post-partum. Ten out of 16 of the kittens born to these queens were virus positive by 8 weeks of age, and remained so up to 7 months. Callanan *et al* (1991) and Sellon *et al* (1994) both postulate that vertical transmission may be more likely in acutely infected queens, as more infectious virus is produced during this stage of infection. Alternatively, Pu *et al* (1995) reported protection of kittens born to FIV infected queens. Protection was primarily by passively acquired colostral antibody but was only seen in kittens born to queens infected for 7 months or more. He postulates that this is due to lack of sufficient antibody response in the dams until this stage of infection. Yamamoto *et al* (1988) failed to report infection in kittens up to six months of age born to chronically infected dams, which is in keeping with Pu's findings .

The virus does not infect human, mouse, sheep or dog lymphocytes *in vitro* (Yamamoto *et al* 1988). No antibody has been detected in people working with infected animals or with the virus (Yamamoto *et al* 1989), suggesting that FIV is host-specific, a property common to most lentiviruses.

1.3.4. DIAGNOSIS

Diagnosis of clinical cases of FIV in the field is by detection of plasma antibody. This is usually by the commercially available concentration immunoassay technology kit (CITE) test consisting of membrane filter paper coated with virus (IDEXX Systems, Portland, ME). Presence of plasma antibody in lentivirus infection is consistent with infection, as the immune response does not result in clearance of the virus. Antibody can also be detected by the enzyme-linked immunosorbent assay (ELISA) or by direct immunofluorescence. Western blotting is considered to be the most reliable and sensitive diagnostic method of detection of plasma antibody, but is not suitable for use in the field (Pedersen *et al* 1987, Hosie & Jarrett 1990).

Virus may be isolated from the peripheral blood lymphocytes of infected cats by culture with mitogens and interleukin-2 (IL-2) (Pedersen *et al* 1987). A cytopathic effect is observed after approximately 7 days. The presence of virus can then be confirmed by immunofluorescence, electron microscopy or reverse transcriptase assay (Hopper *et al* 1994).

The most sensitive method for the detection of FIV in blood or other infected cells is the polymerase chain reaction (PCR). PCR allows the selective amplification of viral nucleic acid sequences to detectable quantities, thus enabling the detection of infection at very low levels. This is a research technique which has demonstrated the presence of FIV in seronegative cats (Dandekar *et al* 1992). *In situ*

hybridisation involves of a radiolabelled FIV probe to detect low levels of FIV RNA in cells.

1.3.5. CLINICAL SIGNS AND PROGRESSION OF DISEASE

1.3.5.1. NATURAL INFECTION

Cats naturally infected with FIV display a variety of clinical signs which may be due directly to the virus or, more commonly, associated with immunodeficiency. Chronic and opportunistic infections are prevalent in the latter stages, and are manifested as malaise, weight loss, lymphadenopathy, gingivitis, stomatitis, upper respiratory infections, gastrointestinal infections and an increased incidence of neoplasia (Grindem *et al* 1989, Hopper *et al* 1989, Ishida *et al* 1989, Yamamoto *et al* 1989). The number and severity of infections tend to increase towards the terminal stages of disease. Neurological disease is also common in infected animals. The virus is known to be neurotropic (Dow *et al* 1992), being found in astrocytes and microglial cells.

An increased incidence of neoplasia is recorded in FIV infected cats, particularly lymphomas, probably due to chronic excessive stimulation of lymphocytes. Shelton *et al* (1990) reported that the relative risk of developing leukaemias or lymphomas is 5.6, 62.1 or 77.3 times greater in cats infected with FIV, FeLV or dual infection respectively, than non-infected cats.

Infection with FIV is lifelong, complete recovery having never been recorded either in natural or experimental infection. Co-infection with other retroviruses is not uncommon, especially feline syncytium-forming virus (FeSFV), but also feline leukaemia virus

(FeLV) (Hosie *et al* 1989, Ishida *et al* 1989, Yamamoto *et al* 1989). An association has been noted between FIV and feline calicivirus (FCV) in cats with chronic gingivitis (Dawson *et al* 1991). Reubel *et al* (1994) also reported that infecting FIV positive cats with FCV resulted in more severe disease than in control animals. Similar results were obtained by infecting FIV positive cats with feline herpes virus (FHV-1) (Reubel *et al* 1992). The activation of chronic toxoplasmosis infection can also be brought about by infecting affected cats with FIV (Lappin *et al* 1992).

The clinical presentation of FIV may be divided into stages as follows: a] the acute phase; b] the asymptomatic phase; c] the so-called AIDS-related complex (ARC) and d] AIDS (Ishida *et al* 1990). The progression of disease is variable, and probably depends on age at infection, infectious dose and state of nutrition, coinfection with other viruses and other cofactors. Following the acute phase seen at four-five weeks post-infection, infected cats undergo a prolonged asymptomatic stage before entering the chronic stages involving lymphadenopathy, wasting and infections. In a study of 11 naturally infected cats in the asymptomatic phase of infection, Ishida *et al* (1992) reported 4 of these animals as having disease progression within 2 years. Two animals died of AIDS-like signs, and a further two developed lymphadenopathy and gingivitis. The remaining animals remained in good health. This was a diverse group of animals with an unknown length of infection and which were exposed to different environments. Their disease progression would therefore be expected to be variable.

1.3.5.2. EXPERIMENTAL INFECTION

Experimental inoculation of cats by the intraperitoneal, intravenous, subcutaneous, intrathecal, oral, intranasal, intravaginal and intrarectal routes with FIV results in an acute phase illness five to eight weeks after inoculation (Yamamoto *et al* 1988). This may be achieved using whole blood, plasma, cell culture supernatants or cell associated virus. Approximately four to six weeks after infection, most cats become depressed, anorexic, pyrexic, develop a lymphadenopathy and may show signs of a flu-like illness (Pedersen *et al* 1987, Yamamoto *et al* 1988, Dawson *et al* 1991, Mandell *et al* 1992, Dua *et al* 1994). At this time a profound but transient neutropaenia develops (Yamamoto *et al* 1988, Mandell *et al* 1992, Dua *et al* 1994). This phase is short lived, lasting for a few days or weeks in most animals, after which most recover and enter a prolonged asymptomatic period. The lymphadenopathy is maintained for up to a year. The immunodeficient stage of disease develops following the prolonged asymptomatic period.

1.3.6. PATHOGENESIS

FIV is found predominantly in primary and secondary lymphoid organs in the early stages of infection, although small quantities of virus can be found in other organs such as liver and kidney (Beebe *et al* 1994). This is consistent with the knowledge that T lymphocytes, macrophages and other mononuclear cells are the primary cellular targets. Numbers of infected macrophages are low in the early stages but rise dramatically during the acute phase of the illness (Beebe *et al* 1994). Widespread dissemination of virus

occurs during the acute phase illness (Beebe *et al* 1994). This may be of significance in that virus disseminated to various organ and cell types may evolve differently and emerge as variants. This is one of the potential mechanisms for the triggering of the terminal stages of infection.

FIV is more cytopathic in lymphocytes than macrophages, and T lymphocyte numbers are reduced during the course of infection. The CD4:CD8 ratio in infected cats decreases gradually as infection progresses (Hoffman-Fezer *et al* 1992, Lehmann *et al* 1992). Low numbers of helper T (CD4) lymphocytes are responsible for the reduced efficiency of the immune response seen in infected animals and the consequent clinical syndrome.

The T-lymphocytes of FIV positive cats have been demonstrated to have persistently elevated levels of MHC class II expression *in vivo* (Rideout *et al* 1992). Lymphocytes from normal animals increase expression in response to infection with a range of viruses, but this is usually accompanied by an increase in IL-2 receptor expression. The alteration in IL-2 receptor expression is not seen in FIV infected animals (Rideout *et al* 1992). The upregulation of MHC class II may occur as a result of chronic immune stimulation, as it is also seen in FeLV positive cats. The role of these molecules in T-cells is unclear, but their increased presence in persistent infections suggests an important role in normal T-cell function. However, Willet *et al* (1991) reported downregulation of MHC class II expression in *in vitro* infected T-cells, along with a transient reduction in the expression of the CD4 receptor. It is postulated that the virus may cause a reduction in the constitutive expression of MHC II as a means of reducing the efficacy of the cellular immune response.

In vitro response tests to mitogens has demonstrated reduced ability of FIV infected lymphocytes to proliferate and respond to these stimulants (Hara *et al* 1990, Lawrence *et al* 1992, Bishop *et al* 1992). The reduced mitogen response is progressive (Taniguchi *et al* 1990), with almost total loss of response by the late stage disease.

It is not known what triggers the chronic stage of the disease, but it may be simply a result of levels of T lymphocytes falling below a critical level (Bennett & Smyth 1992). It has also been suggested that a switch from the cytotoxic T-cell dominated T-helper 1 (Th1) immune response to the humoral mediated T-helper 2 (Th2) response may be responsible (Weiss 1993). Other theories include the development of viral mutants which evade the host immune response, or mild secondary infections themselves causing immune suppression or stimulation of infected cells and thereby enhancing viral replication (Bennett & Smyth 1992).

Infected cats kept in a controlled environment take longer to develop the chronic stages of disease than those in high disease exposure environments (Yamamoto *et al* 1988). This suggests that cofactors, for example extraneous infectious diseases, may be involved in the progression of the syndrome.

1.3.7. HAEMATOLOGICAL CHANGES

1.3.7.1. NATURAL INFECTION

The chronic stages of FIV infection are often accompanied by a range of haematological abnormalities. A number of authors have reported cytopaenias in naturally infected cats in the latter stages of disease (Hopper *et al* 1989, Yamamoto *et al* 1989, Shelton *et al*

1990, 1991, Ishida *et al* 1992, Sparkes *et al* 1993). Cytopaenias are often multiple at this stage, and become more severe as the disease progresses. Hopper *et al* (1989) recorded forty of forty-six cats with a minimum of one haematological abnormality. Eighteen animals had multiple abnormalities. Fifteen animals were anaemic, fifteen lymphopaenic, eleven neutropaenic and seventeen had monocytosis. Thrombocytopaenia was a less common finding. Yamamoto *et al* (1989) surveyed a small number of FIV positive cats with clinical signs of disease and found 12/40 were anaemic, 14/47 were leukopaenic, 18/34 were lymphopaenic, 8/37 neutropaenic and 4/25 mildly thrombocytopaenic. 7/47 cats had a leukocytosis, this was most commonly associated with a neutrophilia. Shelton *et al* (1990) conducted a similar survey and produced similar proportions of cats suffering from these types of cytopaenia. Cytopaenias were seen only in cats suffering from ARC or AIDS, leukopaenia being seen in 35-76% of AIDS cats and 10-20% of ARC cats. Neutropaenia was seldom severe, but lymphopaenia was usually significant. 65-95% of cats in the final stages of disease have haematocrits of less than 40, whilst only up to 15% of healthy seropositive cats or ARC cats has such low values. Observations of cats progressing through ARC to AIDS have demonstrated a gradual fall in PCV to approximately 17.6% at death, with no evidence of regeneration (Ishida *et al* 1992).

At this stage of infection, it is difficult to predict the type and severity of the cytopaenias, such are the complicating factors. These include dietary insufficiencies, treatment regimes, secondary infections, the effects of chronic disease and also an increased frequency of malignancies in infected animals. Cytopaenias are also

recorded in the latter stages of HIV infection, and are comparable with those seen in the cat.

1.3.7.2. EXPERIMENTAL INFECTION

Experimentally infected cats develop a profound leukopaenia during the acute phase of the illness (Pedersen *et al* 1987, Yamamoto *et al* 1988, Callanan *et al* 1992, Mandell *et al* 1992, George *et al* 1993, Dua *et al* 1994, Linenberger *et al* 1995).

Leukocyte counts drop at 5 to 8 weeks post-infection and may remain low for periods up to 40 weeks (George *et al* 1993), although most authors report return to normal ranges before this. The most dramatic change is a profound neutropaenia, which occurs at this time and may last from a few days to several weeks. It usually is accompanied by an acute illness in the cats, characterised by fever, depression, malaise and a generalised lymphadenopathy. The illness begins at around 6 weeks after infection, is generally at its worst by 8 weeks, and recovery is complete by 10-12 weeks. The neutropaenia may be preceded by a mild lymphopaenia at 2-4 weeks post-infection (Callanan *et al* 1992, Dua *et al* 1994). Mandell *et al* (1992) also reports the occurrence of eosinopaenia along with the neutropaenia. Red cell parameters and platelet counts are comparable to control cats at all times during the acute phase. A small proportion of cats do not recover from this stage and are either euthanased on humane grounds or die (Yamamoto *et al* 1988, Callanan *et al* 1992). In these animals, pancytopaenia has been noted (Yamamoto *et al* 1988). George *et al* (1993) report that the haematological events seen in early infection are more severe and of longer duration the younger the cat is at infection. This may relate to the relative activities of bone marrow cells in animals of different

age groups, the more active marrows in younger animals may be more susceptible to perturbation by viral infection than the more quiescent marrow of the aged cat.

1.3.8. PATHOGENESIS OF CYTOPAENIAS

1.3.8.1. NATURAL INFECTION

The pathogenesis of the cytopaenias is a matter of some controversy. Experimental evidence has been conflicting, with no theory being conclusively verified. Most work has been done on the HIV cytopaenias in the late stage of infection. Heterogeneity of the patient groups in terms of stage of infection; drug administration and concurrent infections, as well as differences in *in vitro* culture conditions and in viral isolates are known to affect results. In many cases the cause of the variation is likely to be multifactorial.

Direct infection of marrow precursor cells, leading to compromised function or cell death; growth factor dysregulation (either production of and/or response to); immune mechanisms; opportunistic infections directly affecting marrow; sequestration or consumption of cells; nutritional deficiencies or malignancies have all been implicated as possible mechanisms of cytopaenia.

1.3.8.2. EXPERIMENTAL INFECTION

The pathogenesis of the early, dramatic neutropaenia seen in experimentally infected animals also remains to be determined. The event is consistent and repeatable in the majority of animals. Its occurrence in early natural infections or in HIV is less well documented, but is thought to occur. Complicating factors such as

nutritional deficiencies, therapies and malignancies are not factors at this early stage of infection. Also, since experimental animals are specific pathogen free (SPF) and kept in controlled environments, it is unlikely that secondary or opportunistic infections are relevant to the neutropaenia. The neutropaenia is striking in its profundity, its specificity and in its transience. Once again, several explanations have been offered to clarify this event. Direct infection of progenitor cells; infection of accessory cells causing a growth factor dysregulation; immune mediated mechanisms and sequestration of circulating cells have all been implicated. Sequestration of mature cells in tissues has been investigated by Callanan *et al* (1994). They found evidence of neutrophil accumulation in tissues of selected animals at this time. He suggested that overwhelming infection and toxaemia may be contributory to both sequestration and bone marrow suppression, but this is not common to all affected animals.
It has been established that FIV infection is widespread in lymphocyte populations at this stage, and also, during the phase of acute illness, in monocyte/macrophage cells (Beebe *et al* 1994). These cell lines are known to be potent producers of haemopoietic growth factors. Widespread infection of these cells may therefore disrupt growth factor regulation and upset the delicate balance required for controlled haemopoiesis. At this stage of active viral replication it is also feasible that direct viral infection of progenitor cells may be occurring. A transient phase of replication in these, causing either cell death or a cessation of replicative activity could cause a temporary marrow failure. Similarly, infection of these cells may lead to enhanced self-renewal of this population, with a concomitant reduction in progeny until differentiation is resumed. Following the end of active viral replication, recovery of marrow

activity, with compensation for lost or damaged cells could occur, restoring circulating cell numbers. Surviving infected stem cells could remain in the latent state as an important reservoir of infection in the later stages of disease.

1.3.9. MARROW PATHOLOGY

Bone marrow taken from infected cats in the acute phase of the illness is often hyperplastic, with increased cellularity and a higher proportion of granulocyte and monocyte precursors (Callanan *et al* 1992, Mandell *et al* 1992). It has been reported in some animals (Callanan *et al* 1992) that there is a maturation arrest of the granulocyte line, with increased numbers of immature granulocyte precursors but a relative lack of mature cells and reserve pool of mature granulocytes. Mandell *et al* (1992) reported mild myeloid hyperplasia as the most consistent cytological feature seen in bone marrow at neutropaenia. However, the myeloid response seemed blunted and less vigorous than would have been expected, the shift toward immature cell types not correlating with the degree of the neutropaenia. This suggests a compromised ability of the marrow to respond to neutropaenia. Aggregates of lymphoid follicles have been reported in the marrow at this stage (Callanan *et al* 1992), along with excess numbers of plasma cells, eosinophils and mast cells (Mandell *et al* 1992). Shelton *et al* (1990) found that 13/18 cats in late stage infection had a range of bone marrow abnormalities, whilst only 3/7 of those in the asymptomatic phase had changes. These changes were fewer and less severe, including excess numbers of lymphocytes, plasma cells or eosinophils.

Beebe *et al* (1992) detected FIV RNA in the marrow cells of 8 out of 12 of a diverse group of cats. *In situ* hybridisation revealed the

RNA in megakaryocytes and unidentified mononuclear cells. Positive cells were found most frequently in cats displaying signs of severe illness, those in the acute phase illness and in those with granulocytic myelodysplasia. Even in the sickest animals, the infection rate was low, and RNA could not be detected in those infected cats which were showing no clinical signs. Leukopaenia, usually associated with a neutropaenia, was the most prevalent haematological finding in these cats. Linenberger *et al* (1995) also reported the detection of infected bone marrow mononuclear cells and megakaryocytes by *in situ* hybridisation at weeks 4 to 12 after experimental inoculation (no animals were tested after 12 weeks). The highest viral loads were found in the sickest animals. Soluble p24 antigen could be detected in similar cell types at this time.

1.3.10 TREATMENT

To date, no specific anti-viral drugs have been available to treat FIV. Drugs which have been used in HIV infection have been shown to be effective *in vitro* against FIV. Yates *et al* (1992) reported the use of acemannan in infected cats. Acemannan is a complex carbohydrate which stimulates interleukin-1, tumour necrosis factor-α and prostaglandin e2 production by macrophages. It had shown anti-viral activity against HIV *in vitro*. Treatment over a 12 week period resulted in significant increases in lymphocyte counts, neutrophil counts fell and the incidence of sepsis decreased. Together, these events probably indicate an improvement in the cats' ability to deal with infections. Limited data suggest that survival rates may be improved using this drug.

A second drug, 9-(2-phosphonylmethoxyethyl)adenine, (PMEA), has also been shown to inhibit viral replication *in vitro* (Philpott *et*

al 1992). Egberink *et al* (1991) tested the efficacy of PMEA both *in vitro* and *in vivo* and found that viral replication was inhibited. *In vivo* PMEA alone was found to reduce clinical disease in naturally infected cats with opportunistic infections. The nucleoside analog 3'-azido-3'deoxythymidine (AZT) used in treating HIV in man was used in conjunction with PMEA *in vivo* and was proved to have a therapeutic effect on infected cats (Egberink *et al* 1991). Antiviral drugs can prolong the life of FIV-infected cats, however, none of these drugs has been shown to eliminate infection. Unfortunately, it is unlikely that these or other antiviral drugs will become available to treat cases of disease, due to prohibitive cost.

Treatment of secondary and opportunistic infections suffered by FIV positive cats is important. Specific infections should be treated with appropriate antibiotic therapy, or, if no specific diagnosis is possible, with a broad spectrum bacteriocidal agent. Hopper *et al* (1994) also report the successful use of corticosteroids in some pyrexic or inappetant cats. However, the long-term use of these drugs may be deleterious due to their immunosuppressive effects. Other symptomatic therapies should be undertaken when necessary. It is advantageous to confine FIV positive cats, not only to prevent the transmission of infection to other cats, but also to try to limit their exposure to environmental organisms.

1.3.11. PREVENTION

No effective vaccine against FIV exists to date. Prevention of lentivirus infections is difficult. This is due to the cell-associated nature of the virus, the heterogeneity of the envelope glycoproteins and the relatively poor immunogeneity of the relevant viral antigens

(Hosie 1994). Since FIV infects both lymphocytes and macrophages, vaccination would need to prevent initial entry into these cells to enable an effective immune response to be mounted. It has been shown that maternally derived antibodies have been insufficient to protect new-born kittens from picking up infection from their mothers (Callanan *et al* 1991, Wasmoen *et al* 1992, Pu *et al* 1995). It is likely, therefore that a cell-mediated immune response would be required from an efficient lentivirus vaccine, as well as a strong neutralising antibody response.

Inactivated infected cell vaccines and inactivated virus vaccines have both been shown to provide protection in the experimental situation (Yamamoto *et al* 1991, 1993). These vaccines were found to induce strong antibody responses both to envelope glycoproteins, and also to core proteins. The nature of the mechanism of protection in these studies remains to be clarified. It has been demonstrated in SIV vaccine studies that immune responses may be directed at cell lines used to grow vaccine in, rather than to the virus itself (Hosie 1994). Investigations conducted in FIV have demonstrated that vaccines developed in the FL4 cell line are more successful than those grown in other cells. Whether this is due to immune response directed at the cell antigens or whether the virus grown in this system is antigenically superior is not known. Protection against variant strains of virus is another problem which has yet to be fully addressed.

1.3.12. MANAGEMENT AND CONTROL

In the absence of an effective FIV vaccine, the best way to prevent infection is to avoid contact with infected animals. This may involve confining a cat indoors, or to a restricted area outdoors. If this is not

possible, it is better to have the cat neutered, as this reduces the likelihood of fighting and roaming, especially in males.

In the event of a pet cat being diagnosed as virus positive, it is recommended that it should be isolated from all other cats. If other cats are present in the household they should ideally be kept separately. If contact is unavoidable then the risk of transmission exists, although the risk is likely to be small provided the cats do not fight.

Infected queens should not be bred from, as there is a risk of transmission to offspring. Infected toms should not be bred from although there is no risk of sexual transmission.

Euthanasia is usually advised only when the cat becomes severely ill in the latter stages of disease.

1.3.13. PROGNOSIS

The prognosis given for infected cats must be guarded, given that infection is lifelong. It is not usually possible to pinpoint the time of infection, and the incubation period is most often prolonged. Attempt should be made to stage the illness (Ishida *et al* 1990), and cats showing signs of immunodeficiency be given a poorer outlook compared with those in the asymptomatic stage. Mortality rates for cats in the clinical stage of disease have varied, with estimates of 12 month mortality rates of 15% (Ishida *et al* 1989) to 45% (Hopper *et al* 1989). It is therefore difficult to estimate survival times for individual cases. Euthanasia on humane grounds is usually necessary in the final stages.

1.4. HUMAN IMMUNODEFICIENCY VIRUS (HIV)

1.4.1. GENERAL OVERVIEW

HIV was first isolated as the aetiological agent of the acquired immunodeficiency syndrome (AIDS) in 1983 (Barre-Sinoussi *et al* 1983, Gallo *et al* 1884). It is a lentivirus, and as such, demonstrates many of the characteristics common to the family.

Two subtypes of HIV have been identified; HIV-1, the more widespread of the two, and the quite distinct HIV-2, which was predominantly restricted to West Africa. The major biological and genetic features are essentially similar.

Wong-Staal (1990) reviewed the properties of HIV. The virus has a single-stranded RNA genome contained in a dense protein core and surrounded by a lipid envelope.

HIV replicates in the same way as other lentiviruses, although its entry into cells appears to be by a different method to other retroviruses, in that it is not by receptor-mediated endocytosis. The cellular receptor to HIV has been identified as the CD4 molecule. This receptor is expressed mainly on T-helper lymphocytes and cells of the monocyte/macrophage lineage, hence the viral tropism for these cell lines. HIV also enters cells which do not express the CD4 molecule, for example endothelial cells. The mechanism of entry is not known.

Many variant types of the HIV genome have been isolated, with the envelope genes being the most commonly altered. This causes considerable difficulties in the design of potential vaccines.

1.4.2. PATHOGENESIS AND CLINICAL FEATURES

HIV, in common with other lentiviruses, has a narrow host range, being confined mainly to human cells, although infection of chimpanzees is possible. Hirsch & Curran (1990) have reviewed the major biological properties of HIV.

Transmission is by sex, blood or blood products and perinatally. Needles shared by intravenous drug users are also important in the passage of virus. HIV can be transmitted across the placenta, but there is also evidence for transmission during birth and through breast feeding.

Following infection, HIV is disseminated to target organs in blood. Both the humoral and cell-mediated arms of the immune response are activated in response to infection. Most infected subjects produce neutralising antibody, which is directed mainly at envelope glycoproteins. It is not clear whether levels of antibody drop in correlation with disease progression, as investigations have produced variable results. An antibody-dependent cell-mediated cytotoxic response (ADCC) is mounted, which is also predominantly directed against *env* proteins. There is a drop in titre of these antibodies associated with disease progression. Cytotoxic T-lymphocytes act against a wider range of viral products, produced by the *pol* and *gag* genes, as well as *env*.

1.4.3. PROGRESSION OF INFECTION

The incubation period following infection with HIV is prolonged, averaging 7-8 years in adults (range 1-12) but is much less in perinatally infected infants. Progression is divided into 4 main stages, according to clinical and laboratory findings as follows:

a] Stage 1 - this is the acute phase, sometimes seen soon after infection. Often this is asymptomatic, but some patients suffer a self-limiting illness resembling a mononucleosis or flu-like syndrome. It may be seen from a few days to 3 months after infection. Haematologically there is leukopaenia, lymphopaenia, relative monocytosis and thrombocytopaenia. There are raised numbers of CD8 positive lymphocytes, and abnormal forms may be seen on smears. Recovery takes place in 1-2 weeks.

b] Stage 2 - the so-called asymptomatic phase, which may last for 7-9 years. Clinically the infected subject is normal, but laboratory tests eventually demonstrate a gradual depletion in CD4 positive cells in peripheral blood. The numbers of these cells serve as a prognostic indicator.

c] Stage 3 - persistent generalised lymphadenopathy (PGL). This is not always seen in HIV infection, but sometimes occurs towards the end of the asymptomatic phase. It is not an accurate indicator of progression, but changes in the nature of the lymph node pathology can provide useful indications.

d] Stage 4 - AIDS-related complex (ARC) and AIDS. The factors which contribute to the progression of disease to this clinical stage are not well defined. The role of other infectious agents and genetic predispositions remain to be clarified. It may occur simply as a result of CD4 cell numbers dropping below a critical level. An infected subject displaying two or more relevant clinical symptoms and two or more significant laboratory abnormalities is classified as suffering from ARC. This is the final stage before full-blown AIDS. AIDS patients suffer from a huge variety of chronic and opportunistic infections. These commonly include infections and syndromes which are peculiar to immunologically-compromised

patients such as *Pneumocystis carnii* pneumonia and Kaposi's sarcoma. Symptoms are therefore wide-ranging, depending on the type of infection.

1.4.4. HAEMATOLOGICAL CHANGES

1.4.4.1. CYTOPAENIAS

Haematologically, most AIDS patients suffer from anaemia. Thrombocytopaenia is also common, and lymphopaenia occurs as a result of direct viral lysis (Ho *et al* 1995, Wei *et al* 1995). Cytopaenias of any of the blood cell lineages can occur in any combination, complicating both the clinical picture and the approach to therapy. Lupus-like anticoagulants have also been isolated (Cohen *et al* 1986).

Doweiko (1993) reviewed the cytopaenias in HIV infection. Anaemia is the most common cytopaenia, increasing in severity with progression of disease, up to 70-80% of patients becoming affected in the terminal stages. The aetiology of anaemia in these patients is thought to be multifactorial, with immune mechanisms, defective progenitor cell growth, inhibited maturation, opportunistic infections, nutritional deficiencies, neoplasms or myelosuppressive drugs all thought to play a role. Anaemia is usually normochromic and normocytic, and the lack of an appropriate marrow response suggests a failure of production or maturation (Aboulafia & Mitsuyasu 1991).

Granulocytopaenia and/or monocytopaenia tend to be associated with anaemia, the incidence again rising to a maximum of 75% in AIDS patients. Abnormalities in granulocyte form may be seen, including hypogranularity, hypersegmentation and Pelger-Huet forms (Kaczmarski & Mufti 1993).

Thrombocytopaenia occurs at all stages and independently of other deficiencies in late stage disease. Thrombocytopaenic patients show no signs of disease in many cases, but may present with symptoms similar to those of people suffering from immune-mediated thrombotic disease (Aboulafia & Mitsuyasu 1991).

1.4.4.2. HIV PATHOGENESIS OF CYTOPAENIAS

The multiplicity of haematological abnormalities seen in HIV are multifactorial in aetiology. Although the presence of autoantibodies and repeated infections suggest peripheral destruction or consumption of cells as contributory factors, most evidence points toward inefficient haemopoiesis as the primary cause of low circulating cell numbers. By the terminal stages of disease, when the cytopaenias are most severe and most common, a number of complicating factors exist which compromise bone marrow function. These patients are often on anti-viral and anti-bacterial drugs, many of which are myelosuppressive in their own right e.g. Zidovudine (AZT) (Scadden 1989, Calenda & Chermann 1992a, Doweiko 1993, Kaczmarski & Mufti 1993). Marrow infiltration by opportunistic organisms is not uncommon, infection with agents such as cytomegalovirus and *Mycobacterium avium intracellularae* complex often result in disseminated infection with marrow involvement. Fungal infections are also found in marrow, resulting in suppression of marrow function (Scadden 1989, Calenda & Chermann 1992, Doweiko 1993, Kaczmarski & Mufti 1993). Neoplasms such as B-cell lymphomas have an increased incidence in AIDS patients, again, these tend to be aggressive and disseminated. Kaposi's sarcoma is also seen in marrow in some

cases (Scadden 1989, Calenda & Chermann 1992, Doweiko 1993, Kaczmarski & Mufti 1993).

Nutritional deficiencies in the terminal stages are thought to be significant, for example in some cases there is evidence of vitamin B12 malabsorption, with resultant effects on erythropoiesis (Aboulafia & Mitsuyasu 1991).

The effects of HIV itself on haemopoiesis are controversial, with conflicting experimental evidence for and against direct infection of marrow stem cells by the virus. Explanations such as infection of stromal cells; altered cytokine production by infected lymphocytes and monocyte/macrophages; antibody mediated suppression of progenitor cells or production of inhibitory factors by infected accessory cells all have some experimental evidence to support them.

A number of authors have reported reduced numbers of colony forming cells (CFU-GM, BFU-E, CFU-E, CFU-GEMM and CFU-Meg) in HIV positive patients compared to controls (Leiderman *et al* 1987, Steinberg *et al* 1991, Calenda and Chermann 1992, Louache *et al* 1992, Potts *et al* 1992, Zauli *et al* 1992a, 1992b, 1992c, De Luca *et al* 1993, Re *et al* 1993). This is true of *in vitro* or *in vivo* infection, although the degree of suppression varied according to levels of infection and stage of patient disease respectively. Most authors have failed to find evidence of latent or active infection of CD34+ precursor cells by PCR, or found infection at very low levels (Davis *et al* 1991, Louache *et al* 1992, Zauli *et al* 1992a, 1992b, 1992d, De Luca *et al* 1993). However, Folks *et al* (1988) reported *in vitro* infection of purified myeloid progenitors with HIV and differentiation of these cells in culture. Virus was expressed only after 40-60 days in culture, indicating that

monocyte differentiation is essential for virus production. No cytopathic effect was observed. It was not determined whether the progenitors were infected at onset, or whether contaminating monocytes were responsible for infecting newly differentiated cells. Kojouharoff *et al* (1991) found colonies grown from purified progenitor cells (CD34+) infected *in vivo* were positive for HIV mRNA by in situ hybridisation, indicating active infection of these cells. Steinberg *et al* (1991) were able to detect viral sequences in both CD34+ cells and in T-cell depleted fractions of bone marrow cells. Chelucci *et al* (1995) also found colonies grown from *in vitro* infected circulating CD34+ cells were positive for HIV DNA by PCR and HIV mRNA by RT-PCR, the extent of infection varying with colony type and sequence of DNA searched for. Evidence of viral infection was seen in BFU-E and CFU-GM, but not in CFU-GEMM. This is suggests that infection is dependent on the stage of differentiation of the progenitor cell. The infection rate of the entire CD34+ pool may be relatively low for this reason, thus explaining the failure by other investigators to detect virus. Although Kaczmarski *et al* (1992) were unable to demonstrate differences in colony numbers in asymptomatic HIV patients compared to controls, they were able to detect HIV DNA by PCR in pooled CFU-G and CFU-GEMM.

Sugiura *et al* (1992) found the HIV envelope glycoprotein gp160 could enhance myeloid progenitor growth from cord blood cells. This was not repeatable using purified progenitors, but was in the presence of T-cells. The addition of anti-gp160 antibody abrogated the effect. Calenda & Chermann (1992) were also able to demonstrate a stimulation of CFU-GM growth by infection of progenitor cells with HIV. This was a transient effect, seen at 10

days post-infection, and by 30 days growth had been totally inhibited.

Steinberg *et al* (1991) were able to reverse dose-dependent inhibition of marrow cells by HIV by the addition of anti-gp160 antibodies. Dose-dependent inhibition of colony formation has also been reversed by preincubation of virus with anti-gp120 antibodies (Zauli *et al* 1992a, Re *et al* 1993). These findings suggest that direct virus-cell interactions are required. However, Zauli *et al* (1992b) were also able to reduce CD34+ cell viability by addition of pure recombinant gp120 protein, indicating a direct cytotoxic effect on CD34+ cells.

Leiderman *et al* (1987) found that marrow from AIDS or AIDS-related complex (ARC) patients inhibited growth of GM-CFU from normal individuals. Culture medium derived from HIV-positive marrow also inhibited growth of HIV-negative GM-CFU. Inhibitory activity was associated with a gp84 molecule isolated from liquid culture medium which suppressed colony growth (Leiderman *et al* 1987).

Donahue *et al* (1987) reported that bone marrow progenitors isolated from AIDS or ARC patients respond normally to recombinant growth factors and erythropoietin. This growth was inhibited by HIV antibody positive serum, but this serum had no effect on HIV negative marrow progenitors. It was proposed that progenitor cells in affected marrow were expressing HIV antigens to which the antibody in the serum could attach, whilst the control marrow would have no such antigens and thus be unaffected by the virus positive serum. The virus infection in itself was therefore proposed to be non-cytopathic, as the affected cells could respond

normally to extrinsic growth factors. Rabbit heteroantisera directed against HIV specifically suppressed colony formation.

Louache *et al* (1992) and Zauli *et al* (1992) have both published work implicating the HIV tat protein in the mechanism of cytopaenias. Both groups suggest the effect is an indirect one, induction of the haemopoietic inhibitor TGF-b1 in accessory cell populations being the implied cause.

Infection of T-lymphocytes and cells of the monocyte-macrophage lineage in HIV results in the alteration of cytokine production by these cells (Doweiko 1993). Cell to cell interactions may also be altered in the event of viral infection. Both of these processes may alter haemopoiesis by upsetting the complex homeostatic mechanisms. Doweiko (1993) reviews the range of cytokines produced by HIV infected cells which may be important in the dysregulation of haemopoiesis. IL-1, IL-2, interferons, tumour necrosis factor-a (TNF-a) and transforming growth factor-b (TGF-b) are all potentially suppressive and are produced by virus infected cells or stimulated by products of virus infected cells.

Scadden *et al* (1990) have demonstrated the *in vitro* infection of stromal fibroblasts by HIV. Such infection in vivo, if it exists, could provide an important reservoir of virus and/or a potential source of infective virus for progenitor cells. Since the fibroblast provides a vital source of growth factors for haemopoietic cells, the disruption of function by virus infection could be critical to the continuance of adequate cell production.

Isolated cases of pure red cell aplasia (PRCA) in HIV infected patients with no other apparent aetiology have been reported (Parmentier *et al* 1992, Dervenoulas *et al* 1993). Culture of one patient's marrow failed to elicit any CFU-E and reduced numbers of

BFU-E. This type of pathology has been associated with another retrovirus in cats, that of feline leukaemia virus (FeLV). However, this effect is strain specific, and applies to subgroup C only.

In contrast to the majority of authors, Molina *et al* (1990) compared the marrow of six patients with AIDS with that of four healthy donors and found no statistically significant reduction in colony numbers. No HIV-1 was detected by the polymerase chain reaction (PCR) in these colonies, although it could be detected in peripheral blood cells. *In vitro* exposure of virus to bone marrow cells had no effect on colony growth, nor did it lead to the detection of virus in exposed colony-forming cells.

Immune mediated destruction of peripheral blood cells has been suggested as a cause of cytopaenias. This is based on the detection of red cell, platelet and neutrophil associated antibodies as reviewed by Aboulafia & Mitsuyasu (1991), Calenda & Chermann (1992) and Kaczmarski & Mufti (1993). Despite up to eighty-five percent of AIDS patients having a positive direct antiglobulin test against their red cells, there is little evidence of haemolysis in these patients. Antineutrophil antibodies were detected in twenty-nine percent of HIV positive patients, but no correlation between the presence of these and neutropaenia was found. Similarly, although over ninety percent of patients with HIV have evidence of platelet associated immunoglobulins and circulating immune complexes, there is no clear correlation with the degree of thrombocytopaenia. HIV and FIV are associated with a polyclonal rise in immunoglobulin levels. This may be due to chronic immune stimulation, failure to clear HIV or FIV antigens respectively, opportunistic pathogens or loss of T-cell regulation of B-cell proliferation. Thus, the presence of these

antibodies may result from a non-specific response to a variety of antigens, rather than specific autoreaction.

1.4.4.3. BONE MARROW PATHOLOGY

A clear relationship between marrow cellularity and peripheral cytopaenias has not been established. Abnormalities are frequent in the bone marrows of HIV infected patients, although there are no characteristic changes peculiar to HIV. Increased plasma cells and lymphoid aggregates are seen, which may be a response to abnormal antigenic stimulation or dysregulated B cell proliferation due to HIV (Sun *et al* 1989, Aboulafia & Mitsuyasu 1991, Kaczmarski & Mufti 1993). Bone marrow is generally hyperplastic, although the hyperplasia is not always of haemopoietic cells, e.g. plasma cells or histiocytes (Sun *et al* 1989). There are often elevations in eosinophil numbers. Increased levels of the intercellular matrix protein reticulin have also been reported. Hyaline degeneration of bone marrow stroma may be seen in up to forty percent of HIV infected marrows. Morphological abnormalities are not infrequent. In the late stage disease, hypoplasia and fibrosis are features (Re *et al* 1991).

Conflicting results have arisen from efforts to establish the nature of infected cells in marrow. Busch *et al* (1986) and Zucker-Franklin & Cao (1989) have recorded virus-specific RNA in granulocyte and megakaryocyte precursors respectively. Von Laer *et al* (1990) found only 1 in 14 patients had HIV DNA present in precursor cells. It is clear that if infection of precursor cells exists, that it does so in low frequencies, although the potential for the replicative ability of these cells to increase is not known. Sun *et al* (1989) demonstrated HIV by *in situ* PCR in histiocytes, reticular cells, lymphocytes, immature

myeloid cells (probably myeloblasts), endothelial cells, nucleated red blood cells and red cells. This suggests that HIV can infect a much wider range of cells than was initially presumed, including progenitor cells, making the bone marrow a potentially important reservoir of long-lived infected cells. Wickramasinghe *et al* (1992) identified ultrastructural changes in marrow granulocytes (including eosinophils), erythroid cells, plasma cells and stromal cells which all may have been caused by HIV, supporting the theory that the potential for infection exists in a wide range of marrow cells.

1.5. AIMS OF THE PROJECT

The principal aim of the project was to elucidate the mechanism of neutropaenia in early FIV infection, and to establish whether precursor cells were virus infected. Given the clinical similarities of FIV and HIV, it was hoped that this knowledge would enhance our ability to treat the cytopaenias seen in HIV infected people, and enable more effective use of antiviral products. Much conflicting experimental evidence was available from studies both in cats and in HIV positive people. It was intended to clarify this information by using a uniform group of cases, free from the complications of chronic disease or malnutrition, and which had not been subject to myelotoxic drug therapies.

In order to interpret the haematological data generated during the experiment accurately, it was also considered necessary to re-evaluate "normal" cat haematology values. Existing data for haematological parameters in the cat is known to have been derived from small populations of animals in artificial environments. A database analysis of large numbers of a cross-section of the general

cat population was undertaken to provide more accurate indications of "normal" values and their variations.

Specific intentions of the project were therefore to:-

1. Develop an "expert" system to interpret haematological parameters in the cat by evaluation of data from large numbers of animals in varying states of health.
2. Infect cats with FIV and study haematological parameters over a period of months.
3. Perform bone marrow aspirates on these cats at specific intervals in order to establish numbers of neutrophil progenitor cells and evaluate marrow function. This was to be done by clonal colony assays.
4. To establish, by PCR and by immunohistochemistry, if the clonal colonies grown in the assays were infected with FIV.
5. To establish, by PCR, if mature neutrophils were infected with FIV.

CHAPTER 2

MATERIALS AND METHODS

2.1. ANIMALS

Ten specific pathogen free (SPF) female cats were obtained from a breeding unit and housed at Glasgow University. They were aged between six and eighteen months at the time of infection. The animals were split into two groups, housed in separate rooms and fed a commercial diet.

This study was carried out in accordance with Home Office regulations.

2.2. CHALLENGE VIRUS

The FIV/Glasgow 8 (FIV/GL-8) isolate was obtained from the plasma of a 6 year old entire male domestic cat which had clinical signs of pyrexia, respiratory infection, lethargy, anorexia and weight loss (Hosie 1991). The virus was grown in peripheral blood T cells from SPF cats and the supernatants from the infected culture were filtered through a 0.45μm filter and stored at -70°C.

Uninfected feline thymocytes were incubated overnight with 50ml of this virus stock at a concentration of 2 x 10^6 cells/ml. The following day the cells were washed and the concentration of the cells was adjusted to 5 x 10^5 cells/ml. The culture was monitored for cytopathic effect and when this became apparent (5 days post-infection), the cell suspension was centrifuged at 100rpm for 5 minutes and the supernatant was filtered through a 0.45μm filter and stored in 1ml volumes at -70°C.

The virus titre was found to be 10^4 infectious units/ml in a p51 T-cell assay. This stock was titrated *in vivo* giving a cat infectious dose of 50% (ID50) of 2.2 x 10^4.

Cats in this study were infected by intraperitoneal injection of 4000 ID50.

2.3. CLINICAL EXAMINATION

Cats were clinically examined at times of blood sampling. Demeanour, appetite, coat condition, colour, lymph nodes and gums were all inspected. Any cats appearing unwell were given a more thorough examination, including rectal temperature evaluation.

2.4. HAEMATOLOGICAL PROCEDURES

Cats were manually restrained and 0.5ml samples of blood were taken into potassium-EDTA tubes (0.5ml EDTA Paediatric Push Cap, Bibby Sterilin Ltd., Stone, UK). Cell counts were obtained automatically using the ABX Minos Vet (Roche Products Ltd., Diagnostic Division, Welwyn Garden City, UK). Differential counts were performed manually (200 cells) using May-Grunwald-Giemsa (BDH Laboratories Ltd., Poole, UK) stained smears and cell morphology was assessed at this time.

2.5. BONE MARROW ASPIRATES

Cats were pre-starved and then anaesthetised with 1.5-2ml of alphaxelone/alphadolone acetate (Saffan, Pitman-Moore Ltd, Crewe, UK). Aspirates of marrow were taken from either right or left femur using the Jamshidi disposable 15 gauge sternal/iliac aspiration needle (Baxter Healthcare, Glasgow, UK). Aspirates were collected into 2ml of Iscove's Modified Dulbecco's Medium

(IMDM, Life Technologies Ltd., Paisley, UK) and 200 i.u. preservative free lithium heparin (Sigma Chemical Co., St. Louis, USA).

2.6. FIV ANTIBODY DETECTION

FIV antibody in blood samples was detected by the immunofluorescence test. Ten-fold dilutions of sample serum prepared from heparinised blood samples were added to washed plates containing Crandell feline kidney cells (CRFK) chronically infected with FIV and fixed in methanol. Twenty-five microlitres of sample dilutions were added to separate wells and incubated at 37°C in moisture chambers for 2 hours. The plates were then washed in PBS for 3 minutes and dried. Twenty-five microlitres of FITC anti-cat conjugate was then added to each well and incubated for an hour at 37°C. The plates were then washed in PBS. The plates were examined under ultraviolet light using a x 25 dry objective.

2.7. FIV ISOLATION

Two millilitre samples of heparinised blood were diluted to 8ml in RPMI-1640 medium (Life Technologies Ltd., Paisley UK), layered over 5ml Ficoll-Paque solution (Ficoll-Paque, Pharmacia LKB Biotechnology Inc, New Jersey, USA) and centrifuged at 2000rpm for 10 minutes. The white cells at the interface were removed and pipetted into a clean tube. The solution was diluted to 25ml in RPMI-1640 (Life Technologies Ltd., Paisley UK) and centrifuged at 1000rpm for 5 minutes. The cell pellet was resuspended in 5ml normal T-cell medium (RPMI-1640, 10% BSA (Imperial Laboratories, UK), 2mM glutamine (Life Technologies Ltd.,

Paisley, UK), 100 units penicillin per ml (Life Technologies Ltd., Paisley, UK), 10ug streptomycin/ml (Life Technologies Ltd., Paisley, UK) and 2 x 10^{-5}M mercaptoethanol (Sigma Chemical Co., St. Louis, USA)) with 7.5µg/ml Con A. The cells were cultured for 2 days at 37°C and the unattached cells transferred to a fresh flask containing 5ml normal T-cell medium. The culture medium was changed twice weekly and the cell concentration adjusted to 5 x 10^5 cells per ml. Once a week peripheral blood mononuclear cells were prepared from a normal FIV negative cat by density gradient centrifugation (Ficoll-Paque, Pharmacia LKB Biotechnology Inc, New Jersey, USA) and added to the culture.

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

2.8. MARROW CULTURE TECHNIQUES

2.8.1. PREPARATION OF AGAR

Thirty-three grams of agar (Agar Noble, DIFCO Laboratories, Michigan, USA) was added to 1000ml of distilled water (Life Technologies Ltd., Paisley, UK). The mixture was heated to boiling point, stirring all the time. When the agar had dissolved, the solution was aliquoted into 50ml centrifuge tubes (Falcon, Becton Dickinson UK Ltd., Oxford, UK), cooled and stored at room temperature.

2.8.2. PREPARATION OF METHYLCELLULOSE

Twenty-eight grams of methylcellulose powder (Eastman Kodak Co, New York, USA) were added to 500ml of hand hot water (Life Technologies Ltd., Paisley, UK) and stirred on a hotplate. The mixture was allowed to boil and then simmered for 20 minutes, stirring all the time. After cooling for 90 minutes, an equal volume of IMDM, heated to 37°C, was added and stirred for 20 minutes. The resultant solution was stirred constantly overnight at 4°C, aliquoted into 500ml bottles and frozen at -20°C overnight. Following thawing, 0.2ml of 10^{-5}M $NaSeO_3$/20ml methylcellulose (Sigma Chemical Co., St. Louis, USA) and 0.05ml of 10^{-1}M a-thioglycerol/20ml methylcellulose (Sigma Chemical Co., St. Louis, USA) were added and mixed thoroughly. The methylcellulose was then aliquoted into Oak Ridge centrifuge tubes (BDH Laboratories Ltd., Poole, UK), spun at 17000g for 2 hours and stored at -20°C until ready for use.

2.8.3. CELL PREPARATION

Marrow aspirates were first layered onto a Ficoll-Diatrizoate gradient (Ficoll-Paque, Pharmacia LKB Biotechnology Inc, New Jersey, USA) and centrifuged for 15 minutes at 3000 rpm. Interface cells were then aspirated and washed in an equal volume of IMDM, spun for 5 minutes at 1500 rpm and resuspended in 1-5ml of IMDM, depending on pellet size. Nucleated cell counts were then performed using the ABX Minos Vet automated counter.

2.8.4. GRANULOCYTE-MACROPHAGE COLONY ASSAY

Cells prepared as above were suspended at a concentration of 5 x 10^4/ml in a mixture containing 0.66ml batch-tested foetal calf serum (FCS Advanced Protein Products Ltd., Brockmoor, W. Midlands UK); 0.033ml batch-tested bovine serum albumin (BSA, Sigma Fraction V, Sigma Chemical Co, St Louis, USA); recombinant human growth factors granulocyte-macrophage colony stimulating factor (GM-CSF, Glaxo IMB S.A., Geneva, Switzerland), granulocyte colony stimulating factor (G-CSF, Amgen, Thousand Oaks, USA) or interleukin-3 (IL-3, Sandoz, Basle, Switzerland) at 7.5ng/ml, 10ng/ml and 10ng/ml respectively (diluted in 10% BSA), and IMDM (with 300mg penicillin G (Sigma Chemical Co., St. Louis, USA) and 25mg streptomycin sulphate per 500ml (Sigma Chemical Co., St. Louis, USA)) to a total volume of 2.97ml. 0.33ml of 3.3% agar (Agar Noble, DIFCO Laboratories, Michigan, USA) was then brought to boiling point in a water bath and added to the cell suspension. The resultant mix was then transferred in 1ml aliquots to three 35 x 10mm petri dishes (Falcon, Becton Dickinson UK Ltd., Oxford, UK) allowed to set and incubated in humidified conditions at 37°C, 5% CO2 for 8 days (Heraeus D6450 Hanau, Heraeus Equipment Ltd., Brentwood, UK).

2.8.5. MIX COLONY ASSAY

Cells prepared on the Ficoll gradient were suspended at 5 x 10^4 cells/ml in a mixture containing 0.3ml FCS; 0.1ml BSA; 2 i.u. human recombinant erythropoietin (EPO, Terry Fox Laboratories, Vancouver, Canada); growth factors as for the granulocyte-

macrophage assay and 0.5ml of 2.8% methylcellulose in IMDM (Eastman Kodak Co. Ltd. New York USA) to a total volume of 1ml. Three 0.25ml aliquots of this suspension were added to 3 wells of a 24 well plate (Falcon, Becton Dickinson Ltd., Oxford, UK) and incubated in humidified conditions at 37°C , 5% CO2 for 14 days (Heraeus D6450 Hanau, Heraeus Equipment Ltd., Brentwood, UK).

2.8.6. COLONY SCORING

Unstained colonies (CFU-GM, CFU-G, CFU-M and BFU-E) from both assay systems were counted using a Leitz Labovert FS (Leitz, Wetzlar, Germany) inverted microscope at four times magnification. This is described in more detail in chapter 4.

2.9. POLYMERASE CHAIN REACTION

2.9.1. COLONY PREPARATION

Colonies from both assay systems were picked from the growth medium using a Pipetman (Gilson Medical Electronics, Villiers-le-Bel, France) and washed in 100ml phosphate-buffered saline (PBS) warmed to 37°C. These were spun at 13000rpm for 5 minutes and the supernatant removed using a Pipetman. The colony material was resuspended in 40µl of purified water (Millipore Q50 Water Purification System, Millipore (UK) Ltd., Watford, UK). The resultant suspensions were stored at -20°C until ready for analysis. Immediately before being added to the PCR reaction mix, the colony suspensions were boiled in a water bath for 5 minutes to ensure adequate disruption of cell membranes and DNA, and then immediately cooled on ice.

2.9.2. PRIMER DESIGN

Primers for the long term repeat (LTR) region of the FIV genome were used as this is a well conserved region. These had been previously designed by Fiona Thompson in the Department of Veterinary Pathology.

The sequences were as follows:

LTR a : 5' TGG GAT GAG TAT TGG AAC CCT GAA GA 3'

LTR b : 5' TGC GAA GTT CTC GGC CCG GAT TCC 3'

These primers allowed for the amplification of a 354 base pair fragment. The primers were then manufactured and column purified by Alta Bioscience, Birmingham, UK. Dried primers were reconstituted in 400µl purified water (Millipore Q50 Water Purification System, Millipore (UK) Ltd., Watford, UK), the concentration determined by spectrophotometry (Beckman DU 640, Beckman Instruments (UK) Ltd., High Wycombe, UK), and adjusted to 1µg/µl.

2.9.3. PCR CONDITIONS

PCR reactions were set using reagents from the GeneAmp PCR Reagent Kit and AmpliTaq DNA Polymerase (Perkin-Elmer Ltd, Beaconsfield, UK). 1.25µl of 10 x PCR buffer (15mM MgCl2, 500mM KCl, 100mM Tris-HCl, pH8.3 and 0.01% gelatin); 1µl of each of 4 dNTPs (10mM), dATP, dTTP, dGTP and dCTP; 1µl of each of the 2 preprepared primer solutions (1mg/ml) and 5.25µl of purified water were added to a 0.5µl reaction tube (Treff AG, Degersheim, Switzerland). To each tube was then added a single Ampliwax PCR Gem 100 wax bead (Perkin-Elmer, Beaconsfield, UK). The tubes were closed and heated in a thermal cycler (Omnigene TR3, Hybaid Ltd., Teddington, UK) to

80°C for 5 minutes and allowed to cool back to room temperature. This allowed the wax bead to melt and then solidify to form a wax barrier on top of the reaction mix. To the tube was then added 5µl of buffer as for the lower layer, 0.25µl of AmpliTaq DNA Polymerase, 17.25µl of purified water and 15µl of the colony suspension mix.

The tubes were then transferred to the thermal cycler (Omnigene TR3, Hybaid Ltd., Teddington, UK) and subjected to 1 minute at 91°C, 1 minute at 50°C and 2 minutes at 72°C for a total of 40 cycles. On completion of the programme, the tubes were removed and stored at -20°C until ready for gel analysis.

2.9.4. GEL ELECTROPHORESIS

2.9.4.1. GEL PREPARATION

Four percent polyacrylamide gels were prepared for use in a Mini-Protean II Electrophoresis Cell (Biorad Laboratories Ltd, Hemel Hempstead, UK). A gel mix containing 1.25ml of 10 x Tris-buffered EDTA (TBE); 1.67ml of acrylamide gel mixture (acrylamide : bis-acrylamide = 30% : 0.8%, Scotlab, Coatbridge, UK); 9.47ml purified water (Millipore Q50 Water Purification System, Millipore (UK) Ltd., Watford, UK); 100µl 10% solution of ammonium persulphate (APS, BDH Laboratories Ltd., Poole, UK) and 6µl of TEMED (N,N,N',N'-tetramethylethylenediamine, Sigma Chemical Co., St. Louis, USA) was prepared and poured between the plates. Three-quarter millimeter thick, 15 well combs were used to create wells in the gel. The gel was allowed to set at room temperature for a minimum of 90 minutes.

Combs were removed once the gel had set, and the wells were flushed with 1 x Tris-borate buffer (TBE; 90mM Tris, 90mM

boric acid, 25mM EDTA). TBE buffer (1 x) was also used to fill the electrophoresis tank.

2.9.4.2. PREPARATION OF SAMPLES AND ELECTROPHORESIS CONDITIONS

Five microlitres of the PCR products were then mixed with 1µl of gel electrophoresis dye (12.5 mg bromophenol blue (BDH Laboratories, Poole UK); 12.5 mg xylene cyanol FF (Sigma Chemical Co, St. Louis, USA); 1.5ml 30% glycerol solution (BDH Laboratories, Poole, UK) and 3.5ml water) in a 96 well plate. Five microlitres of the resultant mix was then added to each well. One microlitre of ϕX174 RF DNA/ Hae III fragments (Life Technologies Ltd., Paisley, UK) were diluted in 4µl of purified water and mixed with 1µl of electrophoresis dye. This mixture was added to the wells at either end of the gel to provide markers for rate of movement of DNA fragments of a particular size. The gel was then run at 80 volts for 50 minutes.

2.9.4.3. GEL STAINING

Gels were removed from the cell and immersed in 100ml water containing 6µl of a 3mg/ml ethidium bromide (Sigma Chemical Co, St. Louis, USA) solution. The gels were then agitated on a mixer for 10 minutes. The staining solution was drained and the gels washed in purified water (Millipore Q50 Water Purification System, Millipore (UK) Ltd., Watford, UK). The gels were then visualised using an ultraviolet transilluminator (Ultra-Violet Products Ltd., Cambridge, UK). Hard copies of the gel images were recorded on heat-sensitive paper using a camera and video copy processor (Mitsubishi Electric UK Ltd., Hatfield, UK).

2.10. SOUTHERN BLOTTING

2.10.1. PROBE GENERATION

2.10.1.1. PRIMER DESIGN

A probe was designed internal to the primers used for the LTR sequence for maximum specificity. The sequences were as follows:

PRO 1: 5' GCT TAT GGA CTA AGG ACT GTC 3'

PRO 2: 5' GAG ACC TCA CAG GTA GAA AAG 3'

This allowed for the amplification of a 292 base pair fragment. The primers were manufactured using the Applied Biosystems 432A Peptide Synthesiser (Applied Biosystems Ltd., Warrington, UK)

Primers were dissolved in purified water (Millipore Q50 Water Purification System, Millipore (UK) Ltd., Watford, UK) and concentration established using a spectrophotometer (Beckman DU 640, Beckman Instruments (UK) Ltd., High Wycombe, UK). Primers were then diluted to a concentration of 1mg/ml in purified water.

2.10.1.2. PCR CONDITIONS

PCR reactions were made up using reagents from the GeneAmp PCR Reagent Kit and AmpliTaq DNA Polymerase (Perkin-Elmer Ltd, Beaconsfield, UK). Half millilitre reaction tubes (Treff AG, Degersheim, Switzerland) were set up as follows: 5μl PCR buffer; 4 x 1μl dNTPs; 0.25μl TAQ polymerase; 2 x 1μl primers as above; 28.75μl purified water and 10μl plasmid containing the LTR sequence (obtained from Fiona Thompson).

Two drops of mineral oil (Perkin-Elmer Ltd, Beaconsfield, UK) were added to the top of the mix and they were subjected to 40 cycles of the undernoted PCR conditions in the thermal cycler (Omnigene TR3, Hybaid Ltd., Teddington, UK):

1 minute @ 91°C

1 minute @ 50°C

1 minute @ 72°C.

2.10.1.3. GEL ELECTROPHORESIS

A 1% agarose gel was prepared (1g of agarose powder in 100ml of TBE, boiled and allowed to cool). Six microlitres of PCR product from different reaction tubes were added to 5 lanes, premixed with 1μl of gel electrophoresis dye.

The gel was then run in TBE at 100 volts for 45 minutes and stained using ethidium bromide for 20 minutes. The products were visualised under a ultraviolet transilluminator (Ultra-Violet Products Ltd., Cambridge, UK).

2.10.1.4. PREPARATION OF DNA

Bands of PCR product were excised from agarose gels and put in a 1.5ml plastic tube (Treff AG, Degersheim, Switzerland). Four and a half x volumes of NaI stock solution and 0.5 x volume of TBE modifier (Geneclean II Kit, Bio-01 Inc., La Jolla, USA) were added to the agarose and incubated at 50°C for 5 minutes to dissolve the agarose. Five microlitres of glassmilk suspension (a silica matrix used to bind DNA but eliminate small contaminants) was added and incubated on ice for 15 minutes. This was spun at 13000rpm for 5 seconds. The supernatant was removed and the pellet washed 3 times with New Wash (Bio-01

Inc. La Jolla, USA), spinning at 13000rpm for 5 seconds each time. The supernatant was removed and 20μl of purified water (Millipore Q50 Water Purification System, Millipore (UK) Ltd., Watford, UK) was added to dissolve the pellet. This was incubated on ice for 5 minutes, spun at 13000rpm for 5 seconds and the supernatant containing the DNA removed. The pellet was redissolved in water and the final step repeated to increase the yield.

The product was analysed using a spectrophotometer (Beckman DU 640, Beckman Instruments (UK) Ltd., High Wycombe, UK) and the DNA concentration determined.

2.10.2 GEL PREPARATION

2.10.2.1. DENATURATION AND NEUTRALISATION

Acrylamide gels were denatured (1.5M NaCl, 0.5M NaOH) for 10 minutes on a mixer, then neutralised (1.5M NaCl; 0.5M Tris; HCl to pH 7.4) for 10 minutes and washed in Tris-acetate buffer (TEA: Tris 9.69g; NaOAc($3H_2O$) 5.44g; NaCl 2.34g; EDTA(Na_2) 1.49g; glacial acetic acid to pH 8.15; water to 2000ml).

2.10.2.2. ELECTROBLOTTING

Gels were electroblotted onto nylon membranes (Hybond N, Amersham International plc, Little Chalfont, UK) using the Mini Trans-Blot Electrophoretic Transfer Cell (Biorad Laboratories, Hemel Hempstead, UK). Gels were sandwiched in the holder cassette next to the nylon membrane between 2 fibre pads and 2 sheets of filter paper. The cassettes were then suspended in a tank of chilled (4°C) TEA. A voltage of 20 volts was then

applied across the cassette for a period of 1 hour, the buffer being mixed throughout. The membranes were dried between 2 sheets of filter paper, the DNA crosslinked by a ultraviolet source (Spectrolinker XL-1500, Spectronics Corporation, New York, USA) and stored at room temperature.

2.10.3. RADIATION SOURCE

a-^{32}P-labelled dCTP 370MBq/ml was obtained from Amersham International plc, Little Chalfont, UK.

2.10.4. NICK TRANSLATION

An α-^{32}P-labelled probe was prepared by nick translation using the kit marketed by Amersham International plc, Little Chalfont, UK as follows: 10ng DNA prepared as above was added to a 1.5ml screw-top plastic tube (Treff AG, Degersheim, Switzerland). To this was added 10μl of nucleotide/buffer solution (100mM dTTP, 100mM dATP, 100mM dGTP in concentrated buffer: Tris/HCl, pH7.8; MgCl and 2-mercaptoethanol); 5μl (1.85MBq) of a-32P-labelled dCTP; 5μl of DNA polymerase solution (2.5 units DNA polymerase 1 and 50pg DNase 1 in buffer: Tris/HCl, pH7.5; MgCl; glycerol and BSA) and water up to a total volume of 50μl, the enzyme being added last. This mixture was incubated at 14^{o}C for 60 minutes in a water bath to allow translation to take place.

The resultant labelled DNA was purified on a NICK column (Pharmacia LKB Biotechnology, Milton Keynes, UK) to separate unbound nucleotides. The column was prepared by running two column volumes of 1 x Tris-EDTA buffer (TE; 10mM Tris, 1mM EDTA) through. 50μl of labelled probe was then added to the top

of the column, followed immediately by 400μl of 1 x TE. The displaced 400μl was collected and discarded. A further 400μl of TE was then added to the top of the column and this time the displaced volume was collected into a 1.5ml screw top plastic tube (Treff AG, Degersheim, Switzerland).

One microlitre of this solution was transferred into a scintillation counter vial and 5ml of scintillation fluid (Ecoscint A, National Diagnostics, Manville, USA) was added. The specific activity was then measured in a scintillation counter (Beckman LS1801, Beckman Instruments (UK) Ltd., High Wycombe, UK).

2.10.5. PREHYBRIDISATION AND ADDITION OF PROBE

The nylon membranes prepared from the electroblots were presoaked in 2 x sodium chloride/ sodium citrate buffer (SSC: 0.3M NaCl; 0.03M trisodium citrate) at room temperature and rolled in meshes before insertion into glass tubes. Ten millilitres of prehybridisation buffer consisting of 6 x SSC; 0.5% sodium dodecyl sulphate (SDS); 5 x Denhardts (1% w/v Ficoll; 1% polyvinyl pyrrolidone; 1% BSA fraction V) solution; water and 100ug/ml freshly boiled salmon sperm DNA, preheated to 65°C, was added to each tube. The tubes were incubated in an oven (Hybaid Mini Oven Mk II, Hybaid Ltd., Teddington, UK) for 6 hours at 65°C.

The prepared radioactive probe was boiled in a water bath for 5 minutes and cooled on ice. Probe was added to the prehybridisation solution at a rate of 1-5 x 10^6 cpm per tube (per 10ml prehybridisation buffer), allowed to mix thoroughly and the solution added back into the tube containing the membranes. This

was incubated overnight in an oven at 65°C (Hybaid Mini Oven Mk II, Hybaid Ltd., Teddington, UK).

2.10.6. WASHING

After overnight incubation, the prehybridisation solution and probe were discarded and the membranes rinsed twice in 2 x SSC warmed to 37°C. The membranes were then washed three or four times, depending on the monitored activity of the washing solution, in a 0.1% SSC, 0.5% SDS solution to ensure all non-bound probe was discarded. The membranes were removed from the tubes and rinsed in 2 x SSC and dried between 2 pieces of filter paper.

2.10.7. DEVELOPMENT

Hybridised blots were wrapped between 2 sheets of plastic tubing, placed in screened cassettes (Dupont) containing radiographic film (Biomax MR Scientific Imaging Film, Eastman Kodak Co., New York, USA.) and left overnight at -70oC. The film was developed in an automatic processor (Kodak X-Omat Processor, model ME-3, Eastman Kodak Co, New York, USA) and assessed for activity. The incubation period was extended up to 7 days at -70°C for films with weak activity, and reduced to as little as 6 hours at room temperature in the event of very active films.

2.11. IMMUNOHISTOCHEMISTRY

2.11.1. COLONY PREPARATION

Colonies for immunohistochemistry were picked from the assay plates using a Pipetman (Gilson, Medical Electronics, Villiers-le-Bel, France) and added to 100μl of PBS warmed to 37^{o}C. The 100μl cell suspensions were then cytospun (Shandon Cytospin 3, Life Sciences International (UK Ltd), Basingstoke, UK) at 1500rpm for 5 minutes onto glass slides (Gold Star Micro Slides, Chance Propper Ltd., Warley, UK), rapidly air-dried and fixed in acetone (Fisons Scientific Equipment, Loughborough, UK) for 10 minutes. The individual slides were then wrapped in aluminium foil and stored at -20^{o}C.

2.11.2. METHOD

Slides were allowed to reach room temperature before being unwrapped and placed in a moist chamber. Slides were then flooded with monoclonal anti-p24 antibody (43-IB9, obtained from Professor Niels Pedersen, University of California, Davis, USA) for 30 minutes at room temperature. Antibody was washed off in Tris-buffered saline (TBS: 0.15M NaCl, 0.05M Tris HCl pH 7.6) for 1-2 minutes. Slides were then flooded with a 1/50 dilution of mouse immunoglobulin (Dako Ltd., High Wycombe, UK) for 30 minutes and washed as previously in TBS. A further 30 minute incubation with alkaline-phosphate, anti-alkaline phosphate (APAAP, Dako Ltd., High Wycombe, UK) was then performed, followed by a TBS wash. Fast red substrate (Dako Ltd., High Wycombe, UK) was then added to the slides and left for 20 minutes. This was washed using first TBS, then tap water.

The slides were then counterstained using Mayer's haematoxylin (BDH Laboratories, Poole, UK) for 20 seconds, washed in tap water then in Scott's Tap Water Substitute (STWS: $Na(CO_3)_2$ 3.5g; $MgSO_4$ 20g made up to 1 litre with distilled water) and mounted in an aqueous mounting medium (Glycergel, Dako Ltd., High Wycombe, UK).

2.12. NEUTROPHIL SEPARATION

2.12.1. PERCOLL PREPARATION

Percoll (Pharmacia Ltd, Milton Keynes, UK) solutions of density 1.08 and 1.119g/ml were prepared in saline as follows:

Density (g/ml)	Percoll (ml)	NaCl 1.5M (ml)	HCl (M) (ml)	H_2O (ml)
1.08	11.4	2	6	6.6
1.119	17.4	2	6.5	0.6

4ml of each solution was carefully layered into a 15ml conical centrifuge tube, the most dense layer first.

2.12.2. SEPARATION PROCEDURE

Four millilitres of blood were collected into EDTA tubes (Bibby Sterilin Ltd., Stone, UK) from the jugular veins of manually restrained cats. Two millilitres of the blood was diluted 1:1 in sterile PBS and the resultant 4ml was layered on top of the Percoll gradient in the tube as described by Toth *et al* (1992). The tubes were then spun at 1200rpm for 20 minutes then 1500 rpm for 15 minutes.

Opaque bands containing cells were seen at the plasma/1.080 interface and just above the red cell layer. These were aspirated using a Pasteur pipette and the cells washed in an equal volume of PBS. These suspensions were spun at 1500rpm for 5 minutes, the supernatant discarded and the cell pellet redissolved in 1.5ml of red cell lysis buffer (NH_4Cl 8g; KH_2PO_4 0.1g; EDTA-Na_3 1g in 1 litre). These were allowed to incubate for 4 minutes at 37°C before spinning at 1000rpm for 5 minutes. The supernatant was discarded and the cell pellet redissolved in 1.5ml of PBS.

2.12.3. CONFIRMATION

Aliquots of the cell suspensions were counted in the ABX Minos Vet (Roche Products, Diagnostic Division, Welwyn Garden City, UK) and then spun in the cytospin (Shandon Cytospin 3, Life Sciences International (U.K. Ltd), Basingstoke, UK) for 5 minutes at 1500rpm onto glass slides. The cells were then stained using May-Grunwald-Giemsa and the cell types identified under the microscope at 10-40 x magnification (Leitz Laborlux 12, Wetzlar, Germany).

CHAPTER 3

DATABASE ANALYSIS OF CAT HAEMATOLOGICAL PARAMETERS

3.1. INTRODUCTION

So-called "normal" haematological parameters in cats have been calculated by a number of authors and presented in a variety of papers (Jennings 1947, Johnson & Perman 1968, Penny *et al* 1970, Anderson *et al* 1971, Osbaldiston 1978, Meyers-Wallen *et al* 1984, Earle *et al* 1990). Previous studies have used small numbers of animals from distinct populations. Some were experimental animals kept in closed colonies (Anderson *et al* 1971, Osbaldiston 1978, Meyers-Wallen *et al* 1984, Earle *et al* 1990); whilst others were clinical cases submitted to veterinary clinics (Penny *et al* 1970); and others were derived from subpopulations of field cases (Johnson & Perman 1968). When considering the interpretation of haematological values from a clinical case, the best comparison is that from a large number of animals similar to the one in question. However, a large number of cats of mixed origin and age from the general population has not been studied.

The data obtained in previous studies had all been analysed statistically. This was done by calculating a mean value for each parameter and two standard deviations from that mean on either side to encompass a so-called "normal" range (Tvedten 1981). This type of analysis assumes a normal distribution of values around the mean and includes 95% of the values for that particular parameter. Unfortunately, in the case of some parameters, there is not a normal distribution of data, that is, it is skewed to one side or another. For

example, eosinophil values may tend toward zero and result in a skew to the left. This means that the range calculated on this assumption is inaccurate. This method of analysis also leads to 5% of results from normal animals falling outwith the calculated normal range.

Blood cell numbers can be affected by a number of factors. The age, sex, diet, breed and clinical health of the animal, as well as treatment given, are all significant. As well as this, the cat is particularly susceptible to the stresses of blood sampling. The physiological response to the stress of restraint and sampling results in artificial elevations in neutrophil and lymphocyte numbers (Schalm 1975). This is probably influenced by the large marginal pool of leukocytes in the cat.

Venipuncture in the cat is more difficult than in larger species, and slow sampling times often lead to clumping of platelets. This results in artificially low platelet counts and, in automatic cell counters these clumps may be counted as erythrocytes, leading to artificially high red cell numbers.

Cell numbers and quality are also affected by the quantity and type of anticoagulant used to collect the blood in. Collection into EDTA results in clumping of platelets, particularly in cats (Schalm 1975). It is not known if this is as a result of the EDTA or a characteristic of cat platelets. Penny *et al* (1970) suggested that excessive concentrations of EDTA resulted in lowering of the PCV of the cat, probably by reducing the MCV. Prolonged delay before analysis of samples, leading to excessive exposure to EDTA is implicated in increasing the MCV, and hence the PCV (Hinton & Jones 1978). EDTA, however, gives the best morphological representation of the

white cells for differential counts and is the anticoagulant of choice for haematological samples.

Finally, errors may also arise in the laboratory, by use of inappropriate automatic counters, or inaccurate setting of the machines.

Recently, database analysis has been used to attempt to relate the clinical status of an animal and its biochemical profile (Little *et al* 1994). Suitable databases hold information from large numbers of animals, of assorted age, sex, background and clinical status. Databases have sufficient sample numbers to allow the accurate sorting of results into percentiles. This type of analysis is more accurate for data which does not have a normal distribution, as is common for a number of haematological parameters. Individual results can then be compared with a large population of others, and an indication of the degree of "normality" can be given. The database may then provide information as to the type of disease pattern which is most commonly implicated in any abnormality encountered.

From the basis of the numbers and range of the animals involved in the haematology database at Glasgow University Veterinary School, and the greater accuracy of the database statistical interpretation, it was decided to re-evaluate feline haematological parameters.

3.2. AIM

The objective of this part of the thesis was to re-evaluate feline haematological parameters using "expert" database analysis.

3.3. MATERIALS AND METHODS

The Glasgow Veterinary School Haematology database (Dataflex, Specialist Services Ltd., Glasgow, UK) was initiated in February 1992. Haematology results from all blood samples taken in the veterinary hospital, from research animals and from samples sent from external veterinary practices are stored in the database. The database includes information on signalment; history; source of sample; a clinical summary and comments on the blood smear, as well as the values for the haematological parameters. A representation of the database fields is shown in figure 3.1.

Counts from the samples included in the database were obtained automatically using the ABX Minos Vet (Roche Products Ltd., Diagnostic Division, Welwyn Garden City, UK). Differential white cell counts (200 cells) were then performed manually by experienced technicians using May-Grunwald-Giemsa (BDH Laboratories Ltd., Poole, UK) stained smears.

For the purposes of analysing the haematology data from feline samples, the query facility on the database was used to extract the results and relevant data on age, sex and source of all cat bloods. The samples thus encompassed a range of animals in a variety of states of health, age, sex and backgrounds. Only one result, the first sample recorded, from each animal was included in the analysis to avoid bias from repeated samples. These results were then sorted into two groups of animals by transferring the data into a Microsoft Excel 4 (Microsoft Corporation, Redmond, WA 98052-6399, USA) spreadsheet and using the sort facility to create a separate group of research cat data only. Those research cats which were control or naive animals were manually picked out and used to create a

HAEMATOLOGY DEPARTMENT - SAMPLES

ref <_____> (blank if new)
lab ref <_____ ____>
vet samp <_____>

status _
location _ (RHCEDZ)
charge £_____

date in _____
date out _____

case <_____>
desc <_____>
vet case <_____>

species _____

sex __
dob _____
age ____ _

clinician
<_____> <_____>

source
<_____> <_____>

Sample _ _____
date coll _____ samp _____
tests _____

Clinical diagnosis

Lab ref 0 HAEMATOLOGY DEPARTMENT - SAMPLES - page 2

	result		X
RBC	_____	10E12/l	_
Hb	____	g/dl	_
HT	____	%	_
MCV	_____	fl	_
MCH	____	pg	_
MCHC	____	g/dl	_
PLT	____	10E9/l	_
MPV	____	fl	_
PCT	_____	%	_
PDW	____		_
RETICS	_____%	____	_

	%	absolute	X
WBC (10E9/l)		_____	_
Band neutrophils	_____	_____	_
Neutrophils	_____	_____	_
Lymphocytes	_____	_____	_
Monocytes	_____	_____	_
Eosinophils	_____	_____	_
Basophils	_____	_____	_
_____	_____	_____	
_____	_____	_____	
_____	_____	_____	
_____	_____	_____	
_____	_____	_____	

Film Report _____ Norms _

Figure 3.1. Empty data field from the "Dataflex" programme, showing page for sample and patient details and page for results and comments.

research cat subgroup. The 40 animals in this group were analysed separately from the main "other" group consisting of all cats in the database bar those from the research facility in the Veterinary School.

Platelet counts were excluded from the study due to the unreliability of the counts obtained from EDTA samples. Twelve individual haematological parameters red cell numbers; haemoglobin; haematocrit; mean cell volume; mean cell haemoglobin; mean cell haemoglobin concentration; white cell count; neutrophil count; lymphocyte count; monocyte count; eosinophil count and basophil count were summarised using the 1st, 5th, 10th, 25th, 50th, 75th, 90th, 95th and 99th percentiles. The 50th percentile is more commonly referred to as the median, and is obtained by arranging the data in order and estimating the value of the analyte that 50% of the data are less than. The 1st, 5th, 10th and 25th percentiles can be used to characterise the distribution of low values of the analyte, and the 75th, 90th, 95th and 99th percentiles the high values. The percentile analysis was done using the statistical facility on the Microsoft Excel 5 (Microsoft Corporation, Redmond, WA 98052-6399, USA) software. This software was also used to calculate a mean and standard deviation for each parameter.

The percentile analysis was performed for the research and "other" groups of cats separately. In order to determine whether any significant differences were apparent between the groups, a cumulative frequency distribution curve was created for each parameter for research and "other" cats. Intervals were determined according to the percentile analysis. The intervals were the same for both groups. The Kolmogorov-Smirnov 2-sample 2-tailed test was

then used to compare the frequency distributions (Siegel 1956). The proportion of samples recorded falling into each interval of the cumulative frequency distribution was compared using the equation:

$$D = \text{maximum } |K_{res}(x) - K_{oth}(x)|$$

where K = proportion of samples equal to or less than X

The maximum difference between proportions at each interval (D_{max}) was then tested for significance at the 0.05 level using the equation:

$$1.36 * \sqrt{[(n_{res} + n_{oth})/(n_{res} * n_{oth})]}$$

where n_{res} = number of values from research cats and n_{oth} = number of values from "other" cats

Using this formula, the significant D-value for red cell parameters and the white cell count (n=40 and 992 for research and "other" cats respectively) was **0.219**; and differential white cell counts (n=39 and 962 for research and "other" cats respectively) was **0.222.**

Any value of D_{max} which exceeded the calculated figure from the above equation was deemed significant.

The distribution of the percentile results for research and "other" animals was illustrated using the boxplot facility of the Minitab (Minitab for Windows, Minitab Inc., State College, PA, 16001-3008, USA) statistical software programme.

3.4. RESULTS

There were 40 cats in the research group, consisting mainly of SPF cats which had not been used in any experimental procedures at the time of sampling. These animals were kept under controlled conditions and formed a distinct subpopulation. The "other" category comprised 992 cats from the veterinary hospital or referred

cases from practices, providing a large range of animals in varying states of health, background, age etc.

Not all samples recorded in the database had differential counts recorded, as some samples were for red cell parameters only or had poor cell morphology. These samples were put through the automatic counter and hence had red cell values and a total white cell count but no manual white cell differential count. Thus the total numbers of values in the differential white cell counts was 962 and 39 for the "other" and research groups respectively.

The results of the percentile analysis for the research and "other" groups of cats are shown in tables 3.1(a) to 3.12(a). The calculated mean and standard deviation for each parameter is also shown. These values are most useful for parameters with a normal frequency distribution. Examples of frequency distribution curves for the white cell counts (n=992) and eosinophil counts (n=962) are shown in figures 3.2(a) & (b). The distribution of these counts is not normal, but skewed to the left. Mean and standard deviation interpretations are therefore inaccurate for this type of data.

The results of the Kolmogorov-Smirnov test comparison of the research and "other" groups are shown in tables 3.1(b) to 3.12 (b). The D_{max} value for each parameter is highlighted.

The differences in distribution of percentile results between the two groups is also illustrated by boxplots in figures 3.3 to 3.14. In the boxplots the bottom of the box is drawn at the first quartile value (Q_1) and the top of the box is third quartile value (Q_3).The median is represented by the line drawn across the box. The "whiskers" are the lines extending from the bottom and top of the box to the values determined as follows:

Figure 3.2.(a) Frequency distribution curve for white cell count of "other" cats (n=992) showing skew to left

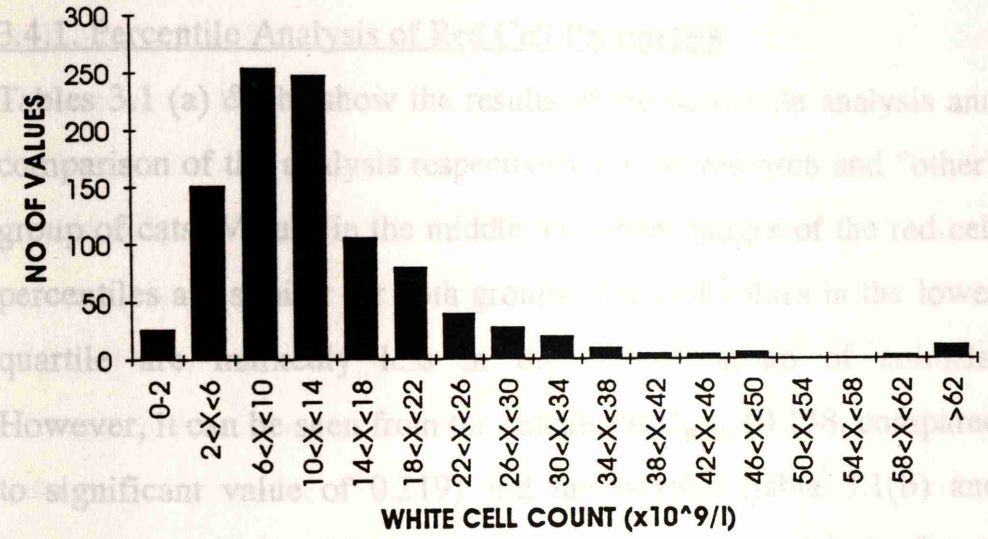
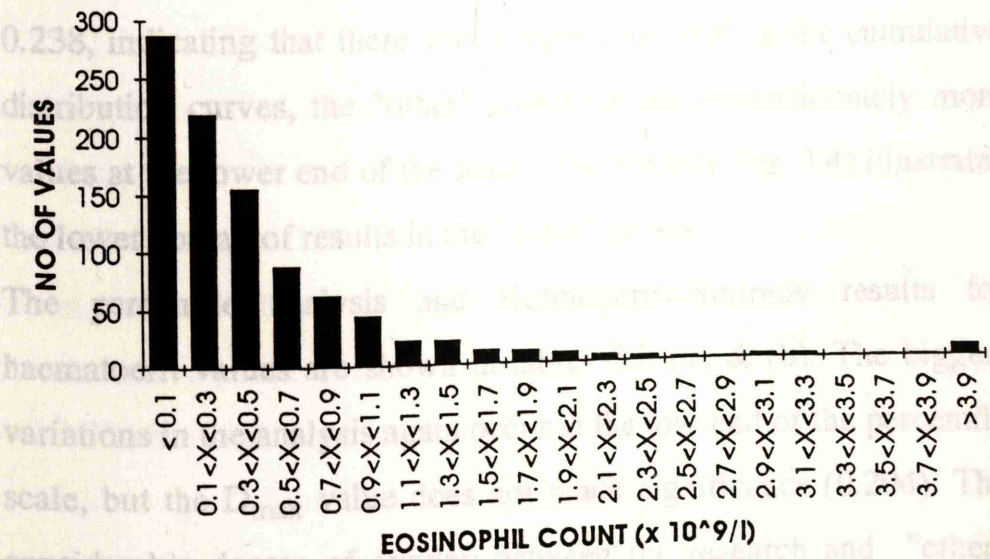


Figure 3.2.(b) Frequency distribution curve for eosinophil counts of "other" cats (n=162) showing skew to left



Lower limit = $Q_1 - 1.5(Q_3 - Q_1)$

Upper limit = $Q_1 + 1.5(Q_3 - Q_1)$

Outliers are values outwith these limits and are shown as asterisks.

3.4.1. Percentile Analysis of Red Cell Parameters

Tables 3.1 (a) & (b) show the results of the percentile analysis and comparison of the analysis respectively for the research and "other" group of cats. Values in the middle and upper ranges of the red cell percentiles are similar for both groups. Red cell values in the lower quartile are markedly less in the "other" group of animals. However, it can be seen from the calculated D_{max} (0.158, compared to significant value of 0.219) and the boxplot (table 3.1(b) and figure 3.3) that the differences between research and "other" cats were not significant.

Tables 3.2 (a) & (b) show the results for the haemoglobin values. The values were again similar in the middle and upper ranges, but differed in the lower quartile. In this case, the calculated D_{max} was 0.238, indicating that there was a significant shift in the cumulative distribution curves, the "other" group having proportionately more values at the lower end of the scale. The boxplot (fig. 3.4) illustrates the lower spread of results in the "other" group.

The percentile analysis and Kolmogorov-Smirnov results for haematocrit values are shown in tables 3.3 (a) & (b). The biggest variations in the analysis again occur at the low end of the percentile scale, but the D_{max} value does not reach significance (0.204). The considerable degree of overlap between the research and "other" groups is illustrated in figure 3.5.

Mean cell volume results are shown in tables 3.4 (a) & (b). The values for both research and "other" groups are largely comparable, with notable differences only taking place at the extreme ends of the scale, at the upper end being more pronounced. The number of outlier values at the upper end are illustrated in figure 3.6. However, the Kolmogorov-Smirnov test results (table 3.4 (b)) indicate that the differences are not significant, $D_{max} = 0.184$.

The percentile analysis of mean cell haemoglobin results are shown in table 3.5(a). There do not appear to be any noticeable differences between the research and "other" animals. Figure 3.7 and table 3.5(b) illustrate the comparison between the two. As for mean cell volume, there are a number of outliers at the upper end of the "other" group scale. However, the Kolmogorov-Smirnov results demonstrate a significant difference ($D_{max} = 0.220$), indicating that there are proportionately more low MCH values in the "other" group of cats.

The results of the percentile analysis and Kolmogorov-Smirnov tests for mean cell haemoglobin concentration are shown in tables 3.6(a) & (b). For MCHC, the percentile values for the "other" group of cats are consistently lower than those of research animals at all but the extreme values at the high end of the scale. The shift is illustrated in figure 3.8, with large numbers of outliers being present at the low end of the scale. The significant shift in the frequency distribution is confirmed by the Kolmogorov-Smirnov results ($D_{max} = 0.394$) shown in table 3.6(b).

3.4.2. Percentile Analysis of White Cell Parameters

The results of the statistical analyses for total white cell counts are shown in tables 3.7(a) & (b), and in figure 3.9. The percentile analysis demonstrates that research cat white cell counts are higher than those of "other" cats at all but the high extreme end of the scale. The difference is confirmed by the Kolmogorov-Smirnov test, with a calculated value of D_{max} = 0.385 being well above the level of significance. The boxplot (figure 3.9) illustrates the poor overlap between the groups for the middle quartiles, research cat values being notably higher.

Values for percentile analysis and D_{max} for neutrophil counts from research and "other" cats are shown in tables 3.8(a) & (b). Neutrophil numbers in research cats are higher than their comparative percentile in the "other" group at all but the high extreme end of the scale, as for total white cell counts. As the numbers of values for differentiated white cell analyses are 39 and 962 for research and "other" cats respectively, the significant value of D_{max} becomes 0.222. For neutrophils (see table 3.8(b)) this value is 0.230, thus confirming a significant shift in the frequency distributions. The boxplot (figure 3.10) shows some degree of overlap between the middle quartiles, and a high number of outliers in the upper ranges of the "other" cat population.

Tables 3.9(a) & (b) contain the results of the lymphocyte statistical analyses. Numbers are consistently higher in the research group of cats. Figure 3.11 demonstrates very little overlap between the percentiles for the two groups. The significant difference is confirmed by the high D_{max} value of 0.592.

Results of analysis of monocyte numbers in the research and "other" groups of cats is shown in tables 3.10(a) & (b). As for other white

cell counts, numbers of monocytes in research cats are higher than in the "other" group. The degree of overlap illustrated by the boxplot in figure 3.12 is greater than that of other white cell types. However, the difference between the groups is still significant, the D_{max} value being 0.247.

Eosinophil count analyses are represented in tables 3.11(a) & (b). Circulating eosinophil numbers in research cats are also elevated in comparison to those of the "other" group. The difference is again significant at the 0.05 level, with $D_{max} = 0.456$. A large numbers of outliers at the high end of the "other" cat scale and a poor degree of overlap of the middle quartiles are obvious from the boxplot (figure 3.13).

In common with the rest of the white cells, basophil counts are shown to be higher in research cats (see tables 3.12(a) & (b)). Although the difference looks dramatic on the boxplot illustrated in figure 3.14, the Kolmogorov-Smirnov test results in a D_{max} of 0.222, right on the borderline of significance.

3.4.3. Mean Values and Standard Deviation

The mean and standard deviations for each parameter are shown in table 3.13, along with sets of accepted traditional "normal" values (Penny *et al* 1970, Anderson *et al* 1971, Schalm 1975, Earle *et al* 1990).

The mean values for red cell parameters obtained for both research and "other" groups of cats are comparable with those published from previous studies (see table 3.13). Only the haematocrit values in the present study are markedly different, in both groups of cats being lower than those published previously. The mean values for

white cell counts for the large group of non-research cats are similar to those published previously. However, the mean total white cell counts, mature neutrophil, lymphocyte and eosinophil counts of the research cats are notably higher than in most other studies.

These results are in keeping with those discussed above in relation to the percentile analysis, although they provide less detailed information.

3.5. DISCUSSION

The figures obtained in this study illustrate the percentile method of analysis of haematology data. Many of the parameters examined do not have a normal distribution, for example white cell count and eosinophil count (see figures 3.2(a) & (b)), so formulating "normal" ranges from mean values and standard deviations is inaccurate.

Percentile analysis gives a full and accurate description of the data provided. The 25th, 50th (median) and 75th percentiles are reliable estimates which are unlikely to be influenced by "outliers", providing enough samples have been used in the analysis. The 1st, 5th, 95th and 99th percentiles are less reliable and depend on the size and status of the population being studied. For example, if a number of animals suffering from lymphoid leukaemia were included in a population then the values of the 90th, 95th and 99th lymphocyte number percentiles would be expected to increase.

The percentile analysis provides a complete breakdown of data from healthy and sick animals. When a single result is compared to the scale, an estimation of the degree of abnormality of the result can be given in relation to a large diverse population. That is, it is not

classified as simply abnormal or normal according to whether it lies within a specified range designated by the calculated mean and standard deviation of a small number of apparently healthy animals. Using the percentile scale, a result may be designated, for example, to be greater than that seen in more than 95% of both sick and healthy cats. This information indicates that there is a serious abnormality which needs urgent attention.

The advantages of the percentile method over the traditional mean and standard deviation method of statistical analysis were demonstrated. Two populations of cats were separately analysed for comparison. The first population consisted of a large number of animals from a variety of backgrounds and in varying states of health. This population consisted of 992 animals and was known as the "other" group. The second population was a much smaller group of animals (n = 40). This consisted of SPF cats which had never been exposed to other animals, were kept in a controlled environment and had not been subjected to any experimental procedures.

Red cell numbers in the research and "other" group of cats were not vastly different by percentile analysis (tables 3.1(a) & (b) and figure 3.3). The main divergence was seen in the first quartile, where the larger, more diverse population of animals had lower values. In disorders involving red cell numbers, deficiencies are far more common than excess numbers. In a population of animals which includes a proportion of unhealthy animals, therefore, it would be expected to find a larger number of cats with low red cell numbers than high. Thus, it is not surprising to find in this case that values in

the lower quartile of the population including sick cats should be lower than those of the small population of healthy animals. However, the accuracy of the percentile system is illustrated in that the 25th, 50th and 75th percentiles are similar, even though there are sick cats in the "other" population. These statistics are not affected by outliers in a population of sufficient size and diversity. The cumulative frequency distribution curves of the two sets of cats were not significantly different.

A similar pattern was noted in the haemoglobin analysis, with values in the first quartile being lower in the "other" group of cats (tables 3.2(a) & (b) and figure 3.4). The reasons for this were as for the red cell counts. In the case of haemoglobin however, there was a significant difference in the cumulative frequency distribution, indicating that the difference between the populations was greater than for red cell numbers.

Haematocrit analysis followed a similar pattern to red cell counts and haemoglobin values, and for the same reasons (tables 3.3(a) & (b) and figure 3.5). The difference in frequency distribution was not significant.

The calculated values for the haematocrit of cats studied in this survey are notably lower than that published by previous authors, approximately 30L/L as apposed to 36-37L/L (see table 3.13). The haematocrits in this survey were calculated values obtained by multiplying the red cell number by the mean cell volume as measured in the automatic cell counter. Most previous surveys have relied on spun haematocrits, or packed cell volumes (PCV). This is a direct measurement of spun blood but invariably includes trapped plasma in the packed red cells. This results in a higher value than

that obtained by the calculation outlined above. The haematocrits in this study are thus lower as a result of a variation in method rather than real differences in value.

The pattern observed in the mean cell volume percentile analysis was different, in that greater divergence between research and "other" cats was seen at the upper end of the scale (tables 3.4(a) & (b) and figure 3.6). Although in sick cats, mean cell volume may be increased or decreased, in the "other" population it is more commonly elevated, the large numbers of outliers in the high extreme values being clearly seen in figure 3.6. This accounted for the differences between the groups, although the Kolmogorov-Smirnov test result indicated the difference did not reach statistical significance.

Mean cell haemoglobin and mean cell haemoglobin concentration are values with upper limits largely determined by red cell volume. For these parameters, values are more widely distributed at the low end of the scale. This is illustrated in tables 3.5(a) and 3.6(a) for MCH and MCHC respectively. Once again in the "other" population of cats these values are lower in the first quartile due to a proportion of ill cats being included in this group. The reliability of the 25th, 50th and 75th percentiles are illustrated as before, with little difference between the groups. Kolmogorov-Smirnov results (tables 3.5(b) and 3.6(b)) indicate that the frequency distribution differences between the groups are statistically significant.

The results of the percentile analysis of total white cell counts and differential white cell counts are interesting. There are significantly

more nucleated blood cells in the research cat group than the "other" group. These differences are not only seen in the first and fourth quartiles, as for red cell parameters, but are apparent at the 25th, 50th and 75th percentiles also (see tables 3.7(a) to 3.12(a)). This indicates that there are fundamental differences between the two groups of animals. The research cat population consisted of a healthy group of SPF cats in a controlled environment, yet their white cell numbers are higher than would be predicted using both percentile analysis and comparing to the "other" group in the study, and also by comparison to previous "normal" ranges (see table 3.13) collected by various authors (Penny *et al* 1970, Anderson *et al* 1971, Schalm 1975, Earle *et al* 1990).

The variations in the respective cumulative frequency distributions for all white cell types (see tables 3.7(b) to 3.12(b)) are statistically significant. The most pronounced differences are seen in lymphocyte and eosinophil counts. Since the cats in the research cat group were clinically normal and were not exposed to any known pathogens, it must be assumed that these are normal cell counts for this group of animals. Most of these cats were young animals, so the influence of age must be considered, although most were over 6 months at the time of sampling.

The danger in using small populations of cats from closed environments for the calculation of haematological "normal" ranges is therefore illustrated by the research cat results shown above. The only published "normal " range for cats with white cell counts similar to those of the research group in this study is one published by Anderson *et al* (1971). Their results were also obtained from a group of young cats in a closed colony.

Both the percentile analysis and the mean data shown illustrate that the red cell parameters are much less variable than those of the leucocytes, the range being narrower. The physical and physiological limits for these parameters are far more rigid. White cell numbers are subject to extreme variation, depending on disease status, but also vary more widely in healthy animals. This is apparent from both percentile analysis and from traditional mean and standard deviation values.

It has been noted by previous authors (Schalm 1975, Osbaldiston 1978) that there are wide variations in leucocyte numbers in healthy cats. This is illustrated by the percentile analysis which demonstrates extensive ranges within the 10-90% limits. The advantage of the percentile system in this instance is that the wide range can be more readily recognised than with the traditional system so the animals can be categorised more accurately and false interpretation is less likely.

The haematology database used in the compilation of data for this study has only been in use for a three year period, but is continuously growing. The analysis of results in the database can therefore be regularly updated with increasing populations of animals. This is true not only of feline haematology but also that of other species. The database also allows for the separation of species populations into subgroups of age or sex for example. Eventually percentile analysis of, for example, castrated male cats over 12 years old will be available. This will enable samples taken from animals of this description to be compared to a large number of similar animals of varying states of health in order to assess its haematological status more accurately.

Further work with the statistical analysis of database-stored haematology could reveal if particular conditions are associated with extreme results. This would be a useful diagnostic aid and prognostic indicator, used in conjunction with clinical information and blood smear analysis.

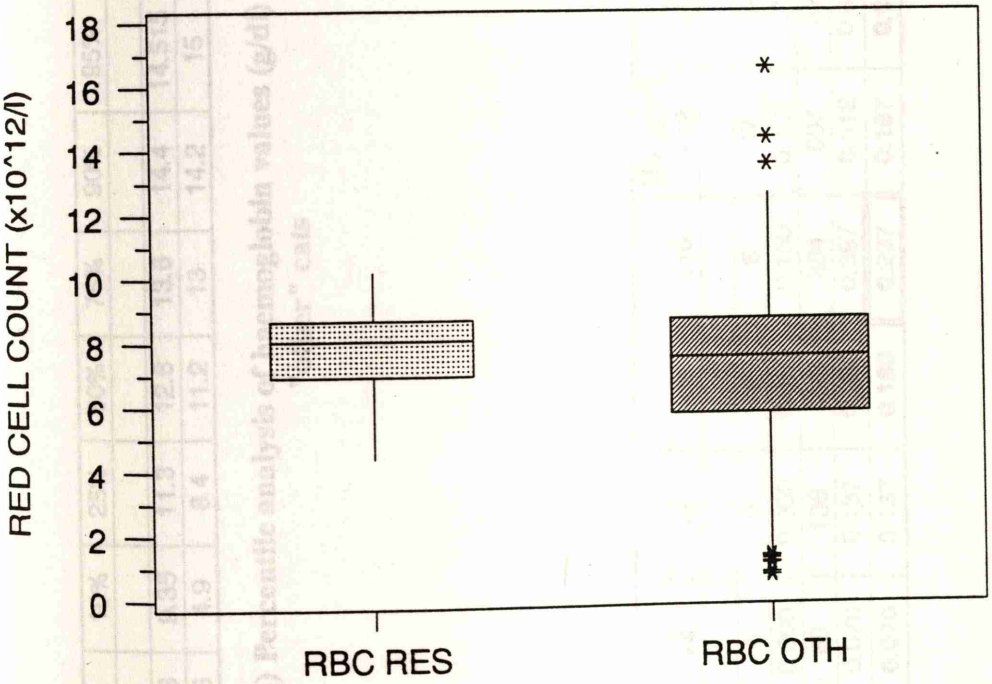
RBC	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	4.35	4.74	5.474	5.786	6.98	8.025	8.53	9.278	9.639	10.03	10.17		7.774	1.315
OTHER	0.58	1.188	2.031	3.168	5.68	7.45	8.65	9.529	10.058	11.223	16.54		6.978	2.404

Table 3.1.(a) Percentile analysis of red cell numbers ($x10^{12}/l$) for research and "other" cats

				RBC (x10^12/l)								
INTERVAL		< 1.5	<3.5	<5.5	<7.5	<9.5	<11.5	<13.5	> 13.5			
NO. VALUES	RES	0	0	3	15	36	40	40	40			
	%	0.000	0.000	0.075	0.375	0.900	1.000	1.000	1.000			
NO. VALUES	OTH	24	112	230	505	890	984	989	992			
	%	0.024	0.113	0.232	0.509	0.897	0.992	0.997	1.000			
	D	0.024	0.113	0.157	0.134	0.003	0.008	0.003	0.000			

Table 3.1.(b) Cumulative frequency distribution of red cell values ($x10^{12}/l$) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.3 BOXPLOT OF RED CELL COUNTS FOR RESEARCH AND OTHER CATS



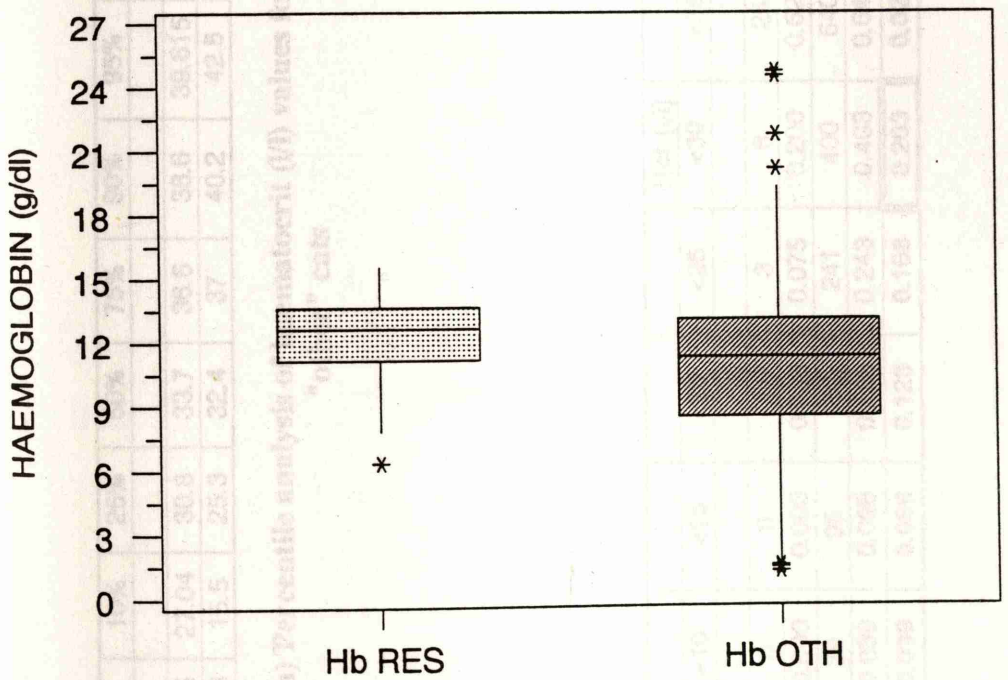
Hb	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	6.3	6.885	8.56	9.35	11.3	12.6	13.6	14.4	14.515	15.227	15.5		12.163	2.013
OTHER	1.1	1.8	3.56	4.9	8.4	11.2	13	14.2	15	17.3	24.7		10.49	3.58

Table 3.2.(a) Percentile analysis of haemoglobin values (g/dl) for research and "other" cats

							Hb (g/dl)					
INTERVAL		< 2	<4	<6	<8	<10	<12	<14	<16	<18	<20	> 20
NO. VALUES	RES	0	0	0	2	6	17	34	40	40	40	40
	%	0.000	0.000	0.000	0.050	0.150	0.425	0.850	1.000	1.000	1.000	1.000
NO. VALUES	OTH	18	69	136	228	384	607	877	964	985	988	992
	%	0.018	0.070	0.137	0.230	0.387	0.612	0.884	0.972	0.993	0.996	1.000
	D	0.018	0.070	0.137	0.180	0.237	0.187	0.034	0.028	0.007	0.004	0.000

Table 3.2.(b) Cumulative frequency distribution of haemoglobin values (g/dl) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.4 BOXPLOT OF HAEMOGLOBIN VALUES FOR RESEARCH AND OTHER CATS



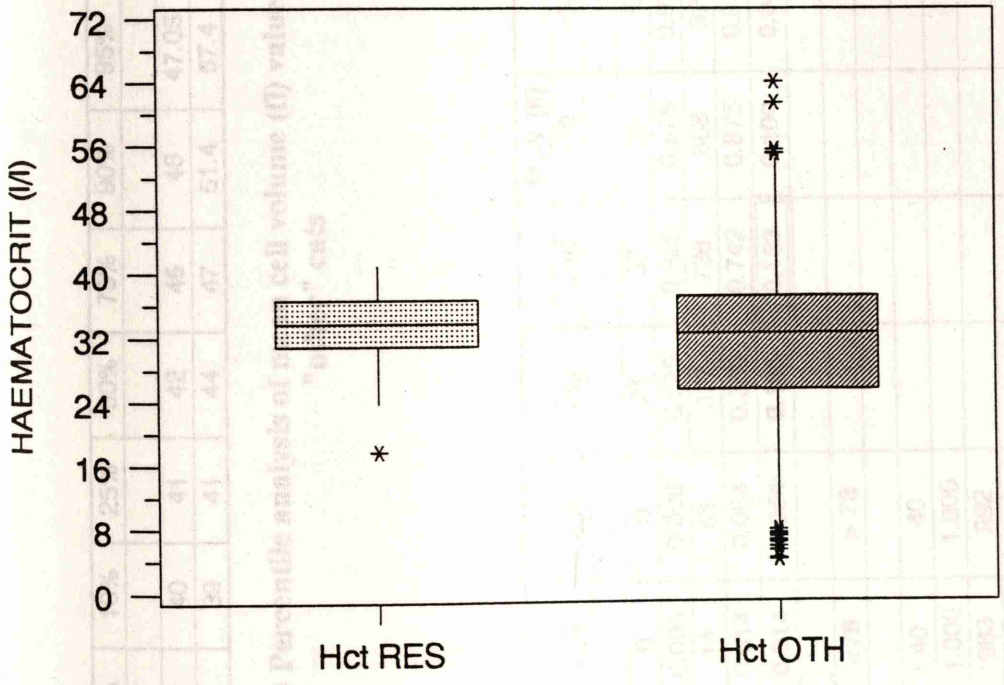
Hct	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	17.6	19.979	24.65	27.04	30.8	33.7	36.6	38.6	39.615	40.449	40.8		33.268	4.899
OTHER	3.9	6.1	11.46	15.5	25.3	32.4	37	40.2	42.5	47.82	64		30.44	9.5

Table 3.3.(a) Percentile analysis of haematocrit (l/l) values for research and "other" cats

							Hct (l/l)					
INTERVAL		< 5	<10	<15	<20	<25	<30	<35	<40	<45	<50	> 50
NO. VALUES	RES	0	0	0	1	3	8	25	39	40	40	40
	%	0.000	0.000	0.000	0.025	0.075	0.200	0.625	0.975	1.000	1.000	1.000
NO. VALUES	OTH	6	39	95	152	241	400	640	883	967	984	992
	%	0.006	0.039	0.096	0.153	0.243	0.403	0.645	0.890	0.975	0.992	1.000
	D	0.006	0.039	0.096	0.128	0.168	0.203	0.020	0.085	0.025	0.008	0.000

Table 3.3.(b) Cumulative frequency distribution of haematocrit (l/l) values for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.5 BOXPLOT OF HAEMATOCRIT VALUES FOR RESEARCH AND OTHER CATS



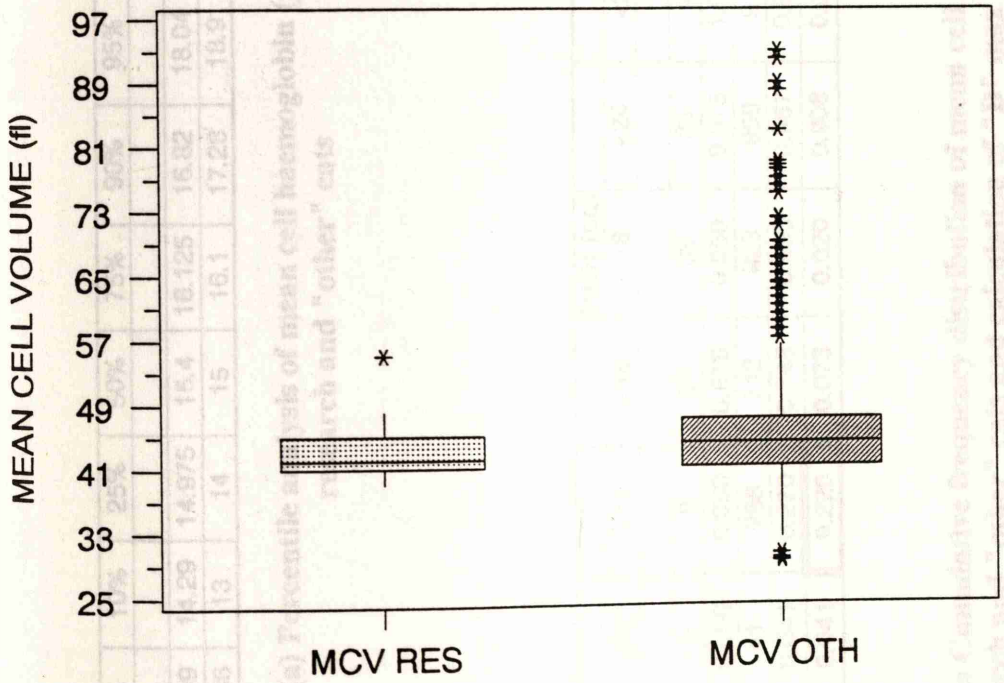
MCV	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	39	39	39	40	41	42	45	46	47.05	52.27	55		43.075	2.999
OTHER	29	34	37	39	41	44	47	51.4	57.4	78	93		45.03	7.36

Table 3.4.(a) Percentile analysis of mean cell volume (fl) values for research and "other" cats

							MCV (fl)					
INTERVAL		< 30	<34	<38	<42	<46	<50	<54	<58	<62	<66	<70
NO. VALUES	RES	0	0	0	21	37	39	39	40	40	40	40
	%	0.000	0.000	0.000	0.525	0.925	0.975	0.975	1.000	1.000	1.000	1.000
NO. VALUES	OTH	4	14	83	377	736	868	928	945	957	966	971
	%	0.004	0.014	0.084	0.380	0.742	0.875	0.935	0.953	0.965	0.974	0.979
	D	0.004	0.014	0.084	0.145	0.183	0.100	0.040	0.047	0.035	0.026	0.021
INTERVAL		<74	<78	> 78								
NO. VALUES	RES	40	40	40								
	%	1.000	1.000	1.000								
NO. VALUES	OTH	977	983	992								
	%	0.985	0.991	1.000								
	D	0.015	0.009	0.000								

Table 3.4.(b) Cumulative frequency distribution of mean cell volume (fl) values for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.6 BOXPLOT OF MEAN CELL VOLUME FOR RESEARCH AND OTHER CATS



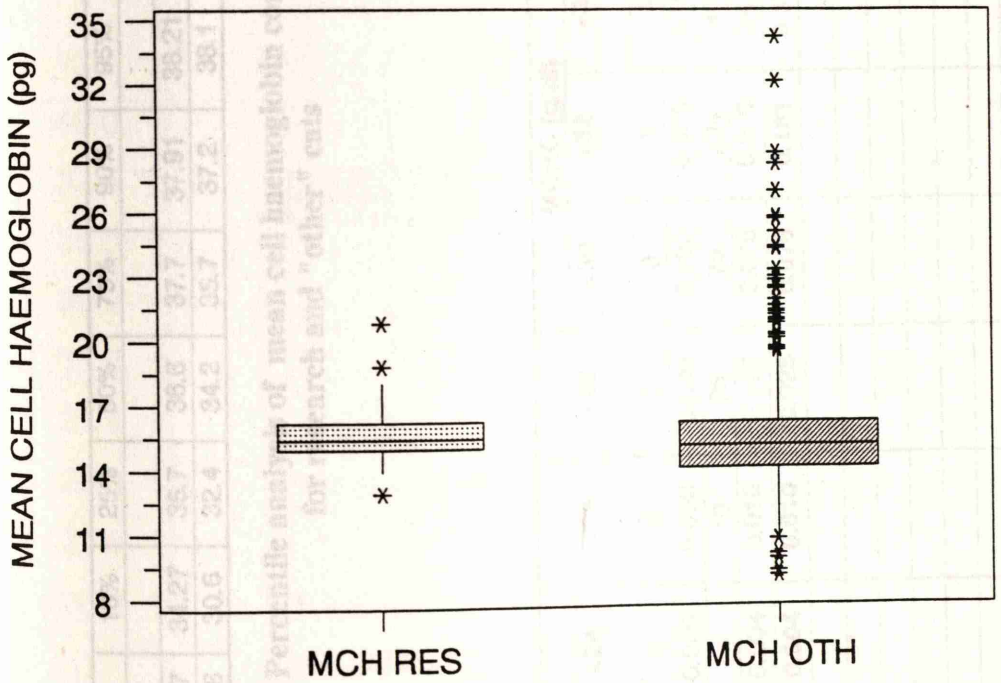
MCH	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	12.9	13.29	14.09	14.29	14.975	15.4	16.125	16.82	18.04	20.02	20.8		15.653	1.378
OTHER	8.9	10.99	12.36	13	14	15	16.1	17.28	18.9	24.3	32.1		15.22	2.23

Table 3.5.(a) Percentile analysis of mean cell haemoglobin (pg) values for research and "other" cats

						MCH (pg)						
INTERVAL		< 10	<12	<14	<16	<18	<20	<22	<24	> 24		
NO. VALUES	RES	0	0	2	27	38	39	40	40	40		
	%	0.000	0.000	0.050	0.675	0.950	0.975	1.000	1.000	1.000		
NO. VALUES	OTH	4	41	268	742	923	959	975	980	992		
	%	0.004	0.041	0.270	0.748	0.930	0.967	0.983	0.988	1.000		
	D	0.004	0.041	0.220	0.073	0.020	0.008	0.017	0.012	0.000		

Table 3.5.(b) Cumulative frequency distribution of mean cell haemoglobin (pg) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.7 BOXPLOT OF MEAN CELL HAEMOGLOBIN FOR RESEARCH AND OTHER CATS



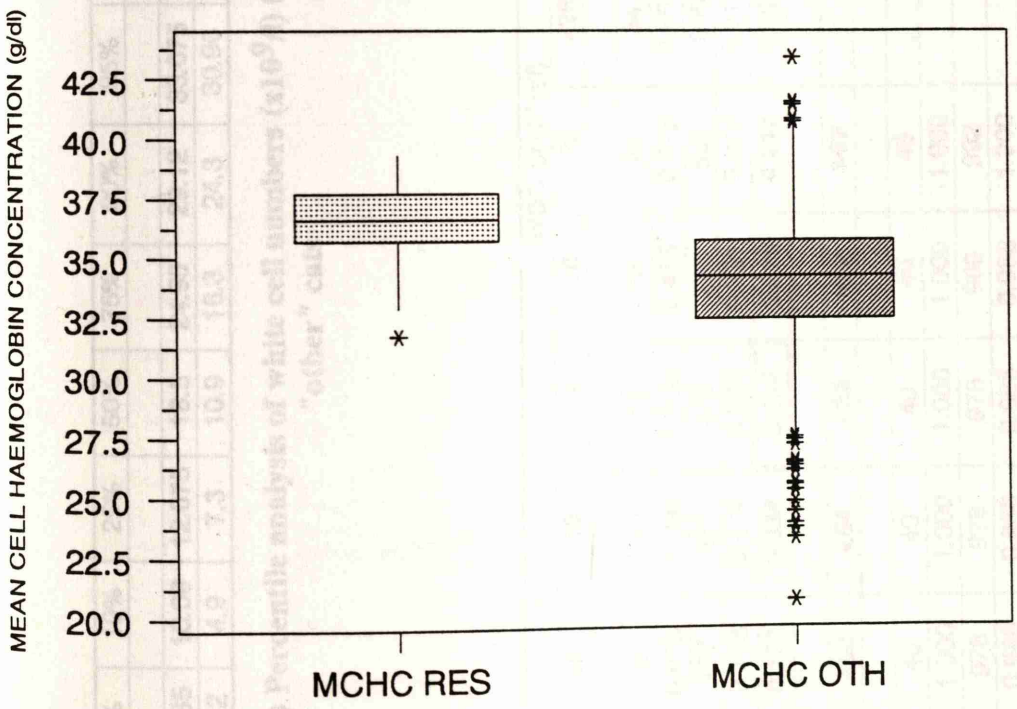
MCHC	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	31.7	32.168	33.47	34.27	35.7	36.6	37.7	37.91	38.21	38.949	39.3		36.423	1.616
OTHER	20.6	26.18	29.16	30.6	32.4	34.2	35.7	37.2	38.1	40.227	43.4		34	2.745

Table 3.6.(a) Percentile analysis of mean cell haemoglobin concentration (g/dl) for research and "other" cats

						MCHC (g/dl)						
INTERVAL		< 22	<24	<26	<28	<30	<32	<34	<36	<38	<40	<42
NO. VALUES	RES	0	0	0	0	0	1	4	16	37	40	40
	%	0.000	0.000	0.000	0.000	0.000	0.025	0.100	0.400	0.925	1.000	1.000
NO. VALUES	OTH	1	4	10	25	75	204	466	788	941	980	991
	%	0.001	0.004	0.010	0.025	0.076	0.206	0.470	0.794	0.949	0.988	0.999
	D	**0.001**	**0.004**	**0.010**	**0.025**	**0.076**	**0.181**	**0.370**	**0.394**	**0.024**	**0.012**	**0.001**
INTERVAL		> 42										
NO. VALUES	RES	40										
	%	1.000										
NO. VALUES	OTH	992										
	%	1.000										
	D	0.000										

Table 3.6.(b) Cumulative frequency distribution of mean cell haemoglobin concentration (g/dl) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.8 BOXPLOT OF MEAN CELL HAEMOGLOBIN CONCENTRATION FOR RESEARCH & OTHER CATS



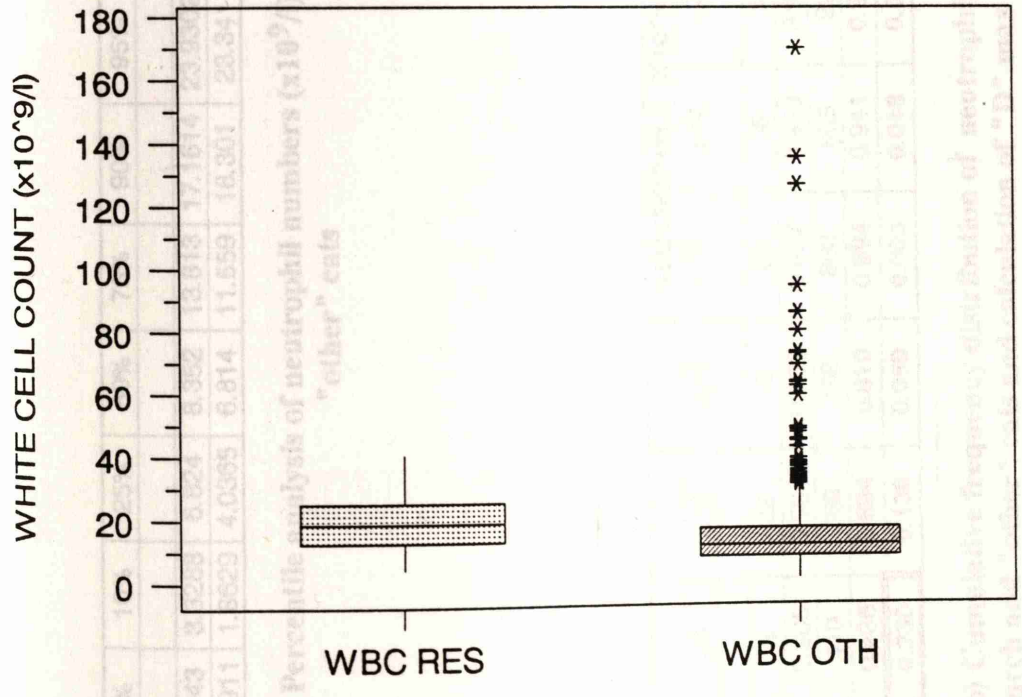
WBC	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	4.2	4.785	5.985	10.08	12.675	18.5	24.95	29.12	33.075	38.038	40.3		19.053	8.298
OTHER	0.4	1.19	3.42	4.9	7.3	10.9	16.3	24.3	30.96	63.43	170		13.77	12.56

Table 3.7.(a) Percentile analysis of white cell numbers ($x10^9$/l) for research and "other" cats

						WBC (x10^9/l)						
INTERVAL		< 2	<6	<10	<14	<18	<22	<26	<30	<34	<38	<42
NO. VALUES	RES	0	3	4	12	19	25	34	36	38	39	40
	%	0.000	0.075	0.100	0.300	0.475	0.625	0.850	0.900	0.950	0.975	1.000
NO. VALUES	OTH	26	177	431	679	785	865	905	933	953	963	968
	%	0.026	0.178	0.434	0.684	0.791	0.872	0.912	0.941	0.961	0.971	0.976
	D	**0.026**	**0.103**	**0.334**	**0.384**	**0.316**	**0.247**	**0.062**	**0.041**	**0.011**	**0.004**	**0.024**
INTERVAL		<46	<50	<54	<58	<62	>62					
NO. VALUES	RES	40	40	40	40	40	40					
	%	1.000	1.000	1.000	1.000	1.000	1.000					
NO. VALUES	OTH	972	978	978	978	980	992					
	%	0.980	0.986	0.986	0.986	0.988	1.000					
	D	**0.020**	**0.014**	**0.014**	**0.014**	**0.012**	**0.000**					

Table 3.7.(b) Cumulative frequency distribution of white cell numbers ($x10^9$/l) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.9 BOXPLOT OF WHITE CELL COUNT FOR RESEARCH & OTHER CATS



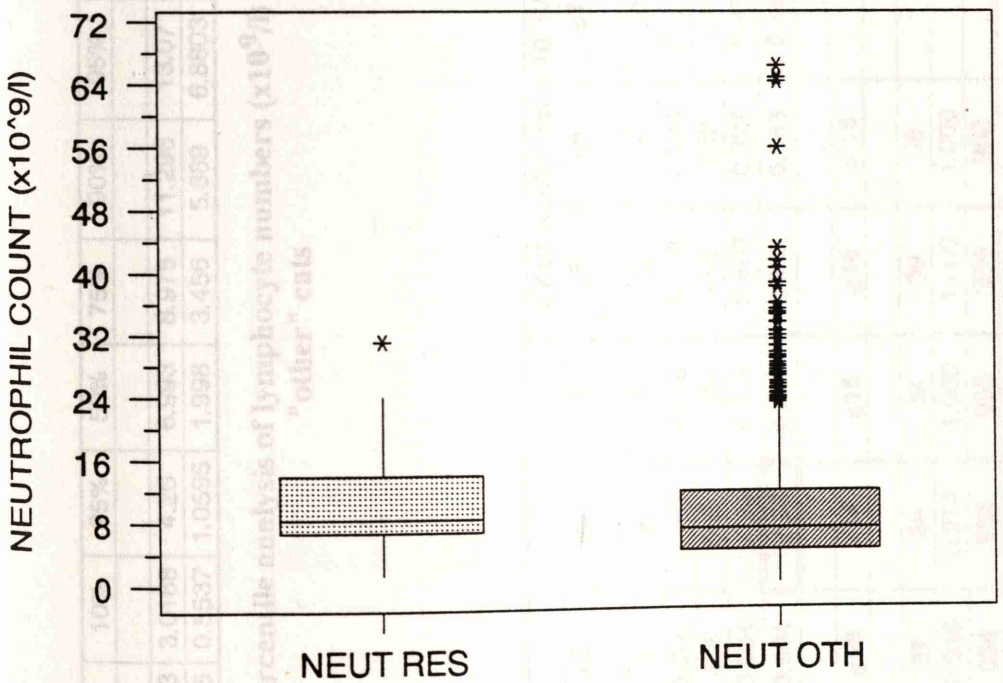
NEUTROPHIL	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	1.344	1.4614	2.343	3.3288	6.824	8.352	13.813	17.1614	23.9302	28.351	31.031		10.376	6.489
OTHER	0	0.1759	0.8911	1.8629	4.0365	6.814	11.559	18.301	23.341	35.233	66.24		6.489	7.7844

Table 3.8.(a) Percentile analysis of neutrophil numbers (x10^9/l) for research and "other" cats

		NEUTROPHILS (X10^9/L)										
INTERVAL		≤ 2	≤6	≤10	≤14	≤18	≤22	≤26	≤30	≤34	≤38	> 38
NO. VALUES	RES	2	8	22	30	35	36	38	38	39	39	39
	%	0.051	0.205	0.564	0.769	0.897	0.923	0.974	0.974	1.000	1.000	1.000
NO. VALUES	OTH	101	419	668	788	860	905	927	939	948	954	962
	%	0.105	0.436	0.694	0.819	0.894	0.941	0.964	0.976	0.985	0.992	1.000
	D	0.054	0.230	0.130	0.050	0.003	0.018	0.011	0.002	0.015	0.008	0.000

Table 3.8.(b) Cumulative frequency distribution of neutrophil numbers (x10^9/l) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.10 BOXPLOT OF NEUTROPHIL COUNT FOR RESEARCH & OTHER CATS



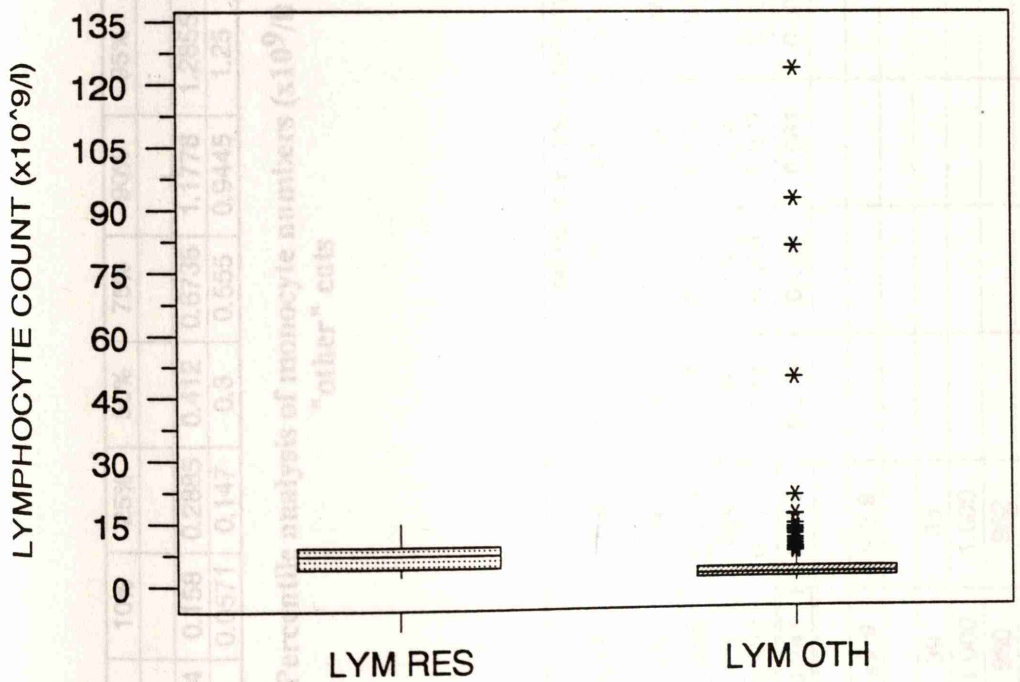
LYMPHOCYTE	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	2.04	2.154	2.6343	3.0168	4.26	6.993	8.975	11.296	13.07	14.317	14.649		7.075	3.326
OTHER	0	0.1261	0.3155	0.5537	1.0595	1.998	3.456	5.389	6.8803	12.4746	123.653		2.9131	6.0732

Table 3.9.(a) Percentile analysis of lymphocyte numbers ($x10^9$/l) for research and "other" cats

						LYMPHOCYTES (x10^9/l)						
INTERVAL		≤ 1	≤2	≤3	≤4	≤5	≤6	≤7	≤8	≤9	≤10	≤11
NO. VALUES	RES	0	0	4	10	13	15	20	24	29	32	35
	%	0.000	0.000	0.103	0.256	0.333	0.385	0.513	0.615	0.744	0.821	0.897
NO. VALUES	OTH	221	485	668	780	852	892	918	936	943	945	948
	%	0.230	0.504	0.694	0.811	0.886	0.927	0.954	0.973	0.980	0.982	0.985
	D	**0.230**	**0.504**	**0.592**	**0.554**	**0.552**	**0.543**	**0.441**	**0.358**	**0.237**	**0.162**	**0.088**
INTERVAL		≤12	≤13	≤14	≤15	≤16	> 16					
NO. VALUES	RES	35	37	38	39	39	39					
	%	0.897	0.949	0.974	1.000	1.000	1.000					
NO. VALUES	OTH	950	954	955	955	956	962					
	%	0.988	0.992	0.993	0.993	0.994	1.000					
	D	**0.090**	**0.043**	**0.019**	**0.007**	**0.006**	**0.000**					

Table 3.9.(b) Cumulative frequency distribution of lymphocyte numbers ($x10^9$/l) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.11 BOXPLOT OF LYMPHOCYTE COUNT FOR RESEARCH & OTHER CATS



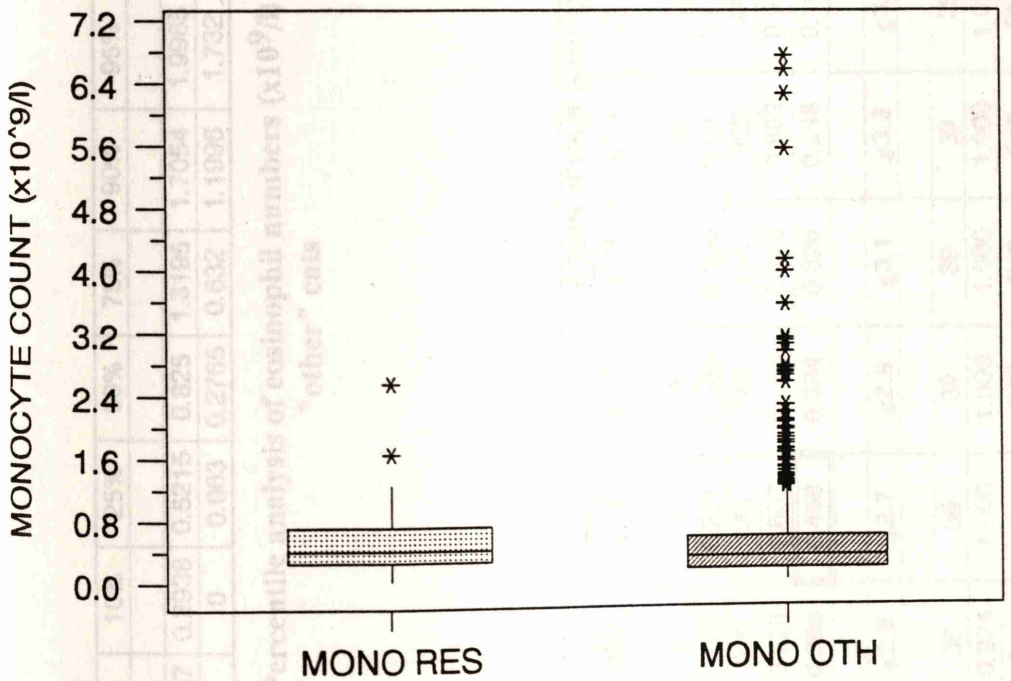
MONOCYTE	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	0.03	0.04	0.0624	0.158	0.2885	0.412	0.6735	1.1778	1.2855	2.208	2.55		0.573	0.491
OTHER	0	0	0	0.0571	0.147	0.3	0.555	0.9445	1.25	3.0349	6.748		0.4591	0.6149

Table 3.10.(a) Percentile analysis of monocyte numbers ($x10^9$/l) for research and "other" cats

						MONOCYTES (x10^9/l)						
INTERVAL		≤ 0.1	≤0.3	≤0.5	≤0.7	≤0.9	≤1.1	≤1.3	≤1.5	≤1.7	≤1.9	≤2.1
NO. VALUES	RES	3	10	23	29	32	33	37	37	38	38	38
	%	0.077	0.256	0.590	0.744	0.821	0.846	0.949	0.949	0.974	0.974	0.974
NO. VALUES	OTH	163	484	684	786	856	892	917	925	932	936	941
	%	0.169	0.503	0.711	0.817	0.890	0.927	0.953	0.962	0.969	0.973	0.978
	D	**0.093**	**0.247**	**0.121**	**0.073**	**0.069**	**0.081**	**0.005**	**0.013**	**0.006**	**0.001**	**0.004**
INTERVAL		≤2.7	≤2.9	> 2.9								
NO. VALUES	RES	39	39	39								
	%	1.000	1.000	1.000								
NO. VALUES	OTH	948	950	962								
	%	0.985	0.988	1.000								
	D	**0.015**	**0.012**	**0.000**								

Table 3.10.(b) Cumulative frequency distribution of monocyte numbers ($x10^9$/l) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.12 BOXPLOT OF MONOCYTE COUNT FOR RESEARCH & OTHER CATS



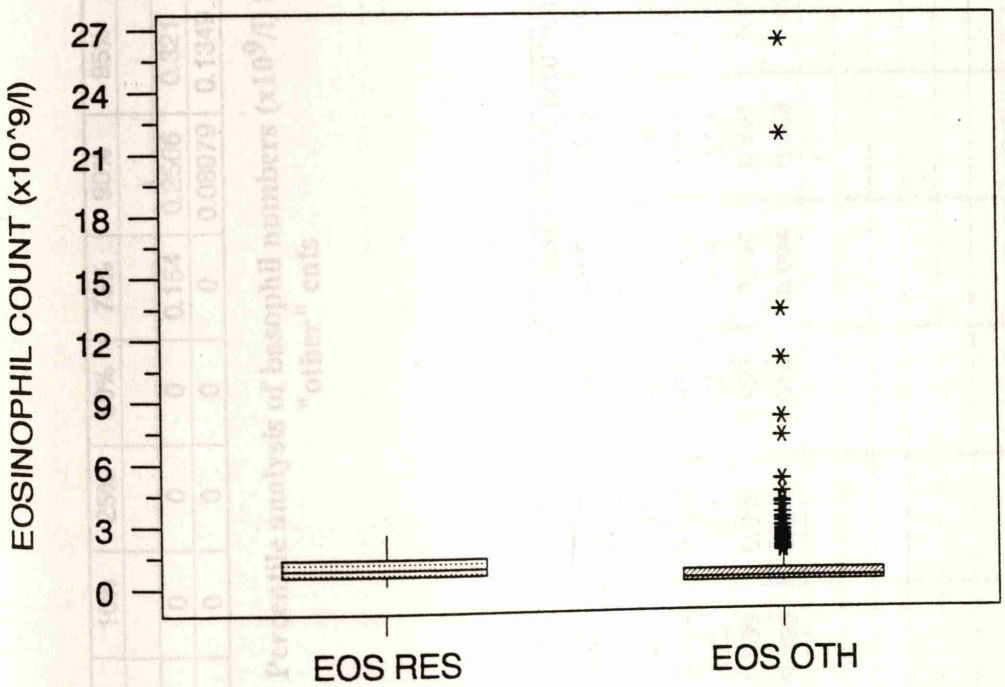
EOSINOPHIL	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	0.105	0.1088	0.2167	0.2938	0.5215	0.825	1.3195	1.7054	1.9965	2.4023	2.56		0.957	0.596
OTHER	0	0	0	0	0.063	0.2765	0.632	1.1996	1.732	3.8693	26.448		0.5551	1.3919

Table 3.11.(a) Percentile analysis of eosinophil numbers (x10^9/l) for research and "other" cats

		EOSINOPHILS (x10^9/l)										
INTERVAL		< 0.1	≤0.3	≤0.5	≤0.7	≤0.9	≤1.1	≤1.3	≤1.5	≤1.7	≤1.9	≤2.1
NO. VALUES	RES	0	5	9	15	23	25	29	30	35	36	37
	%	0.000	0.128	0.231	0.385	0.590	0.641	0.744	0.769	0.897	0.923	0.949
NO. VALUES	OTH	287	506	661	749	881	855	877	898	910	921	930
	%	0.298	0.526	0.687	0.779	0.916	0.889	0.912	0.933	0.946	0.957	0.967
	D	**0.298**	**0.398**	**0.456**	**0.394**	**0.326**	**0.248**	**0.168**	**0.164**	**0.049**	**0.034**	**0.018**
INTERVAL		<2.3	≤2.5	≤2.7	≤2.9	≤3.1	≤3.3	≤3.5	≤3.7	≤3.9	> 3.9	
NO. VALUES	RES	38	38	39	39	39	39	39	39	39	39	
	%	0.974	0.974	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
NO. VALUES	OTH	936	940	942	943	946	949	950	951	952	962	
	%	0.973	0.977	0.979	0.980	0.983	0.986	0.988	0.989	0.990	1.000	
	D	**0.001**	**0.003**	**0.021**	**0.020**	**0.017**	**0.014**	**0.012**	**0.011**	**0.010**	**0.000**	

Table 3.11.(b) Cumulative frequency distribution of eosinophil numbers (x10^9/l) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.13 BOXPLOT OF EOSINOPHIL COUNT FOR RESEARCH & OTHER CATS



BASOPHIL	MIN	1%	5%	10%	25%	50%	75%	90%	95%	99%	MAX		MEAN	ST DEV
RESEARCH	0	0	0	0	0	0	0.154	0.2566	0.321	0.393	0.432		0.089	0.122
OTHER	0	0	0	0	0	0	0	0.08979	0.1349	0.3095	1.38		0.0279	0.0883

Table 3.12.(a) Percentile analysis of basophil numbers (x10^9/l) for research and "other" cats

		BASOPHILS (x10^9/l)										
INTERVAL	BASO	≤ 0.1	≤0.2	≤0.3	≤0.4	≤0.5	≤0.6	≤0.7	≤0.8	≤0.9	≤1.0	≤1.1
NO. VALUES	RES	27	30	36	38	39	39	39	39	39	39	39
	%	0.692	0.769	0.923	0.974	1.000	1.000	1.000	1.000	1.000	1.000	1.000
NO. VALUES	OTH	880	932	951	955	958	959	959	959	959	960	960
	%	0.915	0.969	0.989	0.993	0.996	0.997	0.997	0.997	0.997	0.998	0.998
	D	0.222	0.000	0.065	0.018	0.004	0.003	0.003	0.003	0.003	0.002	0.002
INTERVAL		> 1.1										
NO. VALUES	RES	39										
	%	1.000										
NO. VALUES	OTH	962										
	%	1.000										
	D	0.000										

Table 3.12.(b) Cumulative frequency distribution of basophil numbers (x10^9/l) for research and "other" cats and calculation of "D" max (bordered) for Kolmogorov-Smirnov 2-sample, 2-tailed test

FIG 3.14 BOXPLOT OF BASOPHIL COUNT FOR RESEARCH & OTHER CATS

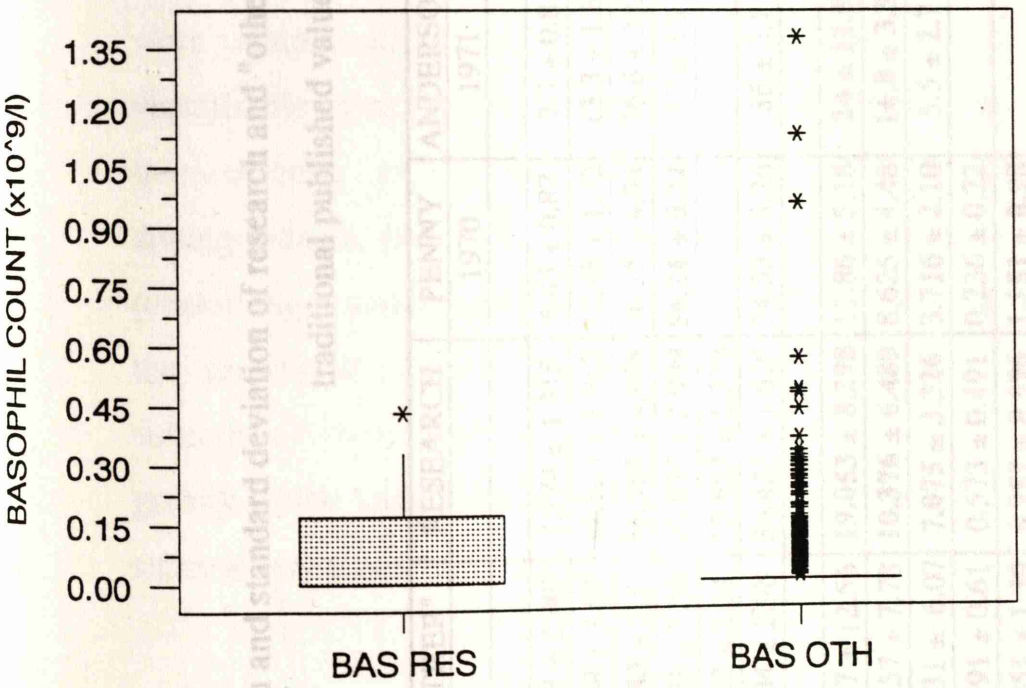


Table 3.13. Mean and standard deviation of research and "other" groups of cats and comparison with traditional published values

	"OTHER"	RESEARCH	PENNY	ANDERSON	SCHALM	EARLE
			1970	1971	1975	1990
RBC(x 10^{12}/L)	6.978 ± 2.407	7.774 ± 1.315	6.45 ± 0.87	7.7 ± 0.8	7.5	7.29 ± 0.80
Hb (g/dl)	10.48 ± 3.58	12.163 ± 2.013	12.48 ± 1.72	13.3 ± 1.8	12	11.38 ± 1.71
Hct (L/L)	**30.43 ± 9.5**	**33.268 ± 4.899**	36.15 ± 4.94	36.6 ± 3.6	37	37.71 ± 4.21
MCV (fl)	45.03 ± 7.36	43.075 ± 2.999	**56.16 ± 6.22**	47 ± 3.9	45	**51.98 ± 5.13**
MCH (pg)	15.22 ± 2.31	15.653 ± 1.378			15.5	15.7 ± 2.38
MCHC (g/dl)	34.04 ± 2.98	36.423 ± 1.616	34.53 ± 3.26	36 ± 3.1	33.2	**30.16 ± 3.00**
WBC (x 10^9/l)	13.77 ± 12.56	**19.053 ± 8.298**	13.86 ± 5.18	**24 ± 12.5**	12.5	13.96 ± 7.16
NEUT (x 10^9/l)	8.9157 ± 7.78	**10.376 ± 6.489**	8.625 ± 4.48	**16.8 ± 3.5**	7.5	
LYMPH (x 10^9/l)	2.9131 ± 6.07	**7.075 ± 3.326**	3.716 ± 2.10	5.5 ± 2.7	4	
MONO (x 10^9/l)	0.4591 ± 0.61	0.573 ± 0.491	0.236 ± 0.22		0.35	
EOSIN (x 10^9/l)	0.5551 ± 1.39	**0.957 ± 0.596**	**1.151 ± 0.98**		0.65	
BASO (x 10^9/l)	0.0279 ± 0.09	0.089 ± 0.122	0.002 ± 0.01		0	

CHAPTER 4

OPTIMISATION OF HAEMOPOIETIC COLONY ASSAYS

4.1. INTRODUCTION

In vitro clonal growth of haemopoietic progenitor cells in semi-solid matrices was first described in the early sixties (Till & McCulloch 1961, Pluznick & Sachs 1965, Bradley & Metcalf 1966). These techniques allowed the quantification of progenitor cells in marrow and spleen by stimulating growth in a medium whereby progeny were maintained around progenitor cells and thus formed a visualisable colony after a period of incubation. It was soon realised that colonies of different cell types were morphologically distinguishable, and that the use of various stimulatory media (containing a variety of cytokines and growth factors) could favour the growth of colonies of particular type. The discovery and subsequent cloning of individual growth factors has contributed greatly to the understanding of haemopoiesis and made the colony assay a very powerful investigative tool.

4.2. AIM

The main aim of this part of the study was to determine optimum cell numbers and concentration of growth stimulants for use in the cat, based on the established values for human and mouse.

Optimum mononuclear cell concentration needed to be determined in order to provide the correct ratio of progenitor cells to stromal feeder cells, so that there were adequate numbers of colonies but not excessive endogenous growth factor production. In the same way, numbers of feeder cells for maximum growth promotion

without masking colony growth had to be determined. It was also necessary to establish the dose of radiation required for mononuclear marrow suspensions to be used as feeder cells. Sufficient radiation had to be provided to stop replication of progenitor cells, but not to inhibit growth factor production by accessory cells in the feeder cell population. Recombinant human growth factors were also titrated to determine optimum concentrations for maximal stimulation of feline progenitors.

4.3. MATERIALS AND METHODS

Bone marrow aspirates were taken from SPF cats or cells were obtained from recently euthanased virus negative cats with healthy bone marrow and prepared for cell culture. A series of experiments were set up to determine optimum values for: concentration of cells per plate; concentration of feeder cells per plate; dose of irradiation for feeder cells; concentration of growth factors to be added for maximum stimulation and type of erythroid stimulant. All the assays were set up in the same way, varying only the factor being titrated in each individual experiment.

4.3.1. Experiment 1 - Optimum Concentration of Cells

Cells were taken from 7 cats and set up in both GM and mix assay systems. Marrow mononuclear cells were plated at the following concentrations with no feeder cells or exogenous growth factors added:

1×10^4 cells/ml; 2.5×10^4 cells/ml; 5×10^4 cells/ml; 7.5×10^4 cells/ml; 1×10^5 cells/ml; 2.5×10^5 cells/ml and 5×10^5 cells/ml.

Colonies were counted at 8 and 14 days for GM and mix assays respectively. The results are represented in figures 4.1, 4.2 and 4.3.

4.3.2. Experiment 2 - Optimum Concentration of Feeder Cells

Cells were taken from 2 cats and set up in a GM assay. Marrow mononuclear cells were added at a concentration of 5 x 10^4/ml, with no exogenous growth factors. Mononuclear cells to be used as a feeder population were exposed to 1600 rads from a Co^{60} source and added to the existing cell suspensions at the following concentrations:

8 x 10^3; 1 x 10^4; 3 x 10^4; 5 x 10^4; 8 x 10^4; 1 x 10^5; 3 x 10^5; 5 x 10^5; 1 x 10^6 and 3 x 10^6/ml.

Colonies were counted at 8 days. The results are represented in figure 4.4.

4.3.3. Experiment 3 - Optimum Irradiation Dose for Feeder Cells

Bone marrow cells were taken from 5 cats and set up in both GM and mix assays. Marrow mononuclear cells were added at 5 x 10^4/ml and feeder cells at a concentration of 1 x 10^5/ml. The feeder cells were subjected to increasing doses of radiation from the Co^{60} source as follows: 0; 200; 400; 600; 800; 1000; 1200; 1400; 1600; 1800 and 2000 rads. No exogenous growth factors were added.

Colonies were counted at 8 and 14 days for GM and mix assays respectively. Results are shown on figures 4.5, 4.6 and 4.7.

4.3.4. Experiment 4 - Optimum Concentrations of Growth Factors

Recombinant human growth factors GM-CSF, G-CSF, SCF and IL-3 were used as growth stimulants in both GM and mix colony assays. Human factors were used as recombinant cat factors were

not available. They were used at various concentrations, based around the known optimum for human cells. In one experiment, (fig. 4.15), medium from FeLV infected cells (FeLV CM), medium derived from culture of spleen cells (spleen CM) and medium from culture of the 5637 bladder carcinoma cell line was used. These cells constitutively produce several growth factors, hence media incubated with these cells is also growth factor rich (Coutinho *et al* 1993).

Mononuclear cells were plated at 5 x 10^4/ml. Feeder cells (irradiated at 1600 rads) were added to some growth factors at 1 x 10^5/ml in order to check for synergy.

Colonies were counted at 8 and 14 days as previously. Results are presented on figures 4.8 to 4.15.

4.3.5. Experiment 5 - Optimum Type of Erythroid Stimulant

Recombinant human erythropoietin (EPO), anaemic mouse serum (AMS), anaemic cat serum (ACS), purified human recombinant erythropoietin and cat conditioned medium (CM) were added to cells from 2 cats along with different batches of both FCS and BSA in a variety of combinations. Cells were plated at 5 x 10^4/ml.

Colonies were counted at 14 days. Results are presented on figures 4.19 and 4.20.

4.4. RESULTS

Unstained colonies were counted using a Leitz Labovert FS (Leitz, Wetzlar, Germany) inverted microscope at 4 x magnification. Colonies were defined as groups of 50 cells or more, and colony types were differentiated morphologically. CFU-G were recognised as loosely distributed colonies of small translucent cells (fig. 4.21).

In contrast, CFU-M (fig. 4.22) were composed of distinctly larger cells, which tended to be packed relatively closer together. CFU-GM (fig. 4.23) were composed of both cell types, having a centre composed mainly of the larger macrophages, and an outer rim of small granulocytes. BFU-E (fig. 4.24) were more easily distinguished, as these were generally haemoglobinised. In addition, the cells were very small and packed together very tightly in groups, or "bursts". True mix cell colonies were not seen in these assays. Mix colonies are composed of all the above cell types in a single colony.

4.4.1. Experiment 1 - Optimum Concentration of Cells

Figures 4.1, 4.2 and 4.3 show the expected relationship between increasing numbers of cells plated and numbers of colonies grown. As no exogenous growth factors or feeder cells were added, the growth was dependent on endogenous growth factor production by accessory cells. Thus, the rates of increase in growth were dependent not only on the number of progenitor cells per well, but also the increasing numbers of accessory cells present.

4.4.2. Experiment 2 - Optimum Concentration of Feeder Cells

Figure 4.4 shows the relationship between increasing numbers of feeder cells and increasing numbers of colonies grown. This was true of both cats studied. Although the numbers of colonies grown continued to increase with increasing numbers of accessory cells, as the concentration rose the feeder cells tended to overcrowd the plate and make counting of colonies difficult. As the feeder cells also had a requirement for the nutrients in the medium, the addition of too

many of these cells resulted in the restriction of essential nutrients to the progenitor cells, with a resultant inhibition of growth.

4.4.3. Experiment 3 - Optimum Irradiation Dose for Feeder cells

Figures 4.5, 4.6 and 4.7 demonstrate the fall in colony numbers produced as the dose of radiation given to feeder cells was increased. At low levels of radiation not all of the progenitor cells in the feeder population were prevented from replicating, thus, the effect was to increase the concentration of cells with multiplicative potential in the assay. At higher doses of radiation the graphs levelled out, indicating that the progenitor cells had been inactivated, but the feeder population remained active and able to support growth. A dose of 1600 rads was chosen as optimum, having considered the results for both GM and mix assay systems. This dose abolished progenitor cell activity without destroying stromal cell ability to produce growth factors. Although the general trend in the BFU-E numbers was similar to that of GM-CFU, growth was less predictable and seemed to be reliant on other factors.

4.4.4. Experiment 4 - Optimum Concentrations of Growth Factors

Figures 4.8 to 4.15 illustrate the results from 8 experiments using recombinant human growth factors at human plateau (P) dose, 3 x plateau (3P) and in combination with feeder cells (F). These experiments were done using different cat cells and at different time points and locations. However, the majority of results show very little effect of human recombinant growth factors on cat cells, little or no stimulation being observed. Human G-CSF was the growth factor with the most consistent effect, a mild stimulatory effect

being seen in a number of experiments. In one case (fig 4.11), IL-3 seemed to inhibit growth. In another experiment (fig 4.12) G-CSF alone and in combination with GM-CSF, SCF or both showed a significant increase in growth. However, colony growth overall was poor in this experiment and the same cell and growth factor combinations in methylcellulose did not produce the same results.

Combining specific growth factors with feeder cell populations also failed to provide consistent results (figs 4.13 and 4.14). In the case of SCF, it seemed to reduce the feeder cell stimulation.

The addition of conditioned media to cat cells failed to result in significant growth promotion. Of the media tested, spleen conditioned medium seemed to provide the best results (fig 4.15).

The titration of recombinant human growth factors, G-CSF, GM-CSF and IL-3 showed mild stimulatory effects in most cases (figs 4.16, 4.17 and 4.18 respectively). There did not appear to be a linear increase in colony growth with increasing dose of cytokine, indeed there was more evidence to suggest that increasing concentrations of these cytokines resulted in growth reduction.

4.4.5. Experiment 5 - Optimum Type of Erythroid Stimulant

As erythroid growth in the experiments done to optimise other parameters was poor and inconsistent, an attempt was made to find a more reliable stimulant. Erythroid growth was poor in both experiments, but cat CM provided the most consistent results. However, since the composition of cat CM is variable and the results not dramatically better, it was decided to use recombinant human EPO in the experimental assays.

4.5. DISCUSSION

The series of optimising experiments demonstrated the extent of inter-assay variability using this system and the strict requirement for control cells to be available for all individual animals and stages of the experiment. The assays used for the infected cats were designed around the existing data available (N. Testa, personal communication, Testa *et al* 1983, Rojko *et al* 1986, Heyworth & Spooncer 1993) and the above results.

The authors mentioned above used concentrations of between 2.5 and 5 x 10^4 cells/ml in their colony assays. This is consistent with the results obtained by the cell titrations described above. In all animals, and for both assay types and colony types, there was a linear relationship between numbers of colonies grown and concentration of cells plated. Although more colonies could therefore be expected by increasing the numbers of cells plated, there would be a corresponding increase in the number of stromal cells per plate, and a consequent increase in endogenous growth factor production. Since one of the intentions of the project involved studying the effects of the addition of exogenous growth factors, this would be undesirable. The best concentration was 5 x 10^4/ml as, at this level, there were adequate numbers of progenitors but not too much endogenous growth factor production to fuel spontaneous growth. This was true of both assay systems, and of both GM-CFU and BFU-E.

The experiments used to determine the optimum concentration of growth factors to add to the cells demonstrated that cat cells fail to respond to recombinant human growth factors to any significant

degree. This was true even when the factors were used at three times the human plateau concentration. This was expected for IL-3, which is known to be a species-specific cytokine. Homology, for example, between human and mouse IL-3 is only 29% (Rasko and Gough 1994). IL-3 stimulates the growth of early stem cells, as well as megakaryocyte and mast cell precursors (Schrader 1994). Its effects are broad-ranging on the haemopoietic system. In colony assays it promotes the growth of CFU-G, CFU-GM and CFU-M (Metcalf and Nicola 1992).

Human G-CSF was expected to be more useful, as this growth factor has been demonstrated to be relatively homologous between species [80% between human and mouse (Rasko and Gough 1994)], although the cat sequence is not yet known. G-CSF is a lineage-restricted growth factor, its effects being limited to relatively mature progenitor cells (Nagata 1994). It is involved purely in the stimulation of neutrophil production and function. The addition of G-CSF to colony assays increases the number of granulocyte colonies grown (Metcalf and Nicola 1992).

GM-CSF sequences have been found to be less homologous than G-CSF but more so than IL-3 [56% between human and mouse (Rasko and Gough 1994)]. GM-CSF is known to be important in the survival, proliferation and differentiation of a number of cell lines (Rasko and Gough 1994). Granulocytes, macrophages, eosinophils, megakaryocytes and dendritic cells are all affected by the cytokine, but have varying degrees of sensitivity. Interactions with other cytokines are important in the regulation of responses. Its use in colony assays results in increased numbers of granulocyte and macrophage colonies.

The poor results achieved using human growth factors on cat cells suggests that it is necessary to have the appropriate growth factor for the species being studied. The fact G-CSF seemed to be the most effective of the cytokines used reflected its greater degree of homology between species. Individual factors were used in the infected cat assays at human plateau doses, as there was no significant increase in growth beyond these concentrations. The titrations represented in figs 4.16 and 4.17 suggested that there may even be a reduction in growth with increasing concentrations of G-CSF and GM-CSF. This may have been as a result of competition for receptor sites with endogenous factors, reducing their efficacy.

In contrast to the poor stimulatory response to exogenous growth factors, the addition of irradiated feeder cells to provide endogenous growth factors proved that the addition of more cells resulted in increasing stimulation (fig. 4.4). However, at higher concentrations, the feeder cells tended to overcrowd the colonies, making counting more difficult, and also restricted availability of nutrients from the medium. For these reasons, a relatively low concentration of 1×10^5 feeders/ml were chosen as optimum. This number was judged to provide enough endogenous growth factors without masking colony growth or competing for available nutrients. These feeders were given an optimum radiation dose of 1600 rads according to results illustrated in figs 4.5, 4.6 and 4.7, and also according to previous experience (N. Testa, personal communication). This was considered to be as far along the plateau phase of the graph as was safe to go before being in danger of destroying the stromal cells themselves, whilst ensuring that all progenitor cells were inactivated.

The reasons for poor erythroid growth in these experiments were unclear. A variety of sources of erythropoietin were used, ranging from anaemic mouse serum, anaemic cat serum, recombinant human erythropoietin and cat conditioned medium. The general lack of response to the assorted sources of erythropoietin alone in various concentrations suggested that it was not this factor which was the limiting step. This was to be expected since there is a high degree of homology [79% between human and mouse (Rasko and Gough 1984)] between erythropoietin of different species, and human would be predicted to work well in the cat. The lack of response to anaemic cat serum supported the idea that erythropoietin was not the limiting factor. The fact that erythroid growth was improved by the addition of cat conditioned medium implied that there was probably a different growth factor involved in the stimulation. Erythroid colony growth is known to be stimulated by a number of cytokines, including GM-CSF and IL-3, with erythropoietin only being involved in the latter stages. However, the variability in the composition of cat conditioned medium made it difficult to predict the nature of the stimulating factor. Due to this, and limited availability, it was decided not to use it in the assays.

In summary, marrow mononuclear cells were to be added at 5 x 10^4/ml; feeders at 1 x 10^5/ml; feeders given a dose of 1600 rads; growth factors added at 10ng/ml (G-CSF and IL-3) and 7.5ng/ml (GM-CSF); and recombinant human erythropoietin was chosen as the erythroid stimulant.

Fig. 4.1. Graph of titration of cell number plated versus mean number of colonies grown for 7 cats (GM-CFU in agar)

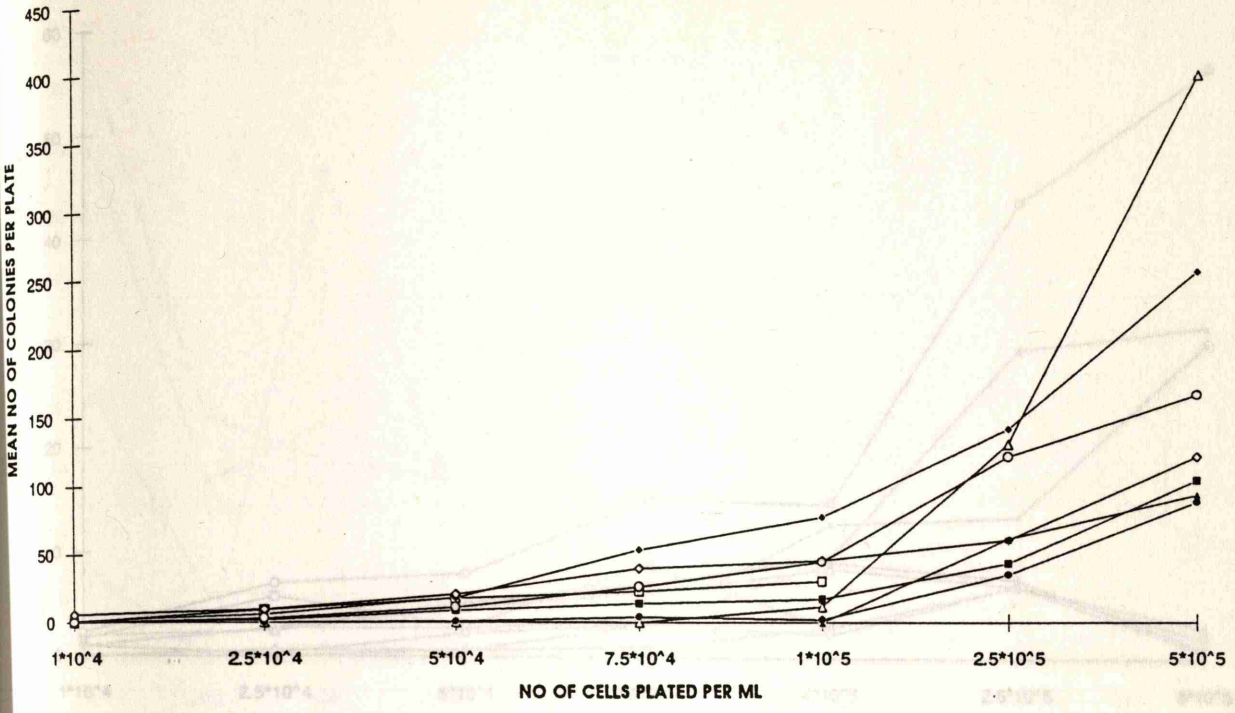


Fig. 4.2. Graph of titration of cell number plated versus mean number of colonies grown per plate for 7 cats (GM-CFU in methylcellulose)

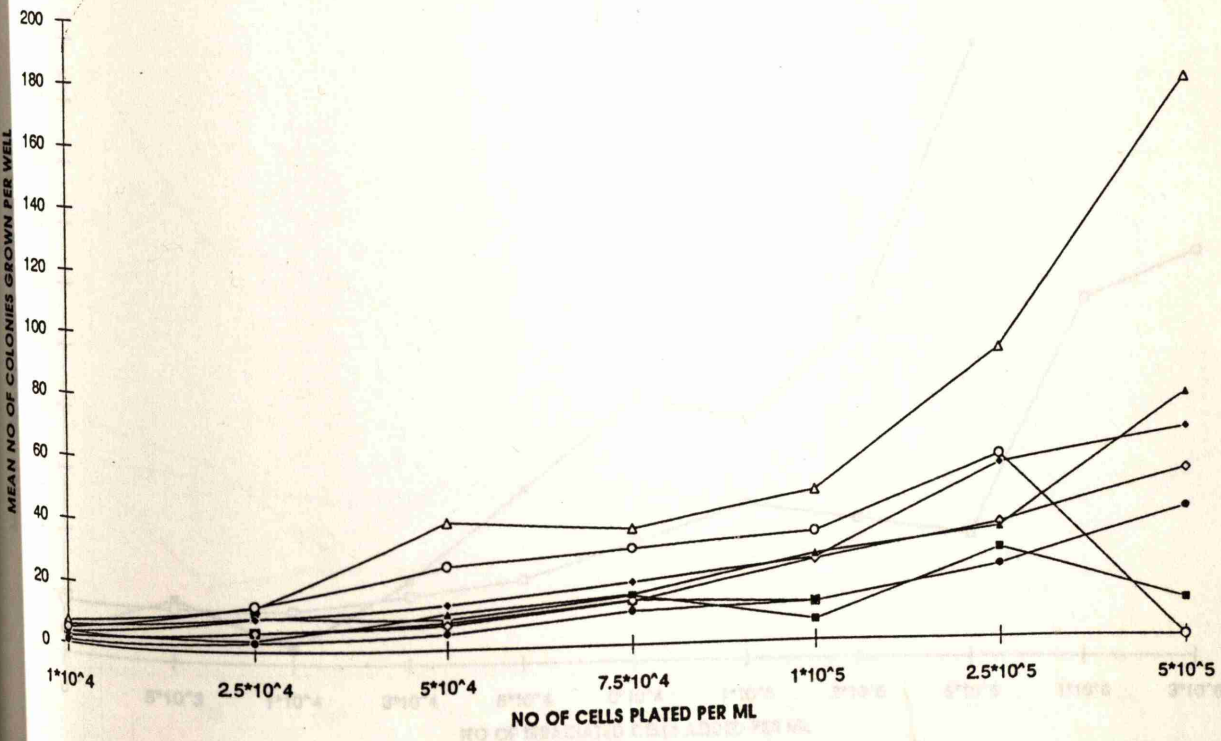


Fig. 4.3. Graph of titration of number of cells plated versus mean number of erythroid colonies grown per well for 7 cats (BFU-E in methylcellulose)

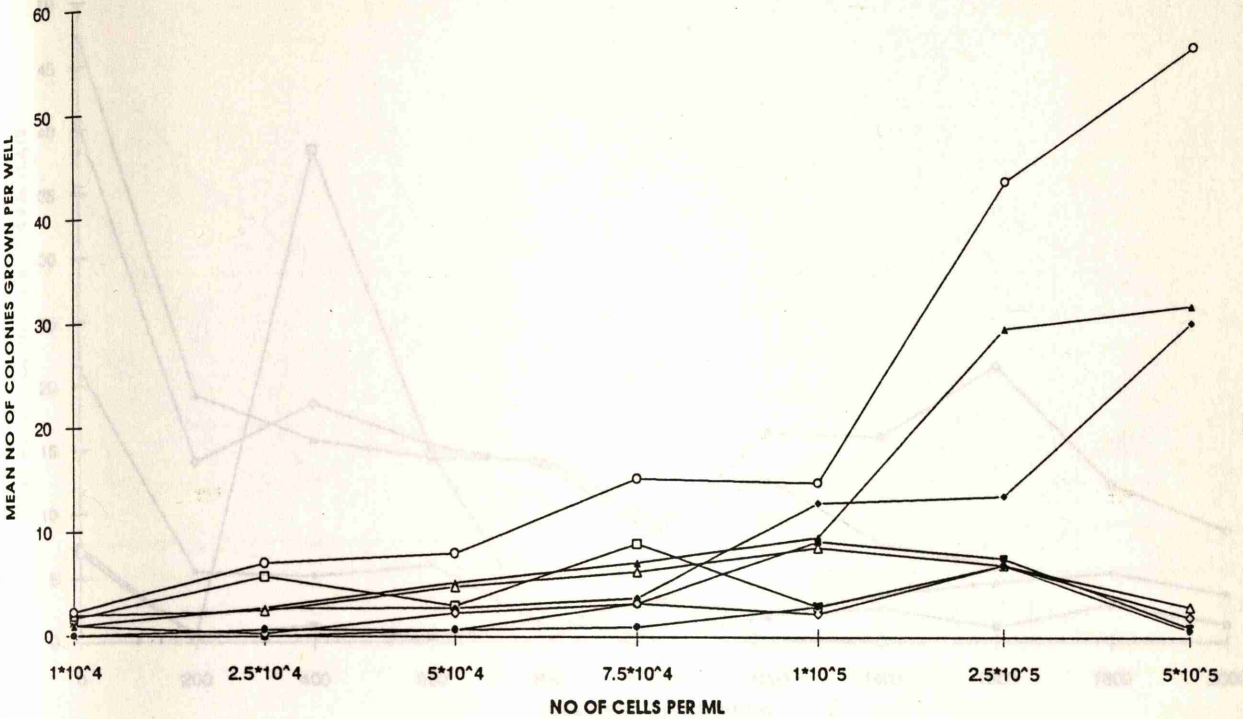


Fig. 4.4. Graph of number of irradiated cells added versus mean number of colonies grown per plate for 2 cats (GM-CFU in agar)

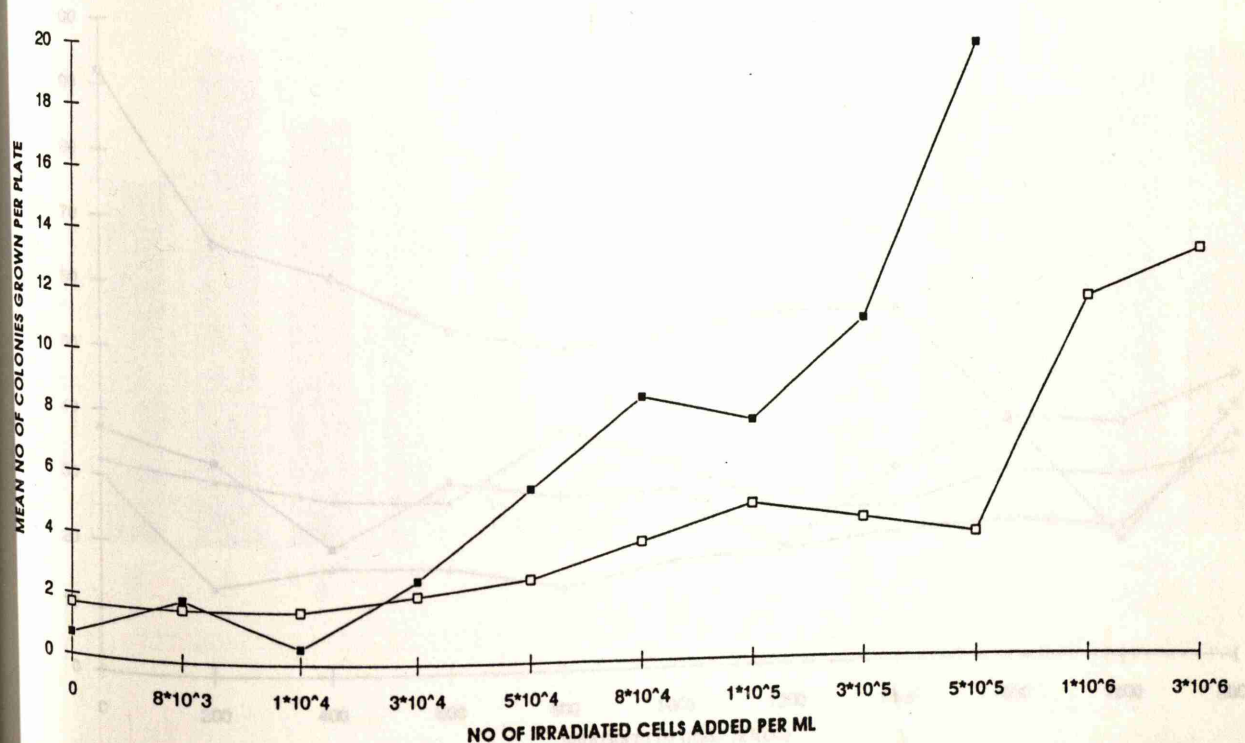


Fig. 4.5. Graph of irradiation dose given to feeder cells versus mean number of colonies grown per plate for 5 cats (GM-CFU in agar)

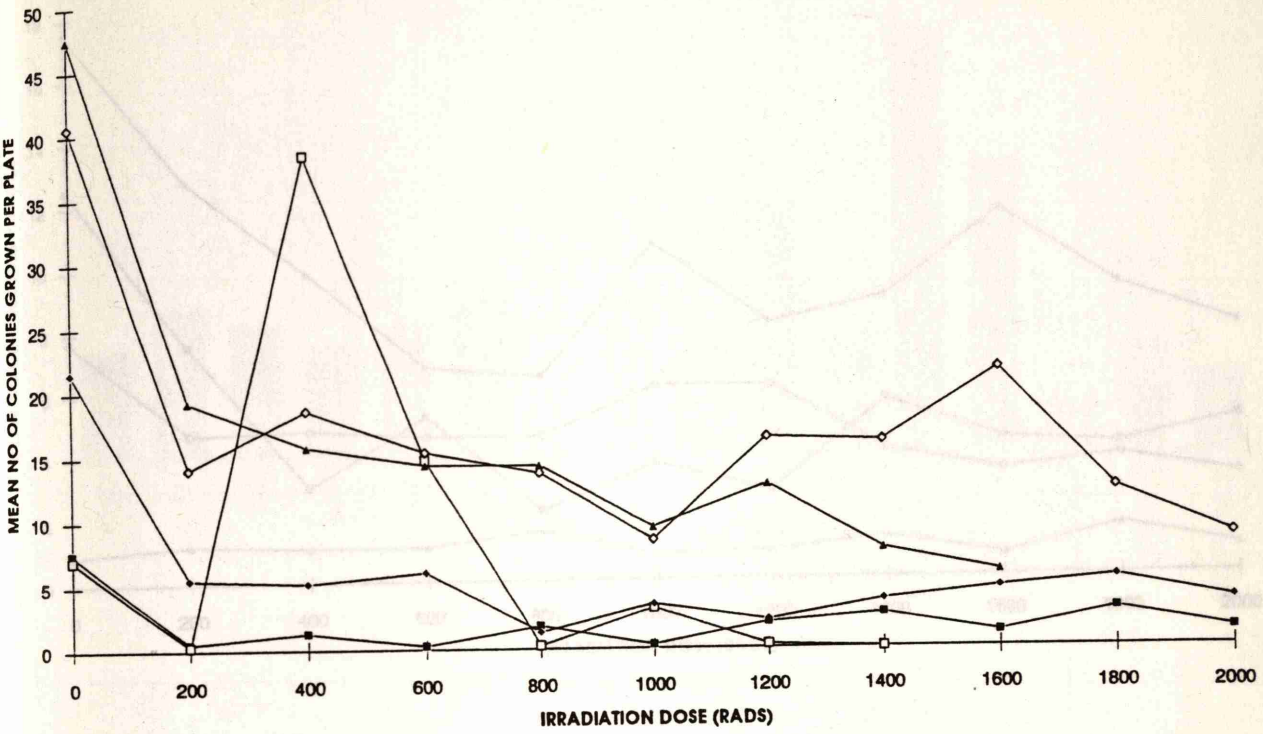


Fig. 4.6. Graph of irradiation dose given to feeder cells versus mean number of colonies grown per well for 4 cats (GM-CFU in methylcellulose)

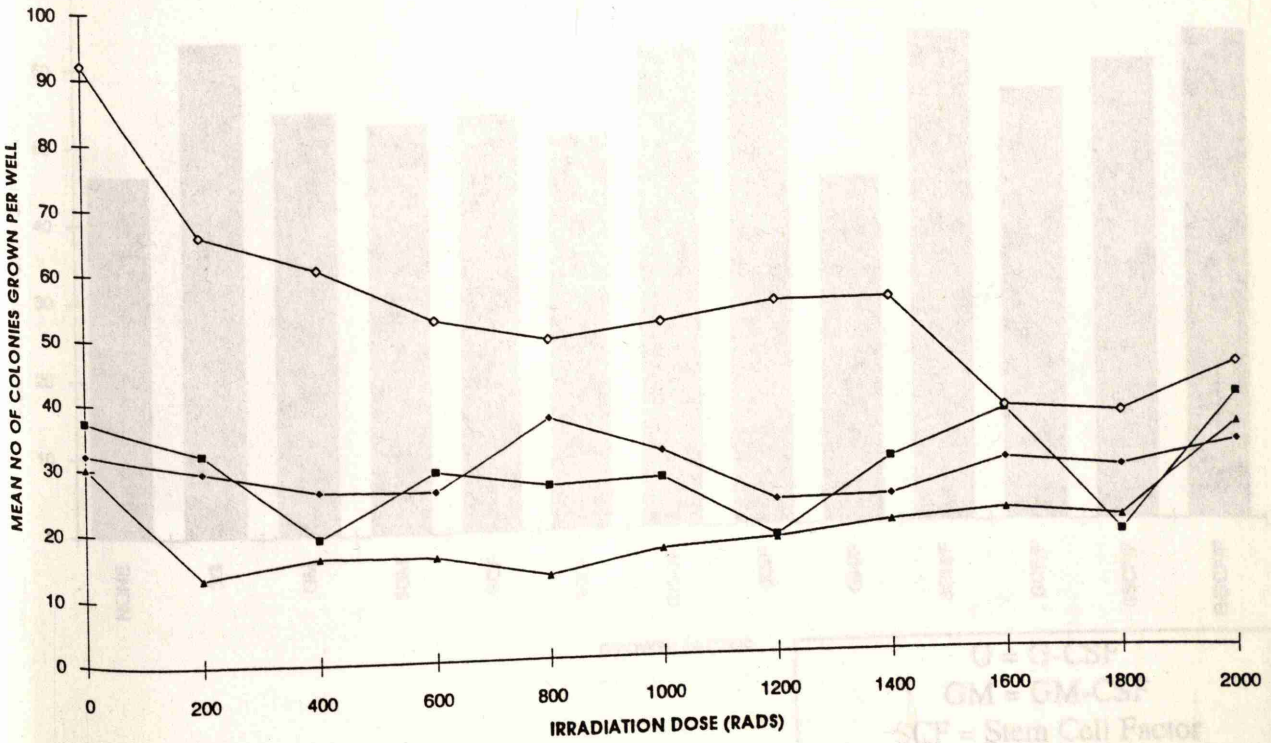


Fig. 4.7. Graph of irradiation dose given to feeder cells versus mean number of colonies grown per well (BFU-E in methylcellulose)

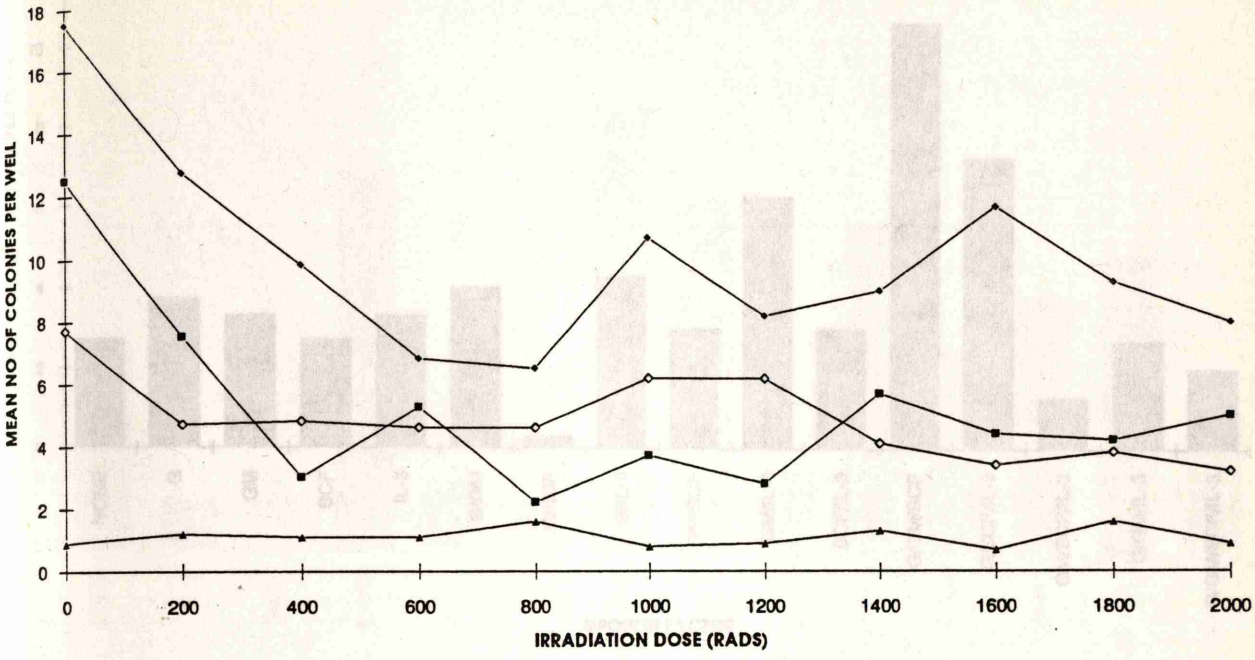
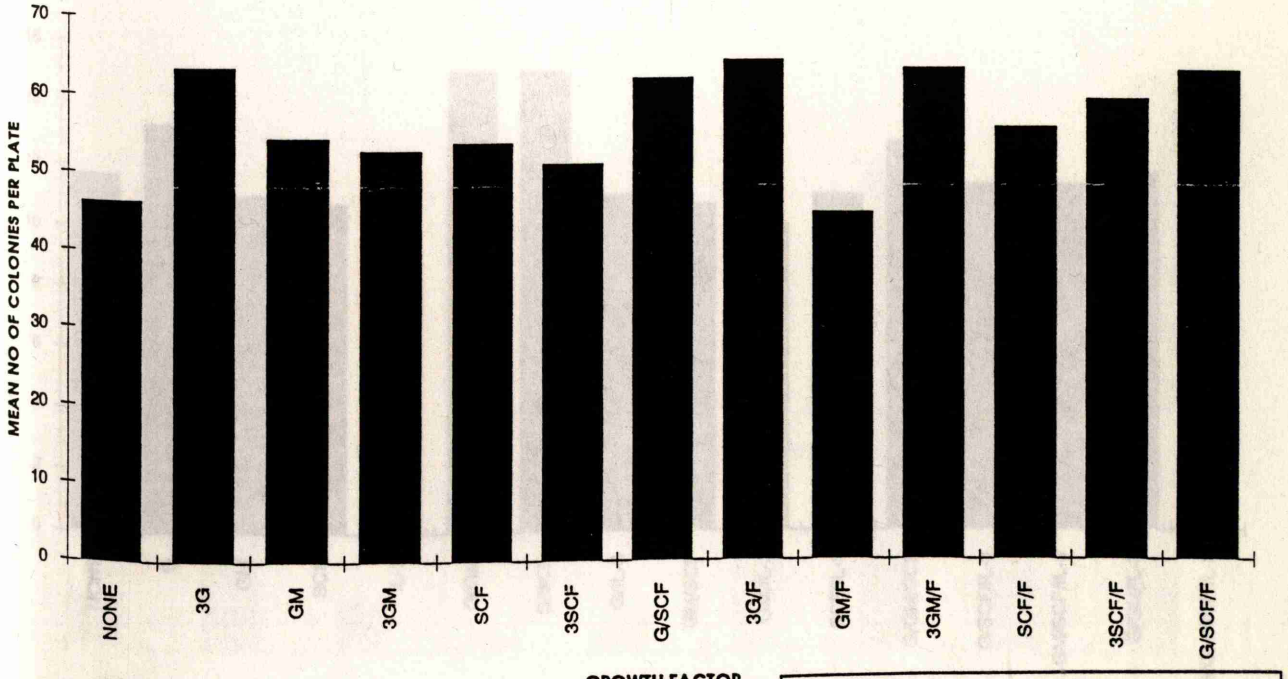
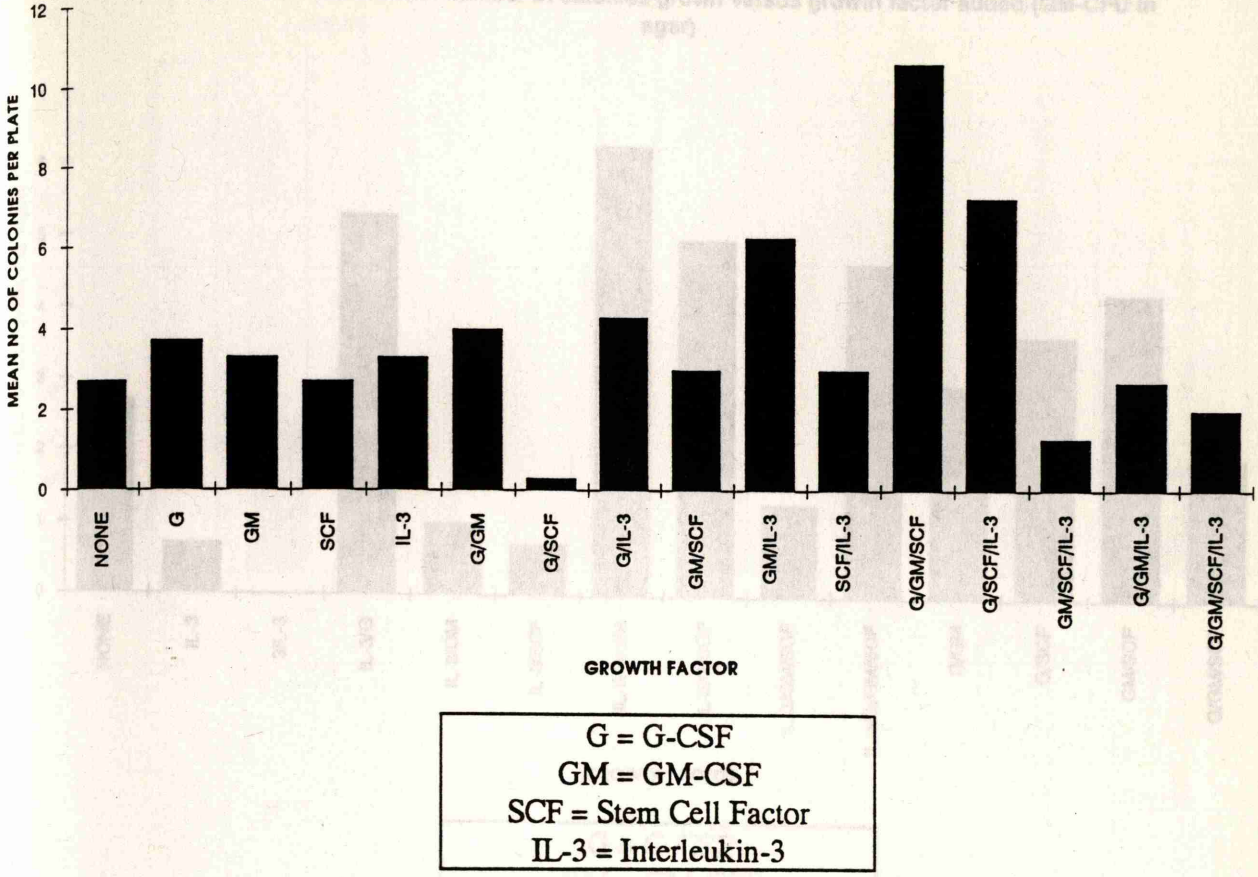


Fig. 4.8. Chart of mean number of colonies grown versus growth factor added (GM-CFU in agar)



G = G-CSF
GM = GM-CSF
SCF = Stem Cell Factor
F = Feeder Cells
3 = 3 x Human Plateau Dose

Fig. 4.9. Chart of mean number of colonies grown versus growth factor added (GM-CFU in agar)



G = G-CSF
GM = GM-CSF
SCF = Stem Cell Factor
IL-3 = Interleukin-3

Fig. 4.10. Chart of mean number of colonies grown versus growth factor added (GM-CFU in methylcellulose)

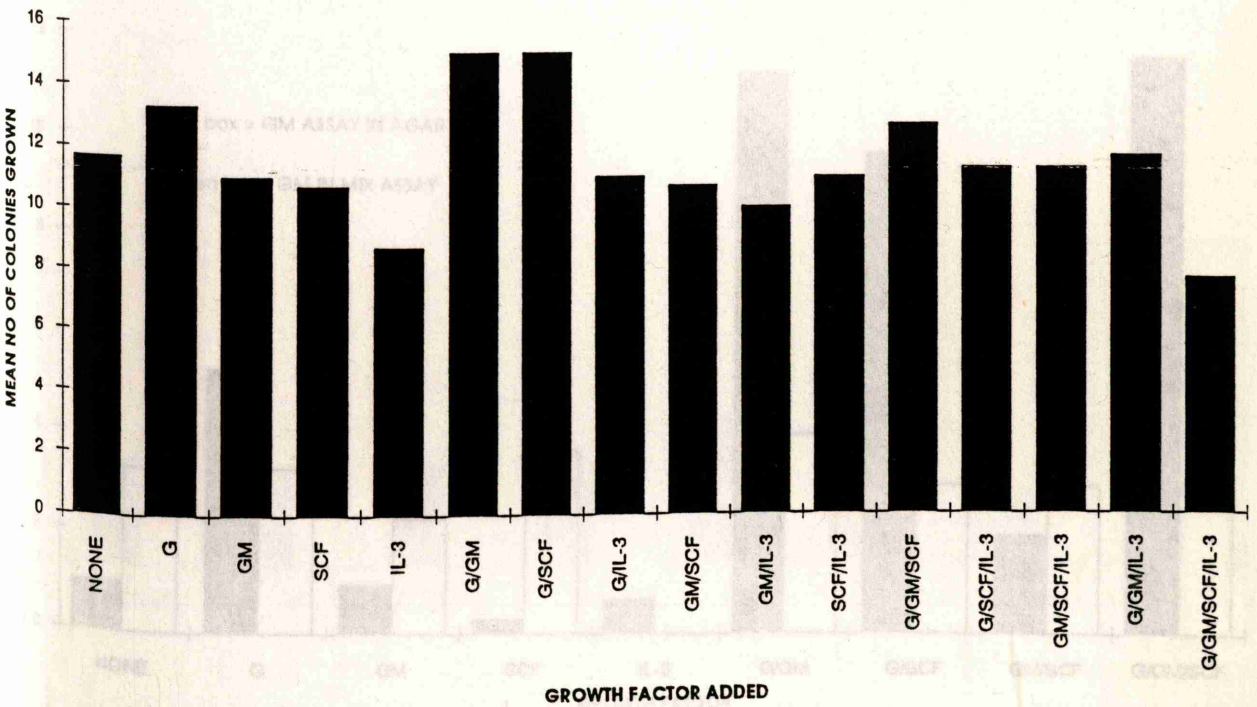
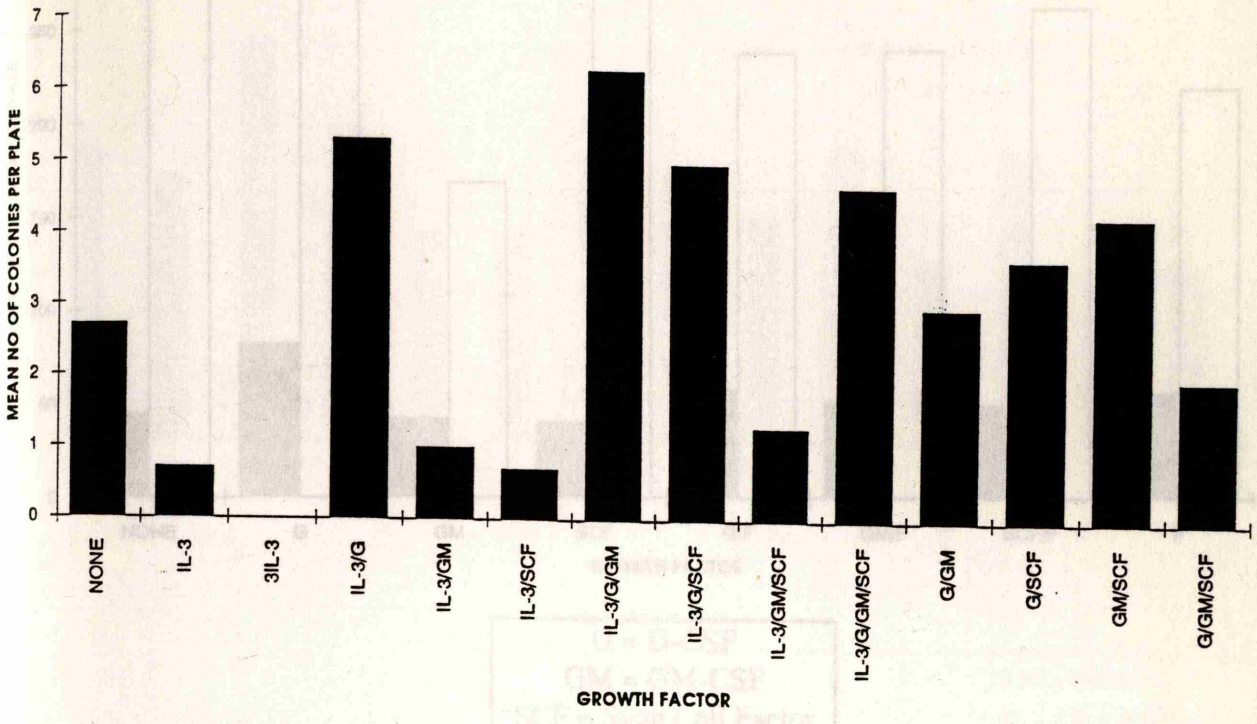


Fig. 4.11. Chart of mean number of colonies grown versus growth factor added (GM-CFU in agar)



G = G-CSF
GM = GM-CSF
IL-3 = Interleukin-3
SCF = Stem Cell Factor
3 = 3 x Human Plateau Dose

Fig. 4.12. Chart of mean number of colonies grown versus growth factor added

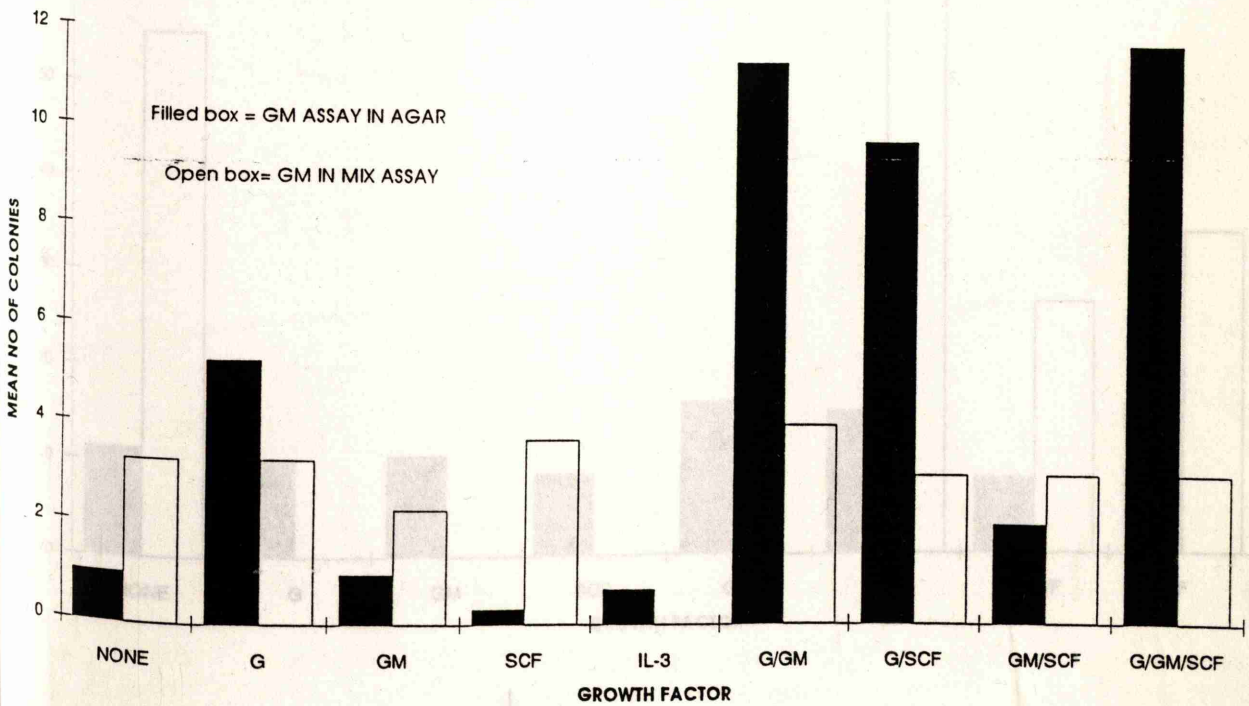
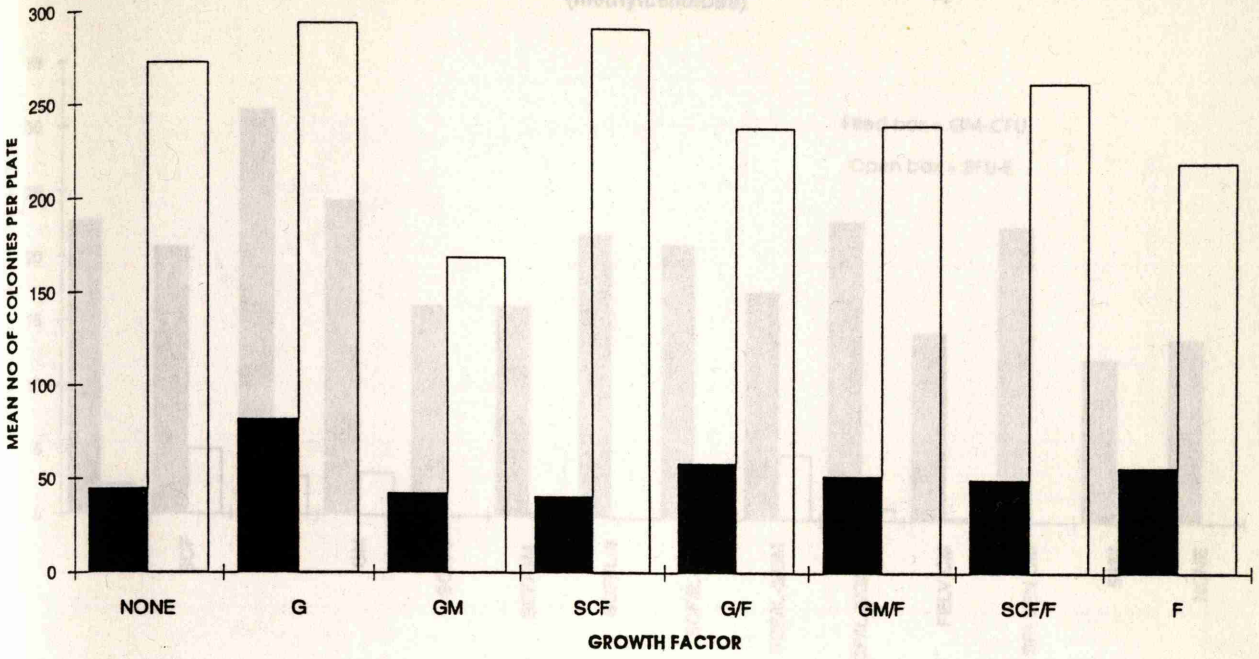


Fig. 4.13. Chart of mean number of colonies grown versus growth factor added for 2 cats (GM-CFU in agar)



G = G-CSF
GM = GM-CSF
SCF = Stem Cell Factor
F = Feeder Cells

Fig. 4.14. Chart of mean number of colonies grown versus growth factor added for 2 cats (GM-CFU in methylcellulose)

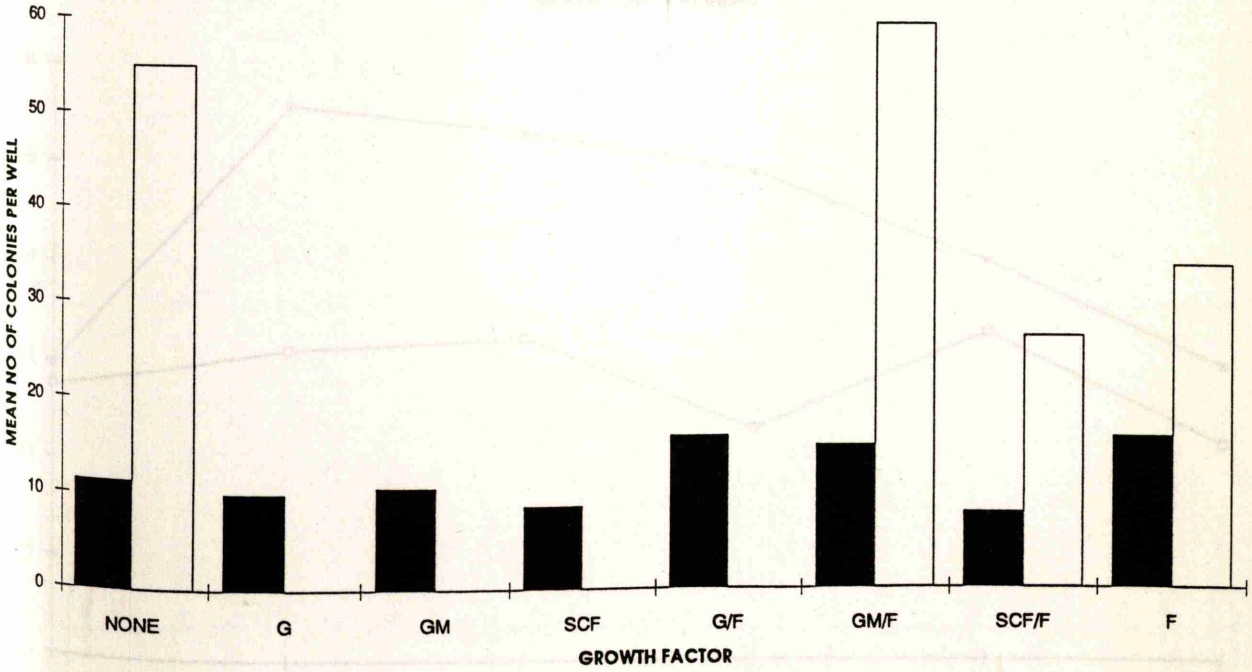
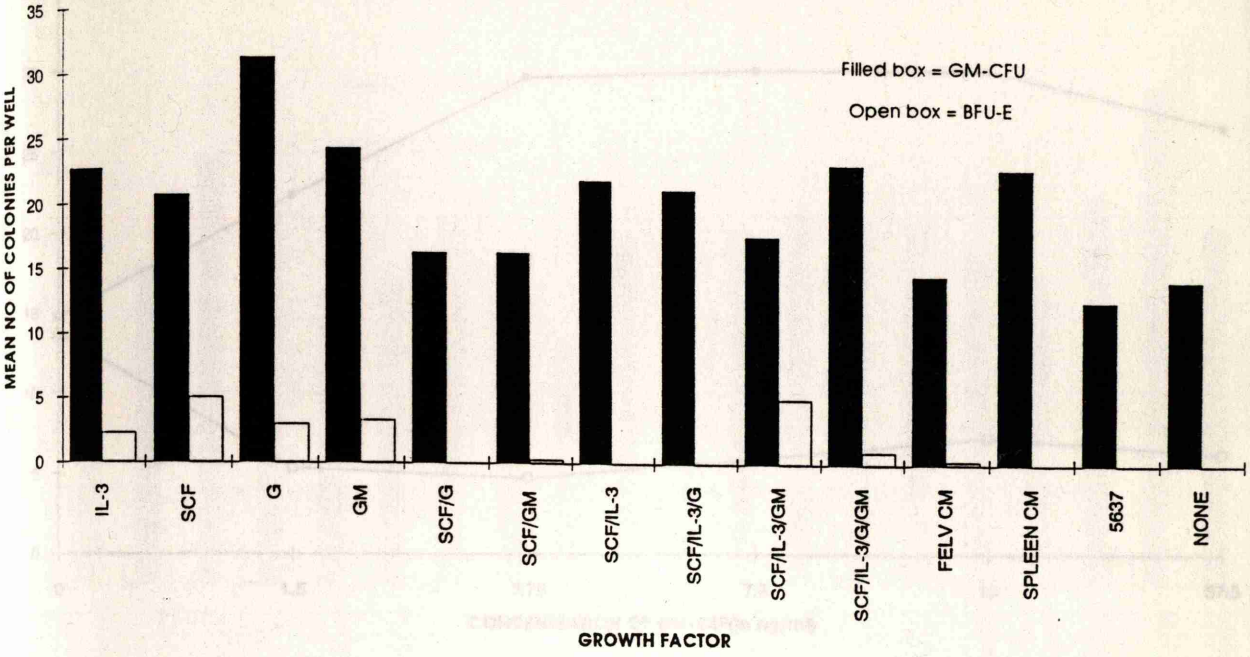


Fig. 4.15. Chart of mean number of colonies grown versus growth factor added (methylcellulose)



IL-3 = Interleukin-3
SCF = Stem Cell Factor
G = G-CSF
GM = GM-CSF
FELV CM = FeLV Conditioned Medium
SPLEEN CM = Spleen Conditioned Medium
5637 = Bladder Carcinoma Cell Line Medium

Fig. 4.16. Graph of mean number of colonies grown versus concentration of G-CSF added for 2 cats (GM-CFU in agar)

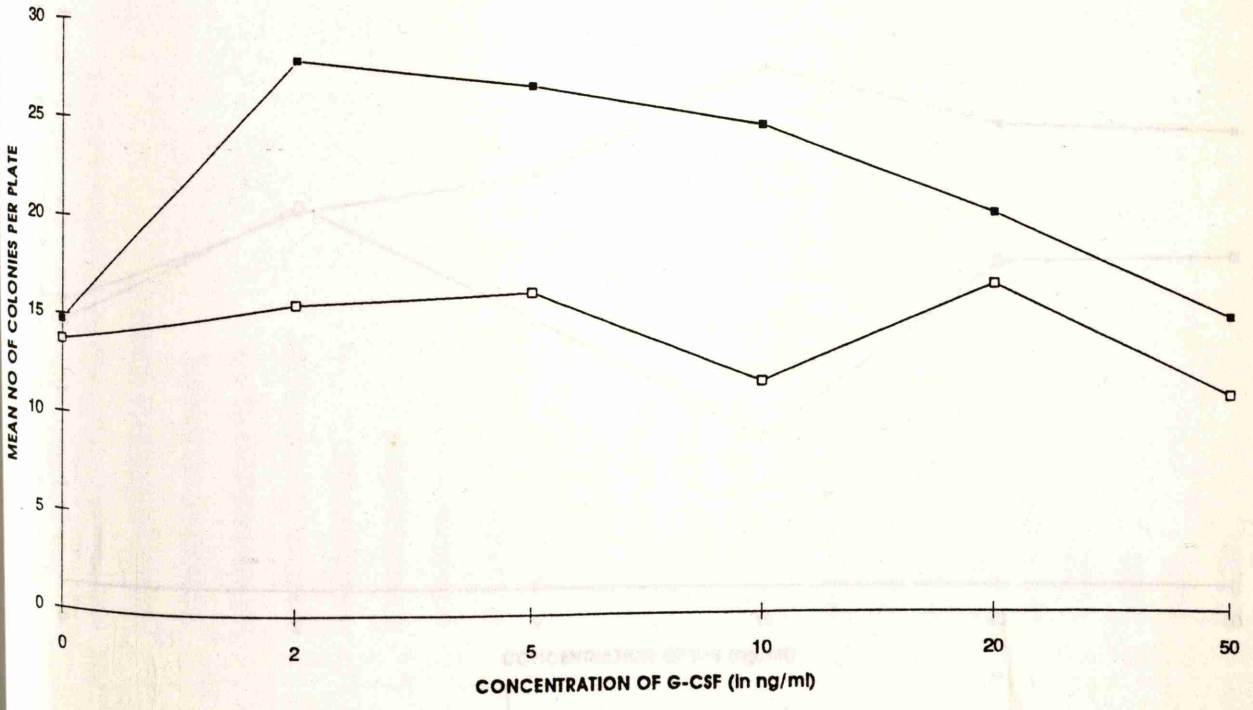


Fig. 4.17. Graph of mean number of colonies grown versus concentration of GM-CSF added for 2 cats (GM-CFU in agar)

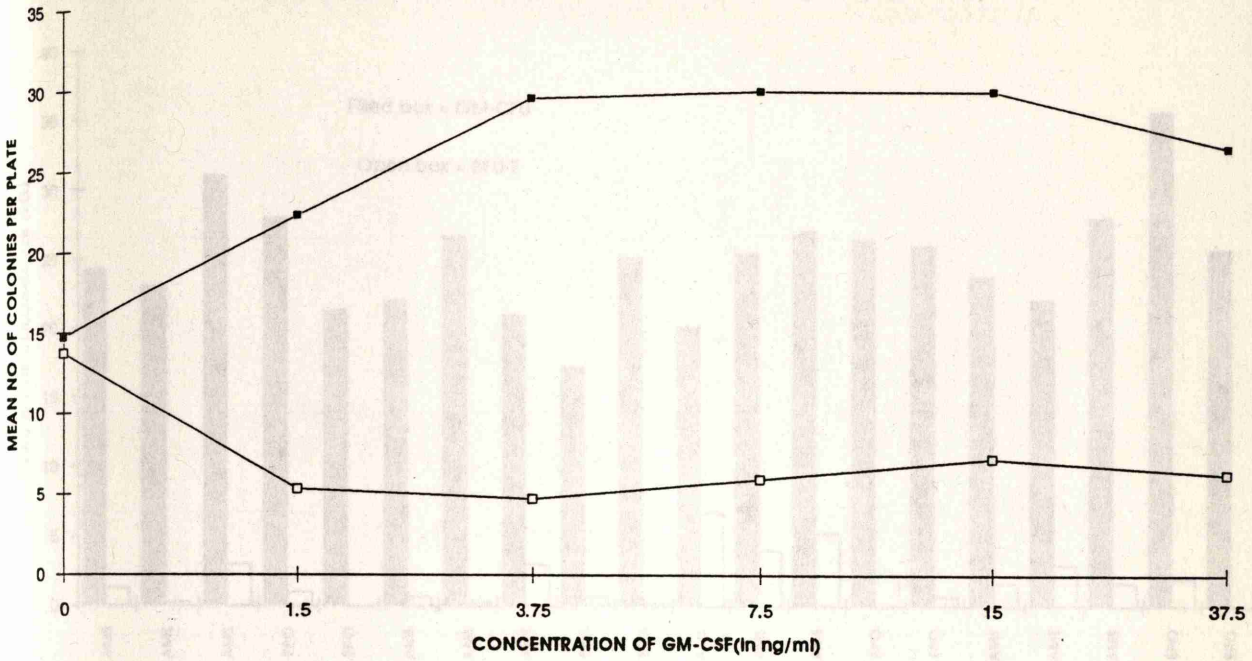


Fig. 4.18. Graph of mean number of colonies grown versus concentration of IL-3 added for 2 cats (GM-CFU in agar)

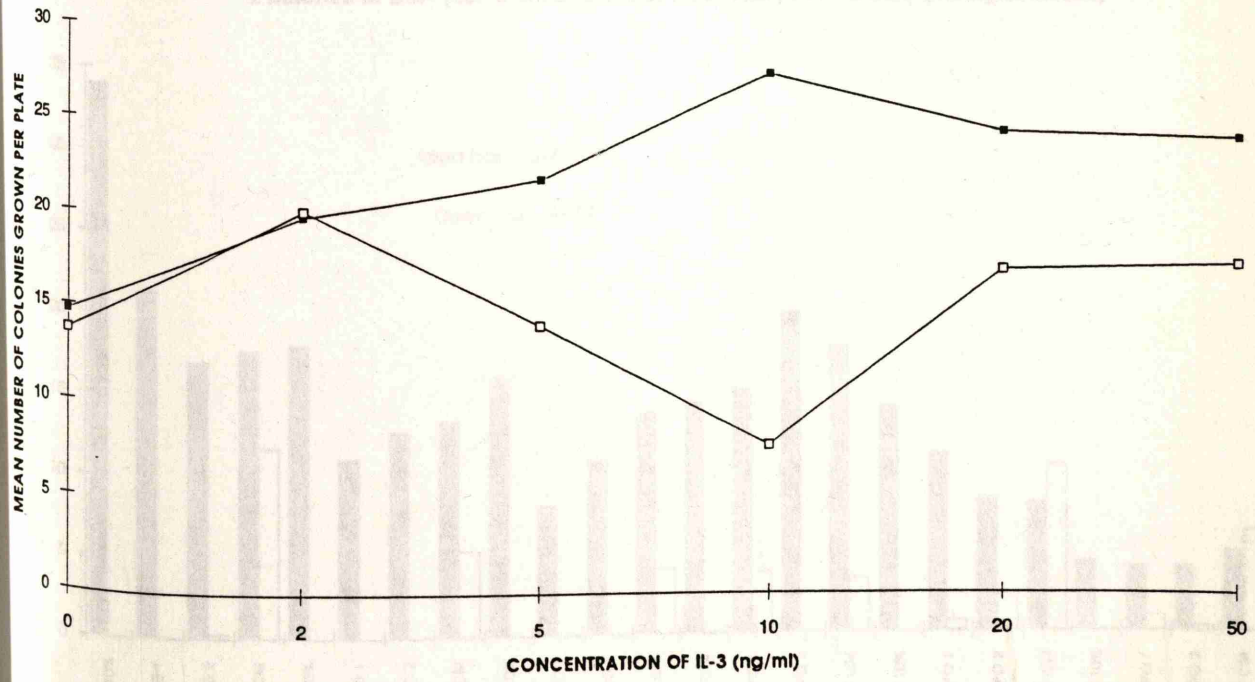


Fig. 4.19. Chart of mean number of colonies grown versus erythroid stimulant for 2 types of BSA (26F & BOE) with and without G-CSF (methylcellulose)

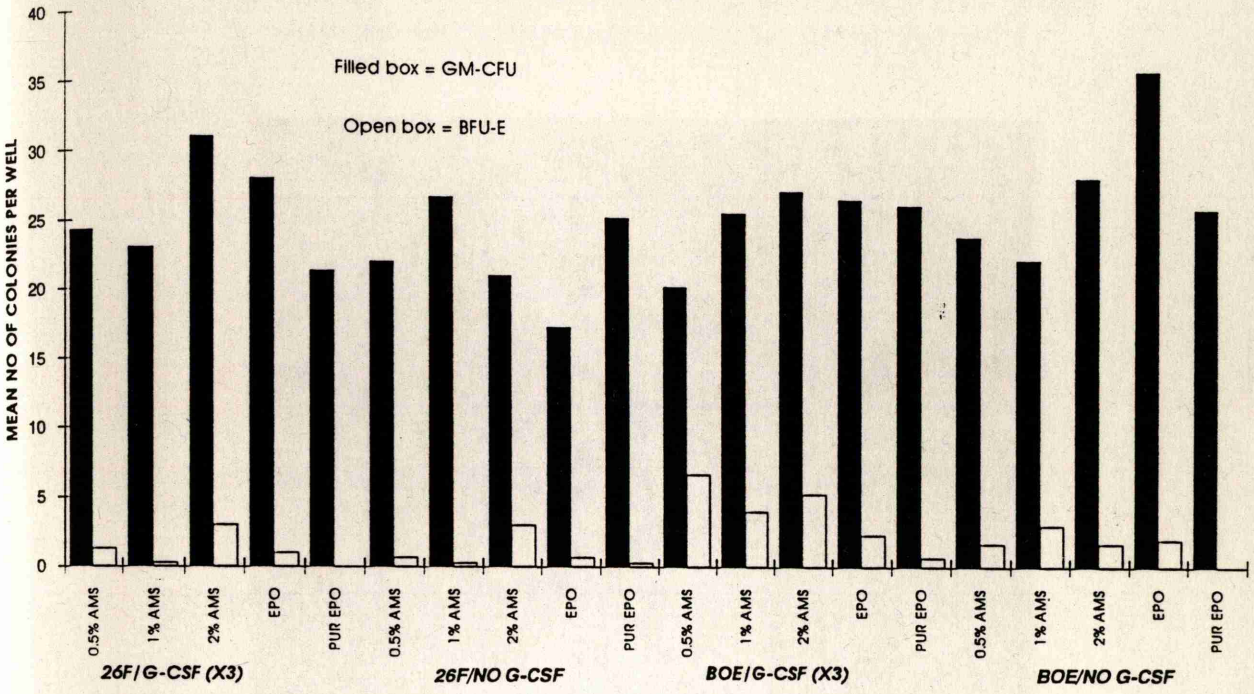


Fig. 4.20. Chart of mean number of colonies grown versus type of erythroid stimulant added for 2 batches of BSA (26F & BOE) and 3 of FCS (1026, 8851 & O24) (methylcellulose)

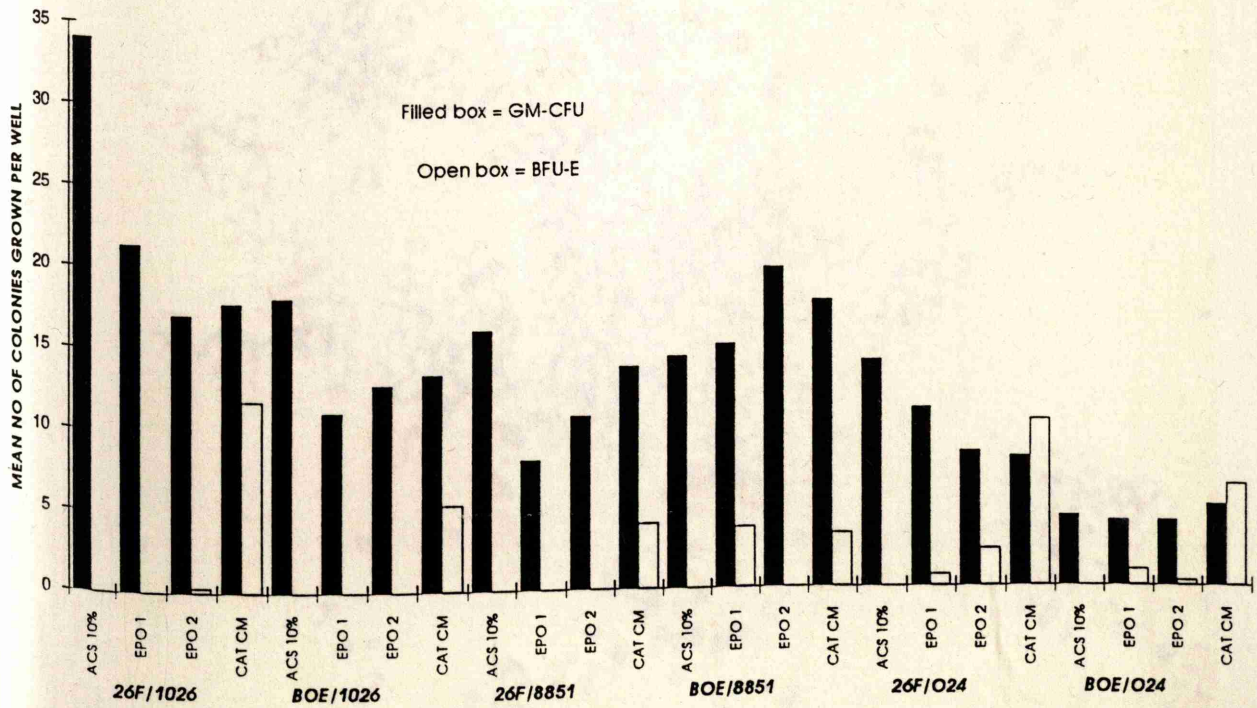


Fig. 4.21. CFU-G composed of small loosely-distributed cells

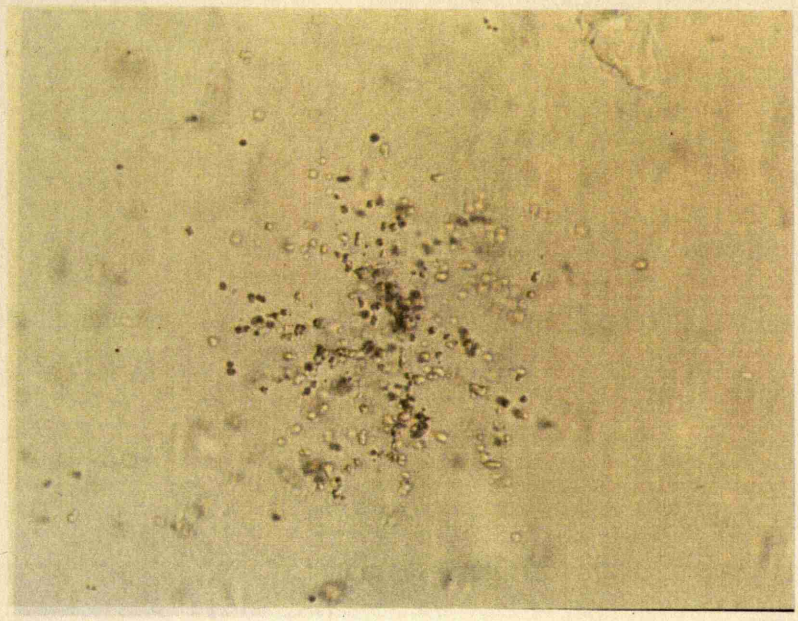


Fig. 4.22. CFU-M composed of large loosely-packed cells

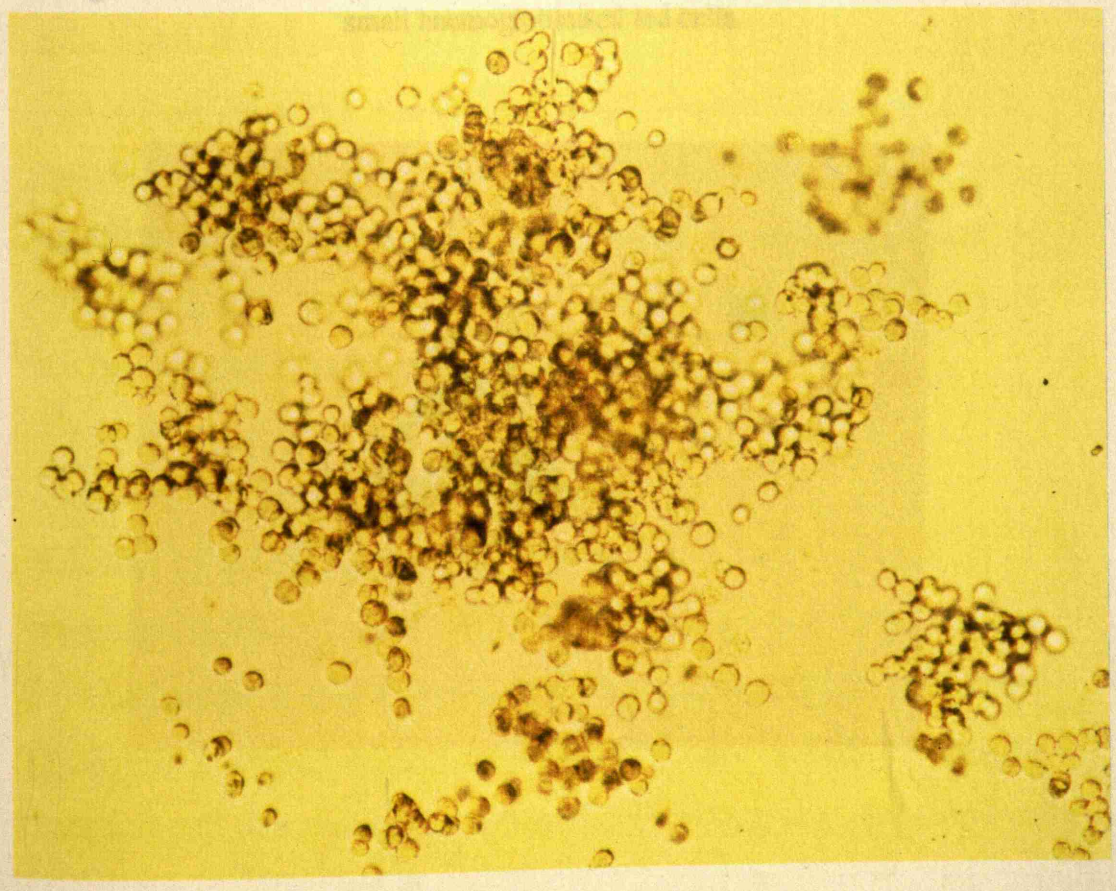


Fig. 4.23. CFU-GM composed of a central zone of large macrophages surrounded by a peripheral zone of small granulocytic cells

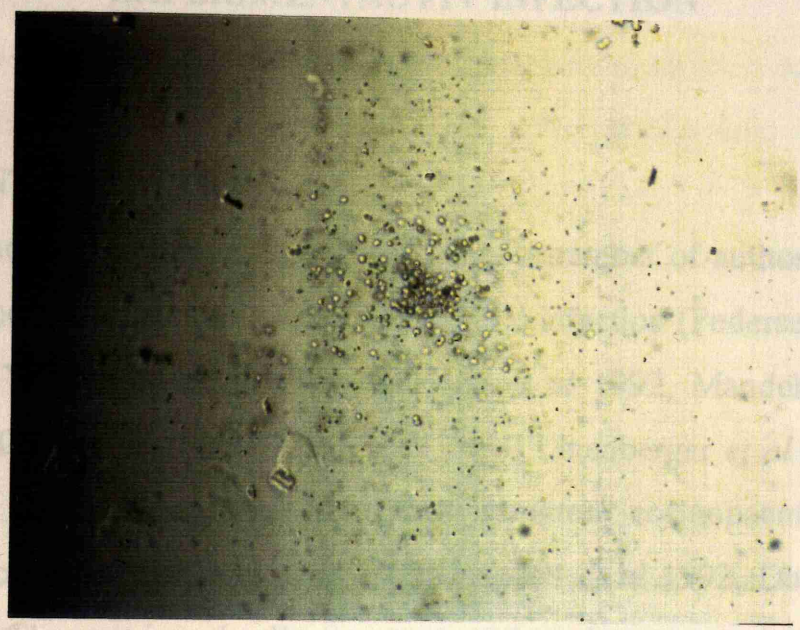
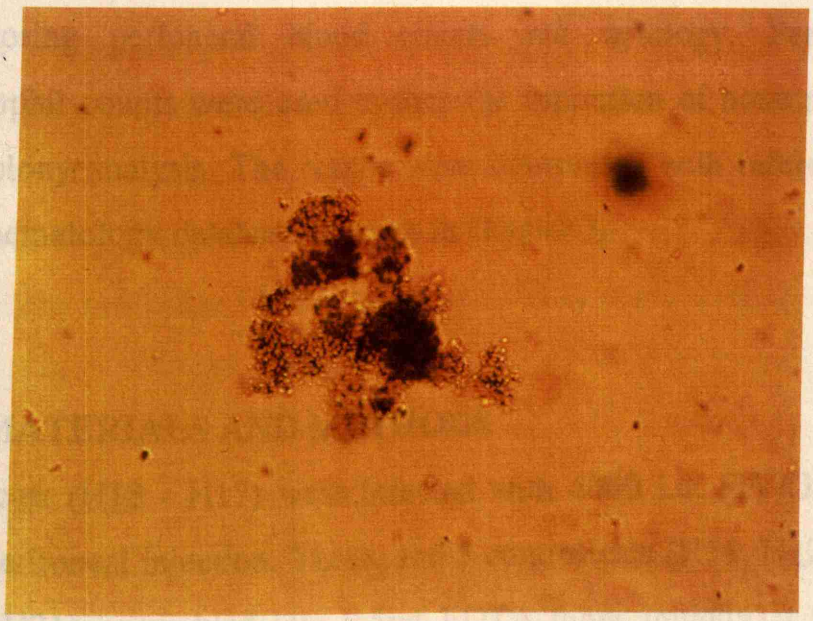


Fig. 4.24. BFU-E composed of several groups - "bursts" - of tightly-packed small haemoglobinised red cells.



CHAPTER 5
HAEMATOLOGICAL OBSERVATIONS IN EARLY EXPERIMENTAL FIV INFECTION

5.1. INTRODUCTION

As described in the general introduction, a number of authors have reported neutropaenia in experimental FIV infection (Pedersen *et al* 1987, Yamamoto *et al* 1988, Callanan *et al* 1992, Mandell *et al* 1992, George *et al* 1993, Dua *et al* 1994, Linenberger *et al* 1995). Some of these authors have also reported eosinopaenia and lymphopaenia (Callanan *et al* 1992, Mandell *et al* 1992, Dua *et al* 1994). Changes in red cell parameters or thrombocyte numbers have not been recorded in early infection. The pathogenesis of these cytopaenias has not yet been established.

5.2. AIM

The aim of this study was to analyse FIV-associated cytopaenias by monitoring peripheral blood counts and cytology. Peripheral neutrophil counts were used to time the aspiration of bone marrow for colony analysis. The results were interpreted with reference to the haematology database analysis in chapter 3.

5.3. MATERIALS AND METHODS

Five cats (H13 - H17) were infected with 4000 i.u. FIV/GL-8 by intraperitoneal injection. These, and 5 control cats (H11, H12, H18, H19, H21), were bled into 0.5ml EDTA tubes initially at weekly intervals (up to 4 weeks post-infection), then at 2 or 3 day intervals

(up to 13 weeks). The frequency of bleeding was then gradually reduced to fortnightly sampling by the end of the study period (27-29 weeks).

Full blood counts and differentials were performed on each sample, any abnormalities in morphology were noted at the time of the manual differential count.

Individual parameters e.g. red cell count, neutrophil count, etc. were compared between the infected and control groups by analysis of variance, and any significant differences were noted. Analysis of variance over time was performed to give 2 values, a group-time interaction and a group interaction. Groups were considered to be significantly different for $p < 0.05$. This was done using the Minitab (Minitab for Windows, Minitab Inc., State College, PA, 16001-3008, USA) statistical computer software. Only time points at which samples were available for all animals were used (16 time points: day 7, 14, 28, 40, 44, 47, 61, 68, 71, 75, 86, 90, 93, 118, 125 and 160) . Values at time points day 0, day 28, day 44, day 86 and day 160 p.i. were also compared between the groups for each parameter by 2 sample t-tests.

Platelets were not included as they were often clumped, making the machine counts inaccurate.

FIV antibody tests were performed at 6 weeks p.i. and 20 weeks p.i. to confirm infection. FIV virus isolation was carried out at 27-29 weeks p.i..

5.4. RESULTS

The sequential haematology results for the five infected cats and controls is represented in tables 5.1 to 5.10 and figures 5.1 to 5.12. Results of the statistical procedures for each parameter are shown in

table 5.11. Two sets of analysis of variance results are recorded. Significant group-time interactions indicate differences in the pattern of response between the two groups between time points. i.e. a lack of parallel response. Group differences signify a difference in the mean values between the two groups over the course of the experiment. These possible outcomes are illustrated in figure 5.13.

FIV antibody tests and virus isolation were positive in all infected cats and negative for all control animals.

5.4.1. Red Cell Parameters - Red cell parameters remained within traditional "normal" limits (5-10 x 10^{12}/l) for both control and infected cats throughout the course of the experiment (see fig. 5.1). These figures were within the control research cat percentile figures derived in chapter 3 (see table 3.1(a)). In both groups of animals nucleated red cells (normoblasts) were recorded, but there was no correlation with red cell counts. Normoblast numbers were low, and their appearance was occasional. Reticulocyte counts were not made.

The samples of cat H16 appeared to be autoagglutinating in the EDTA tube at around week 7 and 8, however a direct Coomb's test was negative on day 49.

Analysis of variance (ANOVA) for red cell numbers suggested there was a significant difference between the counts for the two groups over time (group-time $p = 0.022$), the infected group having slightly lower numbers than the controls (see table 5.11). There was no statistically significant difference in group mean values over time ($p = 0.126$). Figure 5.1 plots mean red cell counts for the infected and control animals over time. There appeared to be a drop in mean

red cell counts of infected cats between days 35 and 55. The mean counts did not, however drop below 5 x 10^{12}/l.

Analysis of variance also showed a significant difference between haemoglobin values (group-time p = 0.007) of infected and control cats (table 3.11). As for red cell numbers, the haemoglobin values for all animals in the study remained within the expected parameters as derived for haemoglobin concentration in chapter 3 (see table 3.2(a)) i.e. between 6.3 and 15.5g/dl. However, figure 5.2 shows that mean values in infected cats were lower than those of controls from approximately day 35 almost throughout the course of the study. The ANOVA group analysis confirmed the difference was significant (table 5.11, p = 0.029). Two-sample t-tests (table 5.11) resulted in significant differences between infected and control cats at days 44 (p = 0.019) and 86 (p=0.01) for haemoglobin concentration.

Group-time interactions for haematocrit (p = 0.064); mean cell volume (p = 0.758); mean cell haemoglobin (p = 0.972) and mean cell haemoglobin concentration (p = 0.261) were not significantly different by analysis of variance (see table 5.11). However, figures 5.3 to 5.6 demonstrate that mean values in infected cats were lower for all parameters in FIV-infected animals. The difference in values reached statistical significance for haematocrit (p = 0.03) and mean cell haemoglobin concentration (p < 0.000). Numbers were maintained within limits calculated by percentile analysis in chapter 3 (see tables 3.3 (a) to 3.6 (a)). Two sample t-tests (see table 5.11) showed that haematocrit levels in the infected animals were significantly less at days 44 (p = 0.02) and 86 (p = 0.012).

The infected cats were not anaemic and there was no evidence of a regenerative response.

5.4.2. White Cell Counts - Individual white cell parameters are considered separately below, but mean overall white cell counts are represented in fig. 5.7. The mean counts of the infected cats fell away dramatically after week 4 and remained below the control values for some weeks. The differences were significantly different by analysis of variance (group-time $p < 0.000$, group = 0.047) (see table 5.11). 2-sample t-tests demonstrate that the differences were significant at day 44 ($p = 0.028$). These differences can be better explained by examination of individual cell types, as demonstrated below.

5.4.3. Neutrophils - Neutrophil counts in the control cats remained above the traditional lower "normal" limit ($2.5 \times 10^9/l$) throughout the course of the experiment, in fact the lowest recorded count was $3.78 \times 10^9/l$. The percentile analysis of control research cats in table 3.8(a) revealed that neutrophil numbers in these animals were very variable, ranging from $1.344 \times 10^9/l$ to $31.031 \times 10^9/l$. Counts in the infected cats began at a level comparable with those of the control cats (see fig 5.8). By days 35 (H13), 47 (H14), 37 (H15), 33 (H16), and 40 (H17) neutrophil numbers in the infected cats had dropped to below $2.5 \times 10^9/l$, and in subsequent samples were often well below this figure. This was associated with an acute clinical illness in cats H13 and H16.

Cat H13 became depressed, pyrexic (40.8°C) and anorexic on day 37. The cat responded to antibiotic therapy (Amfipen LA, Mycofarm UK Ltd., Cambridge, UK.) over the next 3 days but had relapsed by day 44. Amoxycillin (Clamoxyl LA, SmithKline Beecham Animal Health, Tadworth, UK) was given on this occasion, and the clinical signs had resolved by day 47.

Cat H16 became dull and anorexic on day 37. Conjunctivitis and sneezing were also apparent, and the temperature was 40.8°C. Long-acting amoxycillin was given and the cat improved overnight. The clinical signs returned on day 40, the treatment was repeated and chloramphenicol eye ointment was administered (Chloromycetin, Parke, Davis and Co. Ltd., Pontypool, UK). The signs had resolved by day 42.

Neutrophil counts remained low for 43 days (H13), 10 days (H14), 34 days (H15), 25 days (H16) and <2 days (H17). There were no dramatic left shifts associated with the neutropaenia.

The presence of immature cells of the myeloid series was noted in infected cats. Metamyelocytes were recorded in cat H13 on days 35, 51, 57, 63, 68 and 71. Myelocytes were also found on days 51, 57 and 62. Metamyelocytes were also recorded in cat H15 on days 51, 65 and 71; with myelocytes on day 57. Cat H16 had metamyelocytes on day 44, 47, 56 and 61; and myelocytes on day 44, 47 and 50.

Cat H17 suffered an episode of severe leukopaenia on day 153, with a neutrophil count of 0.455 x 10^9/l. This was not associated with clinical signs, and had resolved by the next sampling.

Mean neutrophil counts of the control and infected groups of animals are represented in figure 5.8. A significant divergence in

the graphs was evident from around day 35. Prior to this, and after day 80, the form of the two graphs was similar, with fluctuations in the control group being largely mirrored in the infected cats.

Analysis of variance of the neutrophil data resulted in a group-time p-value < 0.000, suggesting there was a significant difference in the pattern of response between the groups (see table 5.11). The group ANOVA value was 0.029 (table 5.11), revealing a significant difference between the mean values for the two groups. 2 sample t-tests confirmed the differences at day 44 ($p = 0.0051$).

5.4.4. Lymphocytes - Lymphocyte counts in the control group remained above the lower "normal" limit ($1.5 \times 10^9/l$) as traditionally calculated for cats but numbers were frequently elevated above the accepted upper limit ($7 \times 10^9/l$). Figure 5.9 demonstrates that mean counts for the control group were often above $10 \times 10^9/l$. However, the percentile analysis of a comparable group of 40 SPF control research animals (see table 3.9(a)) revealed that lymphocyte counts in these animals were notably higher than that which would be expected from traditional standards, ranging from 2.04 up to $14.65 \times 10^9/l$. Cats H11 and H12 had counts which were often above $14.65 \times 10^9/l$, but the cells were morphologically normal. The high counts may have resulted from a physiological leucocytosis.

In the infected group, lymphocyte counts were within traditional normal limits throughout the course of the experiment, and also within the limits defined by the percentile analysis (table 3.9(a)). Cat H16 had low lymphocyte counts on days 40 ($1.122 \times 10^9/l$) and 47 ($0.994 \times 10^9/l$). Cat H17 suffered a lymphopaenia on day 153, the lymphocyte count being $0.465 \times 10^9/l$.

In all infected cats, especially from weeks 1 to 8, occasional activated lymphocytes, distinguished by their increased basophilic cytoplasmic staining and denser nuclear chromatin pattern, were noted on the blood smears. These were similar to cells described by Femenia *et al* (1994) in experimentally infected cats.

Mean lymphocyte counts in the two groups of cats are represented in fig. 5.9. There is no significant divergence of the two graphs.

Analysis of variance did however, suggest that lymphocyte responses were significantly different between control and infected animals (group-time $p < 0.000$). The results in table 5.11 however, also show that there was not a significant difference ($p = 0.377$) between the mean counts of infected and control cats over the course of the experiment.

5.4.5. Monocytes - Monocyte numbers in control cats were within expected values as calculated for research cats in chapter 3 (table 3.10(a)) i.e. up to 2.55 x 10^9/l. The upper limit is considerably above traditional limits (0-0.85 x 10^9/l) but must be considered "normal" for this group of cats.

Monocyte numbers in the infected group followed a similar pattern. The mean counts are represented on fig 5.10. There is no apparent significant difference between the mean values for the two sets of animals.

Statistical analysis confirmed there were no significant differences between the control and infected cats (table 5.11).

5.4.6. Eosinophils - Eosinophilia was a common finding in control cats, as compared to both percentile analysis (table 3.11(a)) and accepted "normal" ranges (0-1.5 x 10^9/l). Counts were persistently

high in some animals, and reached 4.179 x 10^9/l in one case. Eosinophil numbers were rarely below 0.3 x 10^9/l in this group.

In contrast, infected cats commonly had zero eosinophil counts, lowest numbers coinciding with low neutrophil counts. The analysis performed on 40 research cats in chapter 3 (table 3.11(a)) revealed that none of these animals had zero eosinophil counts, although this is not true of other groups of cats. The relative eosinopaenia broadly mirrored the severity and duration of neutropaenia.

Plotting the mean eosinophil counts demonstrated a pattern of divergence similar to that of neutrophil counts (see fig. 5.11). Whilst counts in the control group remained high, those in the infected group dropped from day 35.

Analysis of variance for eosinophil numbers yielded a p-value of 0.053 for the group-time interaction, which was not statistically significant. The group interaction by ANOVA however had a p-value = 0.007, indicating that the mean counts in infected animals were significantly different over the course of the study. Two sample t-tests suggested that there were significant differences between control and infected cats at days 44, 86 and 160 with p-values of 0.029, 0.0001 and 0.04 respectively (table 5.11).

5.4.7. Basophils - Low numbers of basophils were noted in both control and infected groups intermittently (tables 5.1 to 5.10). the Basophils were detected more often than would normally be expected, a fact possibly related to the elevated eosinophil numbers. Once again, the mean counts in the infected cats appeared to be lower than those in the uninfected group (fig. 5.12).

Analysis of variance confirmed that there was a significant difference in basophil count pattern over time (group-time p =

0.038). The difference in mean values between infected and control cats failed to reach statistical significance (group p = 0.058), nor were there significant differences by t-test at the specific time points investigated (table 5.11).

5.4.8. Platelets - Platelet numbers were difficult to gauge accurately using these low volume EDTA samples, numbers were often misleadingly low due to platelet aggregation. This was noted on smears. There was no evidence of thrombocytopaenia in either group of cats at any stage.

5.5. DISCUSSION

All the cats used in the study were 6 months or over at the point of infection. This eliminated age-related changes as a cause of variation in blood cell parameters.

The red cell parameters in the early stages of FIV infection have been reported to be within normal ranges by a number of authors (Yamamoto *et al* 1988, Callanan *et al* 1992, Mandell *et al* 1992, George *et al* 1993, Dua *et al* 1994, Linenberger *et al* 1995). This study supported these findings. However, despite the red cell numbers, haemoglobin, haematocrit and mean cell haemoglobin concentration remaining within the expected "normal" range for cats (see tables 5.1 to 5.10 and figs. 5.1 & 5.2), statistical analysis suggested that there were significant differences between the infected and control cats (table 5.11). Numbers were comparable at day 0, but numbers of red cells appeared to drop slightly in infected

cats by week 5 or 6 p.i.. This drop was not severe and there was no evidence of clinical anaemia or regeneration. It may be that there is a temporary slight compromise in red cell production in infected cats at this stage, leading to a small fall in red cell numbers and blood haemoglobin concentrations.

The agglutination observed in cat H16 and in one other cat independent of this study suggests some auto-immune mechanism acting at this stage. There was a negative result to the direct Coomb's test. Anti-erythrocyte antibodies can be detected in the serum of HIV-infected persons (Aboulafia & Mitsuyasu 1991, Calenda & Chermann 1992b, Kaczmamrski & Mufti 1993), but the presence of these has not been correlated with haemolysis in these patients. It is possible that these antibodies arise as a result of a non-specific response to a variety of antigens. Further investigation is required to elucidate what may be happening in these cats.
Normoblast release in both groups may have been associated with splenic contraction as a result of stress at the time of sampling.

Significant differences in overall white cell counts were observed between the two groups (table 5.11). These were most evident at 5-7 weeks post-infection (fig. 5.7) and were most closely related to neutrophil counts, although lymphocyte and eosinophil numbers were probably also contributory factors. White counts were significantly different both in their pattern of variation and in mean levels (table 5.11). That is, as well as having lower counts in the infected group, there was also a difference in the fluctuations in cell count over time. White cell numbers in the infected group did not drop concurrently with those in the control group, or vice versa.

This suggested that the events controlling white cell counts were related to virus infection, rather than incidental infections in the cathouse or random variation.

Neutrophil counts in the infected cats dropped dramatically in 3 animals - H13, H15 & H16 - and to the lower limit of the normal range in 2 - H14 & H17 (tables 5.1 to 5.5). As for total white cell counts there were significant differences in both pattern of neutrophil count fluctuations as well as mean numbers over time, strongly implying the differences were directly due to viral infection (table 5.11). This is consistent with the work of previous authors (Pedersen *et al* 1987, Yamamoto *et al* 1988, Callanan *et al* 1992, Mandell *et al* 1992, George *et al* 1993, Dua *et al* 1994, Linenberger *et al* 1995). Those cats which suffered the most dramatic fall in granulocyte numbers also took longer to recover. The two cats which became clinically ill were also those which had the most severe neutropaenias. Lack of band forms and the low numbers of immature precursor cells in the circulation suggested a blunted marrow response in relation to the degree of neutropaenia in these animals.

Previous authors have reported the disappearance of the reserve pool of mature neutrophils from the marrow at the time of neutropaenia (Callanan *et al* 1992, Mandell *et al* 1992). The marrows were reported to be hyperplastic, with an increase in the proportion of immature granulocyte precursors, suggesting a maturation arrest as a possible mechanism for the peripheral cytopaenia. Low numbers of immature neutrophil precursors, metamyelocytes and myelocytes, were found intermittently in the

circulation of three infected cats in this study. These were found in the animals with the most severe and prolonged neutropaenia, their early release possibly being an attempt to compensate for the lack of mature neutrophils. As numbers of mature neutrophils were restored the immature cells disappeared from the circulation.

Lymphocyte responses to early infection were limited in this study. A physiological lymphocytosis was noted in two control cats (H11 & H12, tables 5.6 & 5.7). A mild lymphopaenia was noted in one animal (H16, table 5.4), but was not sustained. Similar results were reported by Mandell *et al* (1992). Reactive lymphocytes were observed in all infected cats from as early as the first week after infection. These were involved in the immune response to the virus. Cat H17 suffered a lymphopaenia along with neutropaenia on day 153 p.i. (table 5.5). There were no clinical signs and all cell lines were within normal limits by the next sampling date.

Callanan *et al* (1992) and Dua *et al* (1994) have reported a mild lymphopaenia at 2-4 weeks post-infection. This was not recorded in these cats, but the phase may have been missed as samples were only being taken once weekly at this stage. The statistical analysis suggested there was a significant difference in the pattern of response between the two groups of cats over time ($p < 0.000$), (table 5.11). This difference was not evident at the time points tested by 2-sample t-tests, but there was a drop in the mean lymphocyte count of infected cats between day 14 and 28 (fig. 5.9). This was missed by the t-test at 0 and 28 days but contributed to the overall difference, the analysis of variance testing the differences between the two populations over the entire course of the

experiment, whilst the t-tests were confined to individual days. The fall at this time also correlated with the lymphopaenia recorded by the above authors. The physiological leucocytosis recorded in cats H11 and H12 may have contributed to the higher mean values of the control group.

However, George *et al* (1994) reported age related differences in the haematological response to experimental FIV infection. Total lymphocyte counts were unaffected in neonatal kittens and in aged cats, but a significant lymphopaenia was recorded in young adults. In all cases, there was a drop in CD4 numbers.

Monocyte numbers were similar in both groups of cats (fig. 5.10). No statistical differences were recorded by analysis of variance or by 2-sample t-tests. No other authors have recorded alterations in the monocyte population at this stage of FIV infection.

Eosinophilia was a consistent finding in the control cats in this study (tables 5.6 to 5.10 and fig 5.11). However, this had also been noted in other studies conducted using cats in this environment and may be explained by a parasitic infection in the cattery. In contrast, the infected cats had noticeably lower eosinophil counts. No eosinophils were counted in a large proportion of infected cat samples, particularly at the time of neutropaenia (tables 5.1 to 5.5 and fig 5.11). Although this was not significant in itself, given the elevated counts in the controls; the high numbers of eosinophils recorded in the percentile analysis of similar cats in chapter 3 (table 3.11(a)); and the statistically significant differences seen in the group interaction ($p=0.007$) by ANOVA and by t-tests at days 44 and 86 ($p = 0.029$ and 0.0001 respectively) (table 5.11.) there appeared to be a relative inhibition of eosinophil production in infected cats.

Mandell *et al* (1992) also noted a concurrent eosinopaenia in early FIV infection.

Basophils were detected surprisingly often in both control and infected groups (tables 5.1 to 5.10 and figure 5.12). This may have been related to the degree of eosinophilia in these cats. However, in the infected group, basophils were less commonly detected, particularly at the time of neutropaenia than at other times (figure 5.12). Comparing response patterns in infected and control groups indicated that there was a statistically significant difference between the two populations (table 5.11). It is possible that the relative loss of circulating basophils was related to reduction in both neutrophil and eosinophil production, although with cells rarely present in normal animals it is difficult to speculate.

These results suggested that in early FIV infection there was a compromise of neutrophil production in bone marrow, leading to a failure to replace the natural daily loss. There was also a concurrent eosinopaenia and possible relative lack of basophils. There may be a common marrow deficit leading to these temporary cytopaenias. It is possible that there was also compromise of erythrocyte and lymphocyte production, but due to the transience of the inhibition or compensatory mechanisms and the relatively long lifespan of these mature cells, deficits were not clinically significant or easily detected as cell numbers only fell slightly. Platelet numbers were difficult to evaluate in this experiment, but there was no evidence of thrombocytopaenia in either control or infected cats.
In order to investigate the pathogenesis of these circulatory deficits, investigation of haemopoiesis was necessary.

Table 5.1. Cat H13 Full Haematology Results

DAY NO	0	7	14	28	35	36	40	42	44	47	49
RBC	8.3	8.05	7.05	7.39	6.55	6.09	5.76	5.97	5.73	5.16	5.04
Hb	12.7	12.1	10.8	11.3	9.9	9.2	8.8	9	8.6	7.9	7.4
Hct	33.8	32.1	28	29.6	26.5	24.7	25.3	25.6	23.5	21.3	21
MCV	41	40	40	40	40	41	44	43	41	41	42
MCH	15.3	15	15.3	15.2	15.1	15.1	15.2	15	15	15.3	14.6
MCHC	37.5	37.6	38.5	38.1	37.3	37.2	34.7	35.1	36.5	37	35.2
WBC	27.5	20.6	19.1	15.9	6	4.8	12.6	9.1	5.4	5.4	3.7
NEUT	14.987	10.403	12.606	6.996	0.18	0.432	5.292	1.092	0.675	3.024	0.111
BAND	0.13	0	0	0.07	0.15	0.33	0.25	0	0.1	0	0
LYMPH	11	8.446	5.443	6.837	4.62	3.504	5.544	6.597	2.835	1.728	3.071
MONO	0.412	1.133	0.477	1.272	0.66	0.432	1.26	1.274	1.782	0.594	0.481
EOS	0.825	0.309	0.573	0.636	0.15	0.096	0.126	0.136	0	0.054	0.037
BAS	0.137	0.309	0	0.079	0.09	0	0	0	0	0	0

DAY NO	51	54	57	61	63	68	71	75	78	82	86	90
RBC	3.9	5.61	4.41	5.07	5.4	5.37	5.42	5.94	5.28	6.09	5.88	6.58
Hb	6	8.2	6.6	7.5	7.8	8.1	7.9	9.1	8.2	9.5	9	9.8
Hct	17	23.1	19.2	22.8	22.8	23.5	23.1	26	24.4	26.2	25.6	27.4
MCV	44	41	44	45	42	44	43	44	46	43	44	42
MCH	15.3	14.6	14.9	14.7	14.4	15	14.5	15.3	15.5	15.5	15.3	14.8
MCHC	35.2	35.4	34.3	32.8	34.2	34.4	34.1	35	33.6	36.2	35.1	35.7
WBC	9.4	6.5	7.4	9.7	5.9	6	5.9	8	12	8.5	9.7	7.1
NEUT	5.405	0.227	0.518	0.873	1.121	0.36	0.295	0.12	0.84	3.442	3.104	1.668
BAND	0.28	0.32	0	0.38	0.11	0.36	0.17	0	0.3	0.04	0	0.14
LYMPH	2.162	4.68	4.218	8.148	3.54	4.02	4.926	1.12	9.48	4.335	5.432	4.118
MONO	0.94	1.202	2.146	0.194	1.062	0.18	0.413	6.08	1.38	0.637	1.018	1.029
EOS	0.047	0	0.148	0	0	0	0.029	0.68	0	0	0.145	0.071
BAS	0	0	0	0	0	0	0	0	0	0	0	0

Table 5.1. Cat H13 Full Haematology Results (cont.)

DAY NO	93	97	100	103	106	114	118	125	132	139	146	160
RBC	6.12	6.49	6.85	6.76	6.33	8.27	7.59	8.02	8.62	7.22	7.31	7.75
Hb	8.9	9.3	9.9	10.1	9.2	11.6	10.8	11.5	12.1	9.9	9.8	11
Hct	25.7	27.7	28.5	29.2	27.1	32.9	32.6	33	35	28	28.3	31.1
MCV	42	43	42	43	43	40	43	41	41	39	39	40
MCH	14.5	14.3	14.4	14.9	14.5	14	14.2	14.3	14	13.7	13.4	14.1
MCHC	34.6	33.5	34.7	34.5	33.9	35.2	33.1	34.8	34.5	35.3	34.6	35.3
WBC	10.5	9.4	9.6	16.6	18.2	12.4	18.4	18.1	18.2	9.6	13.3	13.1
NEUT	3.675	2.256	4.128	4.731	8.099	4.092	5.704	7.421	6.916	3.984	6.118	5.567
BAND	0	0	0	0	0	0	0	0	0	0	0	0
LYMPH	5.985	6.486	4.416	10.209	8.281	7.068	11.316	8.688	9.1	5.04	5.918	6.615
MONO	0.525	0.376	0.864	1.079	1.365	0.744	1.104	0.814	1.638	0.192	0.864	0.786
EOS	0.315	0.282	0.144	0.581	0.273	0.372	0.184	0.995	0.546	0.384	0.332	0.131
BAS	0	0	0.048	0	0.182	0.062	0.092	0.181	0	0	0.066	0

DAY NO	167	181	204
RBC	7.28	7.74	6.29
Hb	10.5	10.9	8.5
Hct	29.5	31.7	25.3
MCV	41	41	40
MCH	14.4	14	13.5
MCHC	35.5	34.3	33.5
WBC	15.9	18.4	13.5
NEUT	7.314	8.096	6.682
BAND	0	0	0
LYMPH	6.996	9.016	6.007
MONO	0.318	0.46	0.135
EOS	1.113	0.736	0.675
BAS	0.159	0.092	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.2. Cat H14 Full Haematology Results

DAY NO		0	7	14	21	28	35	37	40	42	44
RBC		8.46	8.68	8.08	7.42	8.68	9.06	9.09	8.18	8.3	8.37
Hb		12.2	12.5	11.9	10.4	12.5	12.8	12.7	11.4	11.8	11.9
Hct		32.9	33.3	31.1	28.7	32.7	34.4	35	32.1	31.7	32.1
MCV		39	38	38	39	38	38	39	39	38	38
MCH		14.4	14.4	14.7	14	14.4	14.1	13.9	13.9	14.2	14.2
MCHC		37	37.5	38.2	36.2	38.2	37.2	36.2	35.5	37.2	37
WBC		21.2	19.9	17.5	11.1	13.7	15.4	10.8	10.9	12.4	8.8
NEUT		10.494	9.651	11.462	6.826	7.398	6.93	3.564	4.36	4.216	2.816
BAND		0.1	0.29	0	0.5	0.06	0.3	0	0	0.12	0
LYMPH		8.268	7.86	4.55	3.441	4.932	6.468	5.994	5.341	7.068	5.632
MONO		0.636	0.398	0.175	0.166	0.205	1.001	0.486	0.545	0.62	0.22
EOS		1.696	1.592	1.137	0.61	1.027	0.616	0.648	0.599	0.248	0.132
BAS		0	0.099	0.175	0	0.068	0.077	0.108	0.054	0.124	0

DAY NO	47	49	54	57	61	63	65	68	71	75	78
RBC	8.34	8.37	8.62	7.75	9.09	8.96	9.37	8.99	8.93	9.06	8.99
Hb	12	11.6	12	11	12.9	12.6	12.9	12.7	12.4	12.8	12.9
Hct	32	32	32.9	30.1	35.3	34.1	35.3	34.5	34.1	34.5	34.4
MCV	38	38	38	39	39	38	38	38	38	38	38
MCH	14.3	13.8	13.9	14.1	14.1	14	13.7	14.1	13.8	14.1	14.3
MCHC	37.5	36.2	36.4	36.5	36.5	36.9	36.5	36.8	36.3	37.1	37.5
WBC	10	8.6	10.3	11.1	14.1	9.3	11	11.1	10.3	10.6	12.9
NEUT	2.25	2.709	2.575	2.553	5.781	3.115	3.63	3.94	3.862	4.929	4.902
BAND	0	0	0.1	0	0	0	0	0	0.05	0.05	0
LYMPH	7.25	5.375	6.695	8.103	7.966	5.905	5.445	6.438	5.356	5.247	7.288
MONO	0.1	0.215	0.566	0.333	0	0.279	0.22	0.388	0.618	0.318	0.322
EOS	0.35	0.215	0	0.111	0.352	0	0.165	0.333	0.257	0.053	0.387
BAS	0.05	0.43	0	0	0	0	0	0	0.051	0	0

Table 5.2. Cat H14 Full Haematology Results (cont.)

DAY NO	82	86	90	93	97	100	103	106	111	118	125
RBC	9.37	8.24	8.06	8.83	8.18	8.77	9.34	8.71	9.09	8.37	8.96
Hb	13.5	11.5	11.4	12.3	11.7	12.5	13.6	11.9	12.5	11.7	12.8
Hct	35.3	31.4	30.5	33.9	31.7	33.7	35.6	33.2	34.8	32.6	34.5
MCV	38	38	38	38	39	38	38	38	38	39	39
MCH	14.4	13.9	14.1	13.9	14.3	14.2	14.5	13.6	13.7	13.9	14.2
MCHC	38.2	36.6	37.3	36.2	36.9	37	38.2	35.8	35.9	35.8	37.1
WBC	11.5	15.2	10.9	12.5	11	11.5	13.5	13.5	15.8	12.5	15.3
NEUT	6.095	6.916	4.687	5.562	4.18	4.715	5.197	9.855	7.031	5.75	8.109
BAND	0	0	0.05	0	0	0	0	0	0	0	0
LYMPH	4.6	7.6	5.014	6.375	6.38	6.037	7.425	2.835	7.584	5.812	6.273
MONO	0.575	0.228	0.763	0.187	0.22	0.345	0.337	0.405	0.553	0.187	0.229
EOS	0.23	0	0.381	0.375	0.22	0.402	0.54	0.405	0.553	0.75	0.688
BAS	0	0.456	0	0	0	0	0	0	0.079	0	0

DAY NO	132	139	146	160	167	181	204
RBC	8.24	8.34	8.59	8.59	8.24	8.17	6.73
Hb	11.4	11.6	12.1	12.1	11.8	11.4	8.9
Hct	32	32	33.5	33.5	31.7	31.1	25.3
MCV	39	38	39	39	38	38	38
MCH	13.8	13.9	14	14	14.3	13.9	13.2
MCHC	35.6	36.2	36.1	36.1	37.2	36.6	35.1
WBC	12.5	12.1	12.1	12.1	14.2	13	7.5
NEUT	4.312	6.413	5.445	5.082	6.887	7.15	5.362
BAND	0	0	0	0	0	0	0
LYMPH	6.562	4.779	5.445	6.171	5.68	4.355	1.725
MONO	0.25	0.181	0.363	0.363	0.497	0.39	0.112
EOS	1.375	0.665	0.847	0.484	0.994	1.105	0.3
BAS	0	0.06	0	0	0.142	0	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.3. Cat H15 Full Haematology Results

DAY NO	0	7	14	21	28	35	37	40	41	44	47
RBC	8.34	8.27	7.8	7.24	8.3	8.02	7.5	7.62	6.91	7.1	7.16
Hb	13.6	13.2	12.9	11.1	13.3	12.7	11.8	12.3	10.5	11.3	11.3
Hct	35.6	35.4	33.2	30.6	34.8	34.1	32.3	32.7	29.8	30.6	30.8
MCV	43	43	43	42	42	43	43	43	43	43	43
MCH	16.3	15.9	16.5	15.3	16	15.8	15.7	16.1	15.1	15.9	15.7
MCHC	38.2	37.2	38.8	36.2	38.2	37.2	36.5	37.6	35.2	36.9	36.6
WBC	24.9	23.6	17.9	13.5	18.4	10.9	12.4	9	6.3	8.3	10.3
NEUT	12.823	8.496	13.783	6.615	10.58	3.651	2.48	1.125	0.504	1.701	0.412
BAND	0.12	0.11	0	0.06	0.09	0	0	0	0	0	0
LYMPH	8.466	11.328	2.774	5.197	5.888	6.376	9.176	6.93	5.229	5.851	8.755
MONO	1.245	0.944	0.268	0.54	0.736	0.218	0.496	0.45	0.283	0.622	1.03
EOS	1.743	2.478	1.074	0.877	0.92	0.654	0.186	0.495	0.252	0.124	0
BAS	0.249	0.236	0	0.202	0.184	0	0.062	0	0.031	0	0.103

DAY NO	49	51	54	57	61	63	65	68	71	75	78	82
RBC	7.53	6.45	6.73	7.68	7.62	6.91	7.13	6.7	6.52	7.53	7.28	7.16
Hb	11.9	10.1	10.7	12.4	11.8	10.5	11.3	10.5	9.9	11.6	11.5	11.2
Hct	32	28.3	29.2	33.8	33.8	29.5	29.5	29	28	32.7	31.4	30.5
MCV	42	44	43	44	44	43	41	43	43	43	43	43
MCH	15.8	15.6	15.8	16.1	15.4	15.1	15.8	15.6	15.1	15.4	15.7	15.6
MCHC	37.1	35.6	36.6	36.6	34.9	35.5	38.3	36.2	35.3	35.4	36.6	36.7
WBC	5.5	6.7	7.2	9.1	11.3	6.7	10.5	11.1	5.3	12.2	18.6	7.9
NEUT	0.165	1.038	0.288	1.41	2.542	0.134	1.732	3.774	0.106	3.05	7.44	1.066
BAND	0	0.23	0.03	0	0.11	0.2	0	0	0.05	0	0	0
LYMPH	4.785	3.986	6.336	4.14	7.74	5.293	6.037	6.216	4.505	7.747	9.951	5.451
MONO	0.412	0.703	0.432	1.092	0.169	0.837	1.207	0.666	0.503	1.22	1.116	1.145
EOS	0.11	0.1	0.036	0.136	0.169	0.033	0.157	0.333	0.106	0.183	0.093	0.079
BAS	0.027	0.033	0	0	0.113	0	0	0	0	0	0	0.039

Table 5.3. Cat H15 Full Haematology Results (cont.)

DAY NO	86	90	93	97	100	103	106	114	118	125	132	139
RBC	6.88	5.42	6.98	6.79	6.98	6.79	6.91	7.38	7.71	8.06	7.31	8.24
Hb	10.5	8.4	10.5	10.5	10.8	10.6	11.2	11	11.6	12	10.9	12.2
Hct	29.5	23.4	29.6	28.9	29.7	28.9	29.2	30.8	32.3	33.3	29.8	33.8
MCV	43	43	42	43	43	43	42	42	42	41	41	41
MCH	15.2	15.4	15	15.4	15.4	15.6	16.2	14.9	15	14.8	14.9	14.8
MCHC	35.5	35.8	35.4	36.3	36.3	36.6	38.3	35.7	35.9	36	36.5	36
WBC	17.9	10.9	11.4	14.4	15	13.4	11.1	14.5	13.8	11	11.8	13.9
NEUT	9.129	3.924	2.622	5.688	6.6	5.628	4.773	4.785	5.382	5.005	3.953	5.49
BAND	0	0	0	0	0	0	0	0.07	0	0	0	0
LYMPH	7.876	6.104	7.866	7.776	6.9	6.968	6.049	8.917	7.59	4.84	6.962	6.95
MONO	0.805	0.49	0.798	0.576	1.125	0.134	0.222	0.29	0.69	0.77	0.295	1.32
EOS	0.089	0.218	0.114	0.36	0.3	0.67	0.055	0.435	0.138	0.385	0.59	0.139
BAS	0	0	0	0	0.075	0	0	0	0	0	0	0

DAY NO	146	160	167	181	202
RBC	7.75	7.96	7.28	8.11	6.74
Hb	11.3	11.8	10.8	11.7	10
Hct	32	32.9	29.2	32.6	27.7
MCV	41	41	40	40	41
MCH	14.5	14.8	14.8	14.4	14.8
MCHC	35.3	35.8	36.9	35.8	36.1
WBC	12.9	12.8	14.3	15.9	9.4
NEUT	4.708	3.776	4.79	6.28	2.961
BAND	0	0	0	0	0
LYMPH	7.998	8.384	9.08	9.063	5.969
MONO	0	0.32	0.143	0.159	0.282
EOS	0.193	0.32	0.286	0.397	0.188
BAS	0	0	0	0	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.4. Cat H16 Full Haematology Results

DAY NO		0	7	14	21	28	30	33	34	37	40	42
RBC		9.63	8.33	9.06	8.2	8.74	8.12	8.68	7.59	7.59	7.31	7.62
Hb		14.8	12.9	12.9	12.5	13.1	12.4	13.6	11.3	11.4	10.9	11.2
Hct		38.5	32.6	34.8	32.1	34.7	34.1	35.1	30.5	30.6	29.8	30.8
MCV		40	39	38	39	40	42	40	40	40	41	40
MCH		15.3	15.4	14.2	15.2	14.9	15.2	15.6	14.8	15	14.9	14.6
MCHC		38.4	39.5	37	38.9	37.7	36.3	38.7	37	37.2	36.5	36.3
WBC		28.8	27.3	19.3	21.7	17.6	17.7	9.8	7.1	5.7	4.4	5.3
NEUT		14.976	12.967	11.29	10.416	9.68	8.053	1.274	1.029	1.254	1.584	0.795
BAND		0	0	0.38	0.21	0	0	0	0	0	0	0
LYMPH		9.936	12.285	5.693	8.246	6.072	8.584	6.762	5.36	3.705	1.122	2.703
MONO		0.72	0.273	0.675	1.519	0.616	0.531	0.833	0.39	0.342	0.638	0.371
EOS		1.296	1.774	0.965	1.193	1.056	0.531	0.882	0.319	0.057	0.066	0.159
BAS		0.432	0	0.193	0.108	0.176	0	0.049	0	0	0	0

DAY NO	44	47	50	54	56	58	61	64	68	71	75	79
RBC	6.61	6.82	6.49	7.25	7.96	7.16	7.1	7.13	6.33	6.85	7.68	7.31
Hb	9.6	9.9	9.9	10.9	11.6	10.7	10.4	10.2	9.6	10.6	11.9	10.8
Hct	28	28.3	28	31.1	32.3	29.8	28.4	28.9	27.2	30.5	32	29.5
MCV	42	41	43	43	41	42	40	41	43	45	42	40
MCH	14.5	14.5	15.2	15	14.5	14.9	14.6	14.3	15.1	15.4	15.4	14.7
MCHC	34.2	34.9	35.3	35	35.9	35.9	36.6	35.2	35.2	34.7	37.1	36.6
WBC	8.4	3.9	8	7.9	3.6	8.2	5.9	6	9.7	13.7	13.1	6.3
NEUT	0.672	0.819	1.2	1.106	0.342	2.337	3.186	2.88	4.074	7.124	6.812	2.331
BAND	0.63	0.13	0	0.15	0.03	0.08	0	0.06	0	0	0	0
LYMPH	3.15	0.994	5.44	5.767	2.538	4.633	2.035	1.8	4.462	5.959	4.978	3.559
MONO	1.092	0.955	1.08	0.316	0.396	0.984	0.324	0.81	0.921	0.616	1.048	0.378
EOS	0.588	0.058	0.16	0	0	0	0.059	0	0	0	0	0.031
BAS	0.042	0	0	0	0	0.082	0	0	0	0	0	0

Table 5.4. Cat H16 Full Haematology Results (cont.)

DAY NO	83	86	90	93	96	99	104	111	118	125	132	139
RBC	7.28	7.35	7.04	7.81	7.53	7.1	7.31	8.21	8.12	7.75	7.75	6.82
Hb	10.9	11.2	10.4	11.6	11.5	10.5	10.9	11.7	12.1	11.4	11.4	10
Hct	29.8	30.9	30.1	31.6	31.4	29.5	30.2	34.1	33.6	33.2	31.1	29.2
MCV	41	42	43	40	42	42	41	42	41	43	40	43
MCH	14.9	15.2	14.7	14.8	15.2	14.7	14.9	14.2	14.9	14.7	14.7	14.6
MCHC	36.5	36.2	34.5	36.7	36.6	35.5	36	34.3	36	34.3	36.6	34.2
WBC	7.6	10.9	13.6	7.2	12.9	12.7	16.3	17.2	18.2	22.7	15.4	15.1
NEUT	3.116	3.76	5.1	3.06	5.289	6.731	7.824	9.976	8.372	11.35	10.549	5.813
BAND	0.07	0	0	0	0	0	0	0	0	0	0	0
LYMPH	3.496	5.94	7.14	3.744	6.966	4.318	7.987	6.278	8.827	10.555	4.081	7.701
MONO	0.608	0.981	1.02	0.36	0.451	1.397	0.326	0.688	0.637	0.567	0.308	0.981
EOS	0	0.054	0.34	0.036	0.129	0.19	0.163	0.258	0.273	0.227	0.462	0.377
BAS	0	0	0	0	0.064	0	0	0	0.091	0	0	0.075

DAY NO	153	160	174
RBC	6.79	6.52	7.42
Hb	10.2	9.9	11.4
Hct	28.3	28.3	31.1
MCV	42	43	42
MCH	15	15.1	15.3
MCHC	36	34.9	36.6
WBC	8.3	18.2	22.1
NEUT	4.606	9.373	11.16
BAND	0	0	0
LYMPH	3.237	7.462	9.613
MONO	0.415	1.274	0.994
EOS	0.041	0.091	0.331
BAS	0	0	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.5. Cat H17 Full Haematology Results

DAY NO	0	7	14	21	28	30	33	35	37	40	42
RBC	9.25	7.39	8.39	8.05	8.65	7.99	8.43	7.59	9.09	7.78	8.3
Hb	13.3	11	12.8	11.9	12.1	11.6	12.2	11	13	10.9	11.6
Hct	35.8	28.9	33.3	31.2	33.2	32.9	32.7	30.1	34.8	29.5	33
MCV	39	39	40	39	38	41	39	40	38	38	40
MCH	14.3	14.8	15.2	14.7	13.9	14.5	14.4	14.4	14.3	14	13.9
MCHC	37.1	38	38.4	38.1	36.4	35.2	37.3	36.5	37.3	36.9	35.1
WBC	24.2	16	22.4	16	20.3	20.8	15.1	12.9	11.4	8.4	12.3
NEUT	14.52	7.36	17.024	8.4	12.281	12.376	7.172	5.482	4.047	2.016	5.781
BAND	0	0	0	0.08	0.1	0	0	0.12	0	0	0
LYMPH	7.986	7.44	3.248	5.76	6.09	6.968	6.191	5.611	5.7	5.544	5.166
MONO	0.484	0	0.56	0.88	1.116	1.144	0.906	1.032	1.083	0.504	1.107
EOS	0.968	0.96	1.568	0.8	0.71	0.104	0.755	0.645	0.57	0.252	0.184
BAS	0.242	0.24	0	0.08	0	0.208	0	0	0	0.084	0.061

DAY NO	44	47	50	54	56	58	61	64	68	71	75	79
RBC	7.81	7.56	7.56	8.21	8.34	8.21	7.99	8.18	8.3	8.24	9.02	8.43
Hb	11	10.5	10.7	11.6	11.5	11.5	10.9	10.9	11.4	11.3	12.8	11.1
Hct	31.4	28.9	30.1	33.5	32.6	32.9	30.2	30.8	32.7	31.4	34.7	31.4
MCV	40	38	40	41	39	40	38	38	39	38	38	37
MCH	14	13.8	14.1	14.1	13.7	14	13.6	13.3	13.7	13.7	14.1	13.1
MCHC	35	36.3	35.5	34.6	35.2	34.9	36	35.3	34.8	35.9	36.8	35.3
WBC	14.2	7.4	16	16.3	14.8	17.6	10.2	9.3	16.7	12.7	18.6	11.1
NEUT	4.899	2.035	6.8	3.993	4.144	6.424	4.131	3.906	6.179	6.731	6.324	4.329
BAND	0.07	0.07	0.16	0.32	0	0	0.2	0.04	0.08	0	0	0
LYMPH	7.455	4.847	7.84	11.084	9.472	10.208	5.049	4.743	9.101	5.715	10.044	5.772
MONO	1.278	0.407	0.8	0.081	1.184	0.792	0.612	0.372	1.002	0.254	1.302	0.888
EOS	0.497	0.037	0	0	0	0.176	0.204	0.186	0.334	0	0.93	0
BAS	0	0	0	0	0	0	0	0.046	0	0	0	0.111

Table 5.5. Cat H17 Full Haematology Results (cont.)

DAY NO	83	86	90	93	96	99	104	111	118	125	132	139
RBC	7.96	7.87	7.78	7.56	7.5	7.35	7.47	7.31	8.52	8.09	8.15	7.56
Hb	10.7	10.9	10.6	10.4	10.7	10.1	10.4	10.2	12.2	10.9	11.4	10.3
Hct	29.5	31.5	31.4	30.4	29.8	29.8	30.2	29.5	33	30.5	31.7	29.8
MCV	37	40	40	40	40	41	40	40	39	38	39	39
MCH	13.4	13.8	13.6	13.7	14.2	13.7	13.9	13.9	14.3	13.4	13.9	13.6
MCHC	36.2	34.6	33.7	34.2	35.9	33.8	34.4	34.5	36.9	35.7	35.9	34.5
WBC	10.2	20.5	19.5	18	20.4	17.5	17.8	13.5	15.9	12.3	13.9	12.1
NEUT	3.417	8.405	7.02	7.11	7.14	5.862	5.607	4.522	6.042	4.735	6.463	6.473
BAND	0.15	0.3	0	0	0	0	0	0	0	0	0	0
LYMPH	5.559	10.455	11.31	9.45	12.138	10.237	11.481	7.897	8.665	5.842	6.602	4.658
MONO	0.816	1.23	0.78	1.08	0.714	1.137	0.623	0.877	0.954	0.246	0.556	0.363
EOS	0.051	0.102	0.39	0.36	0.408	0.612	0.089	0.202	0.238	1.476	0.208	0.605
BAS	0	0	0	0	0	0	0	0	0	0	0	0

DAY NO	153	160	174	188
RBC	8.71	8.27	8.17	6.96
Hb	12.2	11.8	11.1	9.3
Hct	33.8	32.9	30.5	26.5
MCV	39	40	37	38
MCH	14	14.2	13.5	13.3
MCHC	36	35.8	36.3	35
WBC	1	18.3	21.1	14.2
NEUT	0.455	8.235	12.238	8.66
BAND	0	0	0.21	0
LYMPH	0.465	8.967	6.752	5.25
MONO	0.045	0.183	1.055	0
EOS	0.035	0.915	0.844	0.28
BAS	0	0	0	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.6. Cat H11 Full Haematology Results

DAY NO	0	7	14	28	30	33	34	37	40	42	44
RBC	7.67	7.74	7.98	8.93	7.31	7.5	7.9	8.43	7.87	7.87	8.02
Hb	11.9	11.9	12.4	13.1	11.1	11.5	11.8	12.4	11.7	11.5	12.3
Hct	33.6	32	34.5	36.6	32.6	32.7	34.7	34.8	32.3	32.6	35
MCV	44	41	43	41	45	44	44	41	41	41	44
MCH	15.5	15.3	15.5	14.6	15.1	15.3	14.9	14.7	14.8	14.6	15.3
MCHC	35.4	37.1	35.9	35.7	34	35.1	34	35.6	36.2	35.2	35.1
WBC	25.1	24.9	28.5	18	23.5	27.1	22.2	24.1	18.9	22.1	31.2
NEUT	13.93	12.699	11.15	9.99	8.695	10.433	6.882	12.291	7.182	7.956	14.04
BAND	0	0	0	0.18	0.23	0	0	0	0	0	0
LYMPH	8.91	10.333	14.677	7.02	13.865	14.905	14.652	10.845	10.584	11.934	15.288
MONO	0.376	0.373	2.137	0.18	0.47	0.271	0.222	0.602	0.567	0.552	0.312
EOS	1.631	1.494	0.285	0.45	0.235	1.084	0.444	0.12	0.567	1.657	1.248
BAS	0.251	0	0	0.18	0	0.406	0	0	0	0	0.312

DAY NO	47	50	54	56	58	61	64	68	71	75	79	83
RBC	8.55	8.37	8.02	8.8	8.43	9.09	9.06	8.77	8.09	9.15	8.4	8.06
Hb	12.7	13.3	12.4	13.4	12.8	14	12.9	13.3	12.6	14	12.6	12.3
Hct	34.7	35.9	35.3	36.6	36.6	37.6	37.2	37.6	35.3	37.8	34.7	33.5
MCV	41	43	44	42	43	41	41	43	44	41	41	42
MCH	14.8	15.8	15.4	15.2	15.1	15.4	14.2	15.1	15.5	15.3	15	15.2
MCHC	36.5	37	35.1	36.6	34.9	37.2	34.6	35.3	35.6	37	36.3	36.7
WBC	23.1	29	27.1	18.5	19.8	19.2	17.4	20.1	26.5	21.3	22.3	12.9
NEUT	9.933	12.18	13.008	5.827	9.306	7.488	7.482	7.135	13.912	9.159	7.582	5.095
BAND	0	0	0	0	0.29	0	0.08	0.1	0	0	0	0
LYMPH	11.665	15.225	13.279	11.007	9.405	10.944	8.352	11.055	11.13	10.437	12.599	6.579
MONO	0.346	0.435	0.542	0.555	0.099	0.192	0.261	0.502	0.795	0.106	0.223	0.387
EOS	0.924	1.16	0.271	0.555	0.495	0.48	1.131	1.206	0.662	1.384	1.561	0.774
BAS	0.231	0	0	0	0.099	0.096	0.087	0.1	0	0.213	0.334	0.064

Table 5.6. Cat H11 Full Haematology Results (cont.)

DAY NO	86	90	93	96	99	104	111	118	125	132	139	153
RBC	8.24	7.62	7.78	8.99	6.91	8.77	7.41	9.31	8.46	7.47	6.88	8.15
Hb	13	11.9	12.5	14.4	10.7	13.7	11.8	14.9	13.6	11.5	10.6	12.8
Hct	36.1	34.4	34.9	37.5	31.1	39.1	33.8	41	38.1	31.4	31.1	35.3
MCV	44	45	45	42	45	45	46	44	45	42	45	43
MCH	15.7	15.6	16	16	15.4	15.6	15.9	16	16	15.3	15.4	15.7
MCHC	36	34.5	35.8	38.4	34.4	35	34.9	36.3	35.6	36.6	34	36.2
WBC	26.8	29.4	29.8	22.8	24.4	24.3	28.6	17	25.3	15	22.3	21.7
NEUT	10.854	16.023	14.602	10.26	11.346	11.785	12.87	7.735	12.27	7.5	9.812	12.26
BAND	0	0	0	0	0	0	0	0	0	0	0	0
LYMPH	14.07	11.025	10.728	10.488	8.662	9.477	13.585	7.565	10.246	5.625	10.704	6.51
MONO	0.402	0.735	1.043	0.228	0.976	0.121	0.715	0.765	0.253	0.3	0.334	0.217
EOS	1.474	1.47	3.278	1.596	3.416	2.794	1.43	0.935	2.53	1.5	1.449	2.712
BAS	0	0.147	0.149	0.228	0	0.121	0	0	0	0.075	0	0

DAY NO	160	174
RBC	7.96	8.46
Hb	13.3	13.6
Hct	35.6	36.6
MCV	45	43
MCH	16.7	16
MCHC	37.3	37.1
WBC	21.5	21.8
NEUT	11.18	10.791
BAND	0	0
LYMPH	7.095	8.502
MONO	0.752	0.218
EOS	2.365	2.289
BAS	0	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.7. Cat H12 Full Haematology Results

DAY NO		0	7	14	21	28	30	33	35	37	40	42
RBC		8.33	8.05	7.95	7.49	8.24	7.65	7.59	7.81	7.78	7.99	7.68
Hb		13	13	12.9	12.1	12.9	12.2	12.4	13.1	12.7	13	12.2
Hct		34.8	33.5	33.9	31.8	34.7	34.1	33.9	34.1	33.9	34.7	33.2
MCV		42	42	43	42	42	45	45	44	44	43	43
MCH		15.6	16.1	16.2	16.1	15.6	15.9	16.3	16.7	16.3	16.2	15.8
MCHC		37.3	38.8	38	38	37.1	35.7	36.5	38.4	37.4	37.4	36.7
WBC		32	21.1	33.1	28.1	28.8	29.3	30.8	27.3	28.4	29.9	24.5
NEUT		16.32	10.339	19.694	15.595	12.384	14.21	14.322	13.923	17.324	17.491	9.677
BAND		0	0	0	0.14	0	0	0	0	0	0	0
LYMPH		12.48	8.651	10.923	9.835	14.832	13.624	14.938	12.012	8.662	10.614	12.862
MONO		0.32	0.633	0.827	0.983	0	0.732	0.616	0.955	0.426	0.897	0.245
EOS		2.56	1.477	1.489	1.545	1.152	0.732	0.924	0.409	1.988	0.747	1.347
BAS		0.32	0	0.165	0	0.432	0	0	0	0	0.149	0.245

DAY NO	44	47	50	54	56	58	61	64	68	71	75	79
RBC	7.56	8.09	7.9	8.87	8.9	8.55	8.3	8.3	8.43	8.18	7.99	8.3
Hb	12.4	13.1	13.1	14.5	14.2	13.7	13.3	12.7	13.6	13.2	13.3	13.2
Hct	34.1	35	35	39.6	38.1	36.6	35.1	35	36.7	36.6	34.1	35.3
MCV	45	43	44	45	43	43	42	42	44	45	43	43
MCH	16.4	16.1	16.5	16.3	15.9	16	16	15.3	16.1	16.1	16.6	15.9
MCHC	36.3	37.4	37.4	36.6	37.2	37.4	37.8	36.2	37	36	39	37.3
WBC	35.3	32.3	39	30.3	34.3	34.4	27.9	24.5	37.9	44.1	35.5	42.2
NEUT	14.12	16.473	24.96	9.847	11.662	15.824	10.881	9.555	16.676	19.845	11.537	19.412
BAND	0.17	0	0	0	0	0	0	0	0	0	0	0
LYMPH	18.885	13.404	11.895	19.846	18.179	16.34	12.834	12.495	15.349	19.624	19.347	18.568
MONO	0.529	1.13	0.39	0.151	1.372	1.204	0.558	0.857	1.326	2.425	0.887	1.688
EOS	1.588	1.13	1.365	0.303	2.744	0.516	3.069	1.347	4.169	2.205	3.372	2.532
BAS	0	0.161	0.195	0.151	0.343	0.516	0.558	0.245	0.379	0	0.355	0

Table 5.7. Cat H12 Full Haematology Results (cont.)

DAY NO	83	86	90	93	96	99	104	111	118	125	132	139
RBC	7.5	8.68	7.96	8.3	7.75	8.09	7.99	7.99	8.24	8.27	8.68	8.06
Hb	11.8	13.7	12.8	13.2	12.5	12.9	12.5	12.8	12.9	13.3	13.8	12.5
Hct	31.7	36.7	34.7	35.2	32.9	34.7	34.5	35.3	35.4	36.6	36.6	34.7
MCV	42	42	44	42	42	43	43	44	43	44	42	43
MCH	15.7	15.7	16	15.9	16.1	15.9	15.6	16	15.6	16	15.8	15.5
MCHC	37.2	37.3	36.8	37.5	37.9	37.1	36.2	36.2	36.4	36.3	37.7	36
WBC	30.9	27.8	39.3	37.6	34.2	31.1	34.6	37.6	29.5	44.1	27	25.7
NEUT	17.304	13.483	18.667	18.612	16.758	18.038	20.068	21.432	15.34	30.429	14.445	11.693
BAND	0	0	0	0	0	0	0	0	0	0	0	0
LYMPH	12.051	11.676	16.702	15.416	14.877	11.507	11.072	13.348	11.652	10.804	9.99	11.308
MONO	0.309	1.112	0.786	0.752	0.513	0.622	1.038	0.94	0.295	0.661	0.54	1.028
EOS	1.236	1.529	3.144	2.444	2.052	1.71	2.422	1.504	2.212	2.205	2.025	1.542
BAS	0	0	0	0.376	0	0.155	0	0.376	0	0	0	0

DAY NO	153	160	174	188
RBC	8.83	7.62	7.17	6.12
Hb	13.6	11.8	10.9	9.3
Hct	36.9	30.1	30.5	25.3
MCV	42	40	43	41
MCH	15.4	15.4	15.2	15.1
MCHC	36.8	39.2	35.7	36.7
WBC	26.7	30.6	31.9	23.9
NEUT	9.612	18.819	18.98	15.29
BAND	0	0	0	0
LYMPH	13.216	10.098	11.324	7.17
MONO	0.801	0.306	0.159	0.23
EOS	3.07	1.377	1.276	1.19
BAS	0	0	0.159	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.8. Cat H18 Full Haematology Results

DAY NO	0	7	14	21	28	35	37	40	41	44	47
RBC	8.43	7.33	7.89	7.39	8.39	8.68	7.81	7.93	7.71	8.02	8.15
Hb	12.9	10.5	11.7	10.8	12.5	12.5	11.2	11.4	10.7	11.6	12
Hct	34.1	29.3	31.1	29	32.4	33.5	31.1	31.5	29.2	31.2	31.4
MCV	40	40	39	39	39	39	40	40	38	39	39
MCH	15.3	14.3	14.8	14.6	14.8	14.4	14.3	14.3	13.8	14.4	14.7
MCHC	37.8	35.8	37.6	37.2	38.5	37.3	36	36.1	36.6	37.1	38.2
WBC	22.2	12.2	19.5	12.7	17	16.2	13.3	13.9	10.3	13	13.3
NEUT	14.43	6.405	10.042	5.588	7.565	8.1	6.184	7.158	4.738	6.37	6.65
BAND	0	0	0	0	0.08	0.08	0	0	0	0	0.06
LYMPH	6.549	4.758	7.312	6.286	7.905	6.642	5.386	5.351	4.789	5.59	5.586
MONO	0.555	0.427	0.292	0.063	0.425	0.081	0.332	0.486	0.051	0.195	0.199
EOS	0.666	0.61	1.852	0.762	0.935	1.215	1.396	0.903	0.669	0.845	0.731
BAS	0	0	0	0	0.085	0.081	0	0	0.051	0	0.066

DAY NO	49	51	54	57	61	63	65	68	71	75	78	82
RBC	7.96	7.65	7.9	7.68	7.84	7.87	8.37	7.62	7.28	7.78	7.28	8.43
Hb	11.3	11	11.1	11	11.2	11.2	11.9	10.7	10	10.9	10.1	12
Hct	30.8	30.8	30.8	30.5	31.7	30.5	32.9	29.3	28	30.9	27.7	32.3
MCV	39	40	39	40	40	39	39	38	38	40	38	38
MCH	14.1	14.3	14	14.3	14.2	14.2	14.2	14	13.7	14	13.8	14.2
MCHC	36.6	35.7	36	36	35.3	36.7	36.1	36.5	35.7	35.2	36.4	37.1
WBC	12.4	14.9	13.5	15.9	13.7	12.2	12.3	10	11.1	11.1	11.7	12.2
NEUT	4.588	6.109	5.737	7.155	7.192	5.673	5.719	5.05	5.55	5.328	4.621	5.307
BAND	0	0	0	0	0.2	0	0.12	0	0	0	0	0
LYMPH	6.262	7.077	6.817	6.837	5.069	5.429	5.166	4.05	4.551	4.717	5.908	5.795
MONO	0.496	0.298	0.27	0.636	0	0.427	0.246	0.25	0.166	0.333	0.117	0.244
EOS	0.806	1.341	0.675	1.192	1.233	0.671	0.984	0.6	0.832	0.055	0.994	0.854
BAS	0.248	0	0	0.079	0	0	0.061	0.05	0	0	0.058	0

Table 5.8. Cat H18 Full Haematology Results (cont.)

DAY NO	86	90	93	97	100	103	106	111	118	125	139	146
RBC	7.78	7.62	7.13	7.19	7.25	7.16	7.07	7.47	7.9	7.16	8.46	7.59
Hb	11	10.5	10.2	9.9	10.2	10.3	9.9	10.2	10.9	9.8	11.4	10.2
Hct	30.5	28.9	28.4	27.4	28.5	28.3	28.6	29.3	31.1	27.2	31.7	28.6
MCV	39	38	40	38	39	40	40	39	39	38	37	38
MCH	14.1	13.7	14.3	13.7	14	14.3	14	13.6	13.7	13.6	13.4	13.4
MCHC	36	36.3	35.9	36.1	35.7	36.3	34.6	34.8	35	36	35.9	35.6
WBC	17.4	10.4	12.2	12.1	12	14.9	17.3	12.4	12	12.1	11.9	8.3
NEUT	9.222	5.824	5.734	6.11	5.58	7.301	12.456	6.262	5.52	6.776	5.117	4.814
BAND	0	0	0	0	0	0	0	0	0	0	0	0
LYMPH	5.916	3.9	5.307	4.84	4.92	6.556	3.46	4.712	5.04	4.356	4.938	2.822
MONO	0.696	0.052	0.305	0.121	0.24	0	0.519	0.372	0.48	0.363	0.297	0.083
EOS	1.566	0.624	0.854	0.847	1.26	0.894	0.865	0.992	0.84	0.605	1.547	0.581
BAS	0	0	0	0.181	0	0.149	0	0.062	0.12	0	0	0

DAY NO	160	167	181	202
RBC	8.24	7.25	7.36	8.9
Hb	11	9.7	9.7	11.9
Hct	30.8	27.4	27.4	32.6
MCV	37	38	37	37
MCH	13.3	13.3	13.1	13.3
MCHC	35.7	35.4	35.4	36.5
WBC	9.3	11.7	11.2	8.9
NEUT	5.115	4.855	5.712	4.005
BAND	0	0	0	0
LYMPH	3.301	5.148	4.2	4.138
MONO	0.232	0.234	0.224	0.267
EOS	0.651	1.462	1.064	0.4
BAS	0	0	0	0.089

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.9. Cat H19 Full Haematology Results

DAY NO	0	7	14	21	28	35	36	40	42	44	47
RBC	7.99	8.11	8.24	7.17	8.24	7.84	7.01	8.21	8.4	8.43	8.24
Hb	14.4	14.1	14.3	12.4	14.6	13.2	11.8	14.2	14.5	14.4	14.1
Hct	37.8	37.6	37.2	32.7	37	35.6	32.4	37.9	38.4	38.2	36.9
MCV	47	46	45	46	45	45	46	46	46	45	45
MCH	18	17.3	17.3	17.2	17.7	16.8	16.8	17.2	17.2	17	17.1
MCHC	38	37.5	38.4	37.9	39.4	37	36.4	37.4	37.7	37.6	38.2
WBC	40.3	32	32.7	28.3	27.9	28.1	24.7	38.3	23.9	27	22.4
NEUT	31.031	22.08	18.639	20.093	17.716	15.736	14.202	29.874	16.969	17.28	15.008
BAND	0	0	0	0.28	0	0	0	0	0.11	0	0
LYMPH	6.448	5.76	10.464	5.66	6.835	7.727	7.163	4.979	4.302	5.94	5.152
MONO	1.209	0.48	0.163	0.566	0.279	0.562	0.37	0.766	0.358	0.81	0.224
EOS	1.612	3.52	3.106	1.698	2.65	3.934	2.964	2.298	2.151	2.97	2.016
BAS	0	0.16	0.327	0	0.418	0	0	0.383	0	0	0

DAY NO	49	51	54	57	61	63	65	68	71	75	78	82
RBC	8.74	7.9	7.35	8.12	8.87	8.77	8.74	7.59	8.12	8.46	8.3	8.49
Hb	14.7	13.3	12.4	14.1	15	14.6	14.7	13	13.1	13.6	13.9	14.7
Hct	39.3	35.9	33.2	36.9	40.8	39	39	33.6	35.9	37.3	36.9	37.8
MCV	45	45	45	45	46	44	45	44	44	44	44	45
MCH	16.8	16.8	16.8	17.3	16.9	16.6	16.8	17.1	16.1	16	16.7	17.3
MCHC	37.4	37	37.3	38.2	36.7	37.4	37.6	38.6	36.4	36.4	37.6	38.8
WBC	23.4	26.4	31.4	26.2	26.2	29.5	26.2	28.1	31.1	25.8	28.9	29.1
NEUT	14.391	13.992	25.748	17.423	18.864	19.617	12.969	19.67	22.858	20.124	16.762	19.206
BAND	0	0.52	0	0	0	0	0.13	0	0	0	0	0
LYMPH	5.499	8.448	3.14	5.24	4.978	6.637	5.502	4.636	5.131	4.386	8.381	5.965
MONO	0.585	0.264	0.314	0.917	0.262	0.442	0.917	1.124	0.466	0.387	0.578	0.436
EOS	2.808	3.036	2.198	2.358	2.096	2.507	2.096	2.669	2.643	0.903	3.179	3.492
BAS	0.117	0	0	0	0	0.147	0	0	0	0	0	0

Table 5.9. Cat H19 Full Haematology Results (cont.)

DAY NO	86	90	93	97	100	103	106	114	118	125	132	146
RBC	8.15	8.3	8.09	8.09	8.09	7.93	8.09	8.52	8.18	7.59	8.68	8.15
Hb	13.3	13.7	13.3	13.3	13.3	13.5	13.4	13.6	13.4	12	14	12.9
Hct	36.2	36.6	36.1	35.9	35.8	35.9	36.6	37.2	36.9	33.3	38.4	35
MCV	44	44	45	44	44	45	45	44	45	44	44	43
MCH	16.3	16.5	16.4	16.4	16.4	17	16.5	15.9	16.3	15.8	16.1	15.8
MCHC	36.7	37.4	36.8	37	37.1	37.6	36.6	36.5	36.3	36	36.4	36.8
WBC	36.2	30.3	27.2	21.9	26.5	30	25.4	19.9	23.9	21.9	26.9	21.7
NEUT	27.331	25.149	19.176	13.359	15.502	17.55	17.018	12.636	13.145	14.235	17.485	13.652
BAND	0	0	0.13	0	0	0	0	0	0	0	0	0
LYMPH	6.697	3.939	4.896	5.694	6.625	9.3	5.207	4.378	6.214	4.161	5.38	5.316
MONO	0.181	0.454	0.408	0.219	0.927	0	0.381	0.298	0.717	0.438	0.269	0.651
EOS	1.81	0.757	2.584	2.409	3.71	3.15	2.794	2.388	3.824	2.956	3.497	2.061
BAS	0.181	0	0	0.219	0	0	0	0.199	0	0	0.269	0.108

DAY NO	160	167	181	204
RBC	8.62	7.96	8.02	7.7
Hb	13.8	12.7	12.6	12.3
Hct	37.5	34.4	34.7	33.2
MCV	44	43	43	43
MCH	16	15.9	15.7	15.9
MCHC	36.8	36.9	36.3	37
WBC	17.2	20.3	22.5	12.1
NEUT	8.084	12.484	12.712	6.897
BAND	0	0	0	0
LYMPH	5.848	5.988	5.737	4.84
MONO	0.516	0.203	0.112	0
EOS	2.752	1.624	3.937	0.363
BAS	0	0	0	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

Table 5.10. Cat H21 Full Haematology Results

DAY NO	0	7	14	21	28	35	37	40	42	44	47
RBC	9.81	9.25	9.34	8.46	9.28	9.21	8.68	8.77	8.09	8.68	8.68
Hb	15.5	14.2	15	13.3	14.9	14.4	13.5	13.8	12.9	13.7	13.8
Hct	40.8	37.9	38.7	35.1	37.9	38.4	35.9	37.6	35.3	36.1	35.6
MCV	42	41	41	41	41	42	41	43	44	42	41
MCH	15.8	15.3	16	15.7	16	15.6	15.5	15.7	15.9	15.7	15.8
MCHC	37.9	37.4	38.7	37.8	39.3	37.5	37.6	36.7	36.5	37.9	38.7
WBC	14.9	14.3	16.3	15	17.2	18.3	11.2	17.9	15.1	15.8	13.8
NEUT	7.301	7.65	8.231	7.65	9.374	9.333	5.152	11.277	8.909	9.085	7.452
BAND	0	0	0	0	0	0	0	0	0.07	0	0
LYMPH	6.705	5.148	6.927	6.45	6.106	6.862	4.928	4.922	5.134	4.898	4.554
MONO	0.521	0.429	0.163	0.225	0.774	0.366	0.168	0.537	0.075	0.553	0.138
EOS	0.298	1.001	0.978	0.675	0.688	1.555	0.952	1.074	0.755	1.185	1.242
BAS	0.074	0.0071	0	0	0.258	0.183	0	0.089	0.151	0	0.345

DAY NO	49	51	54	57	61	63	65	68	71	75	78	82
RBC	9.12	8.55	9.31	9.12	9.24	9.78	9.43	9.12	9.56	9.18	8.74	9.5
Hb	14	13.6	14.6	14.4	14.2	15.4	14.7	14	14.4	14	13.3	15.1
Hct	37.8	36.2	38.4	38.4	39.3	40.8	39	37.3	39	37.9	35.6	38.1
MCV	41	42	41	42	43	42	41	41	41	41	41	40
MCH	15.3	15.9	15.6	15.7	15.3	15.7	15.5	15.3	15	15.2	15.2	15.8
MCHC	37	37.5	38	37.5	36.1	37.7	37.6	37.5	36.9	36.9	37.3	39.6
WBC	11.9	17.2	13.8	16.1	16.3	16.5	13.7	10.7	10.1	16.5	17.1	10.1
NEUT	5.831	8.772	5.451	9.016	8.476	7.92	6.576	5.243	5.555	9.652	9.832	5.454
BAND	0	0	0	0.16	0.16	0	0	0	0.05	0	0	0
LYMPH	4.879	7.052	7.176	6.279	6.683	7.095	5.891	3.959	3.282	5.775	5.386	3.585
MONO	0.119	0.086	0.069	0.161	0	0.495	0.548	0.535	0.151	0.082	0.342	0.303
EOS	1.071	1.29	1.104	0.483	0.815	0.825	0.548	0.856	1.01	0.99	1.539	0.757
BAS	0	0	0	0	0.163	0.165	0.137	0.107	0.05	0	0	0

Table 5.10. Cat H21 Full Haematology Results (cont.)

DAY NO	86	90	93	97	100	103	106	111	118	125	132	139
RBC	9.27	8.8	9.31	8.83	9.34	9.56	8.87	9.21	9.5	9.24	9.34	10.07
Hb	13.7	13.1	13.9	13.1	13.6	14.5	13.1	13.3	13.9	13.5	13.6	14.6
Hct	37.2	34.7	37.6	35.6	37	38.4	36.2	37.3	38.1	36.7	37.2	39.3
MCV	40	39	40	40	40	40	41	40	40	40	40	39
MCH	14.7	14.8	14.9	14.8	14.5	15.1	14.7	14.4	14.6	14.6	14.5	14.4
MCHC	36.8	37.7	36.9	36.7	36.7	37.7	36.1	35.6	36.4	36.7	36.5	37.1
WBC	13.3	12.9	16.8	16.5	13.7	16.3	14.2	14.6	13.5	14.5	12.4	12.7
NEUT	6.118	6.321	8.568	9.57	8.425	9.209	7.881	8.176	8.37	7.395	7.378	6.794
BAND	0	0	0	0	0	0	0	0	0	0.07	0	0
LYMPH	5.719	5.16	6.888	5.362	3.767	5.705	4.899	4.891	3.51	5.22	3.906	4.572
MONO	0.133	0.193	0.252	0.33	0.342	0.163	0.497	0.146	0.27	0.145	0	0.444
EOS	1.33	1.161	0.924	1.155	0.959	1.222	0.923	1.095	1.282	1.667	0.93	0.635
BAS	0	0.064	0.168	0.082	0.205	0	0	0.292	0.067	0	0.186	0.254

DAY NO	146	160	167	181	204
RBC	9.18	10.29	9.37	8.65	7.26
Hb	13	14.6	13.7	12.2	10
Hct	36.2	40.2	36.9	34.1	28
MCV	39	39	39	39	39
MCH	14.1	14.1	14.6	14.1	13.7
MCHC	35.9	36.3	37.1	35.7	35.7
WBC	9.4	10	11.2	12.7	7.2
NEUT	4.465	5.05	5.264	5.588	3.78
BAND	0	0	0	0	0
LYMPH	4.042	4.05	3.92	5.588	3.06
MONO	0	0.05	0.672	0.317	0
EOS	0.705	0.85	1.12	1.143	0.36
BAS	0.188	0	0.224	0.063	0

RBC (x 10^{12}/L)
Haemoglobin (g/dl)
Haematocrit (l/l)
MCV (fl)
MCH (pg)
MCHC (g/dl)
WBC, Neut, Lymph, Mono, Eosin, Baso (x 10^{9}/l)

TABLE 5.11 P-values for Statistical Analyses of Individual Parameter Data as Compared Between Infected and Control Groups of Cats (Significance < 0.05)

	ANOVA Group-Time	ANOVA Group	T-TEST day 0	T-TEST day 28	T-TEST day 44	T-TEST day 86	T-TEST day 160
RBC	**0.022**	0.126	0.47	0.45	0.097	0.051	0.25
Hb	**0.007**	**0.029**	0.78	0.10	**0.019**	**0.010**	0.086
Hct	0.064	**0.030**	0.61	0.086	**0.020**	**0.012**	0.21
MCV	0.758	0.323	0.11	0.15	0.17	0.80	0.82
MCH	0.972	0.144	0.18	0.24	0.11	0.27	0.37
MCHC	0.261	**<0.000**	0.55	0.74	0.28	0.065	0.067
WBC	**<0.000**	**0.047**	0.74	0.18	**0.028**	0.092	0.54
NEUT	**<0.000**	**0.029**	0.49	0.36	**0.0051**	0.14	0.29
LYMPH	**<0.000**	0.377	0.51	0.19	0.17	0.47	0.32
MONO	0.162	0.277	0.65	0.087	0.13	0.21	0.39
EOSIN	0.053	**0.007**	0.92	0.48	**0.029**	**0.0001**	**0.04**
BASO	**0.038**	0.058	0.42	0.062	0.44	0.60	(1)

Fig.5.1. Mean Red Cell Counts for Control and Infected Groups

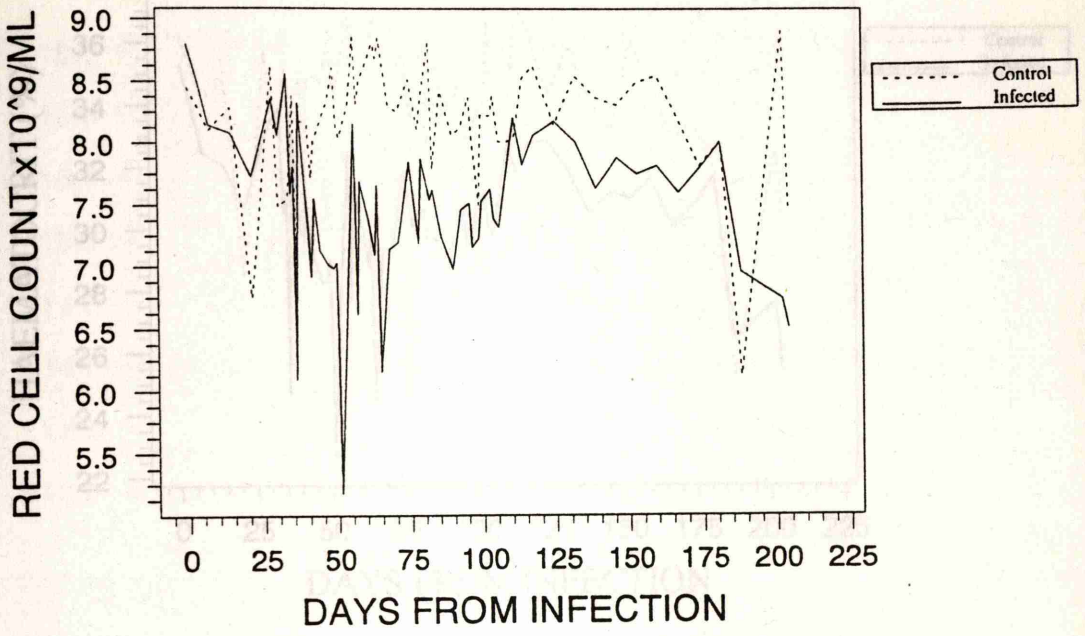


Fig. 5.2. Mean Haemoglobin Values for Control and Infected Groups

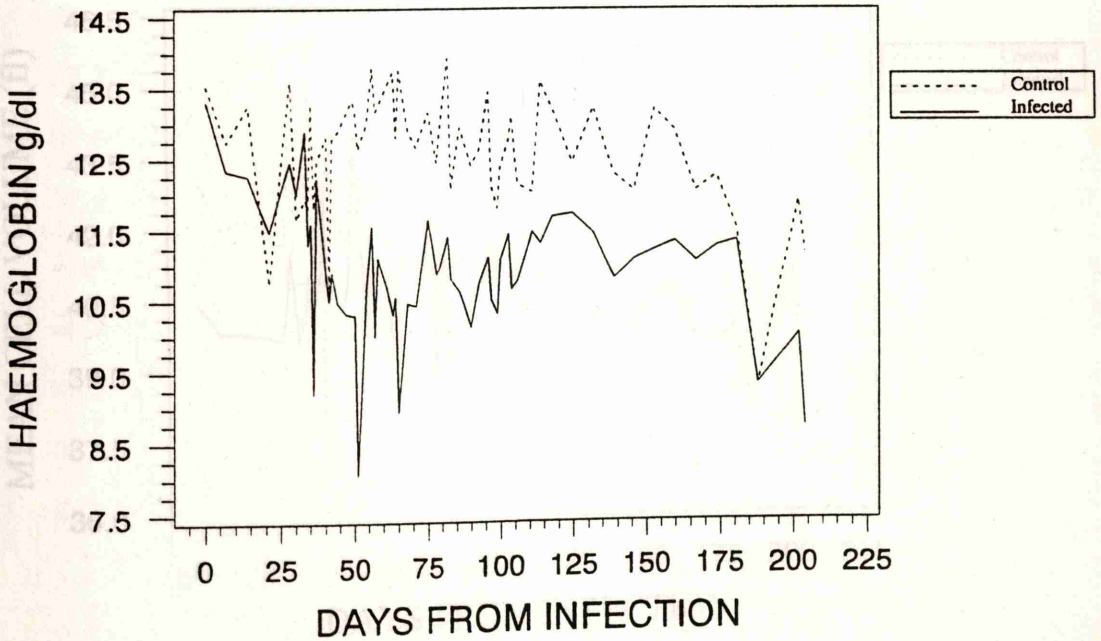


Fig 5.3. Mean Haematocrit for Control and Infected Groups

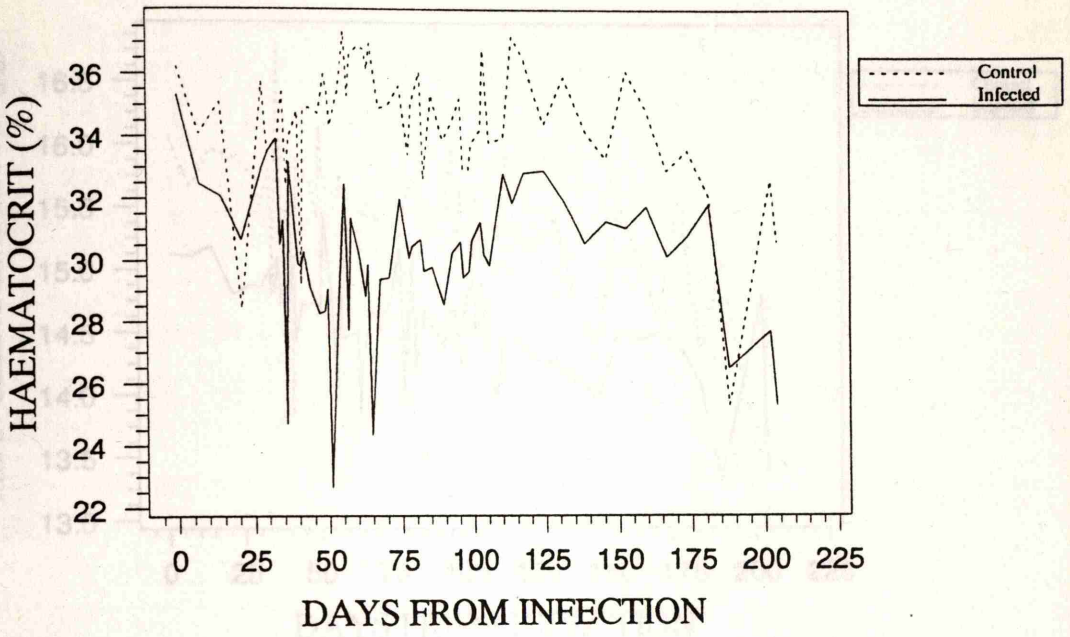


Fig 5.4. Mean Mean Cell Volumes for Control and Infected Groups

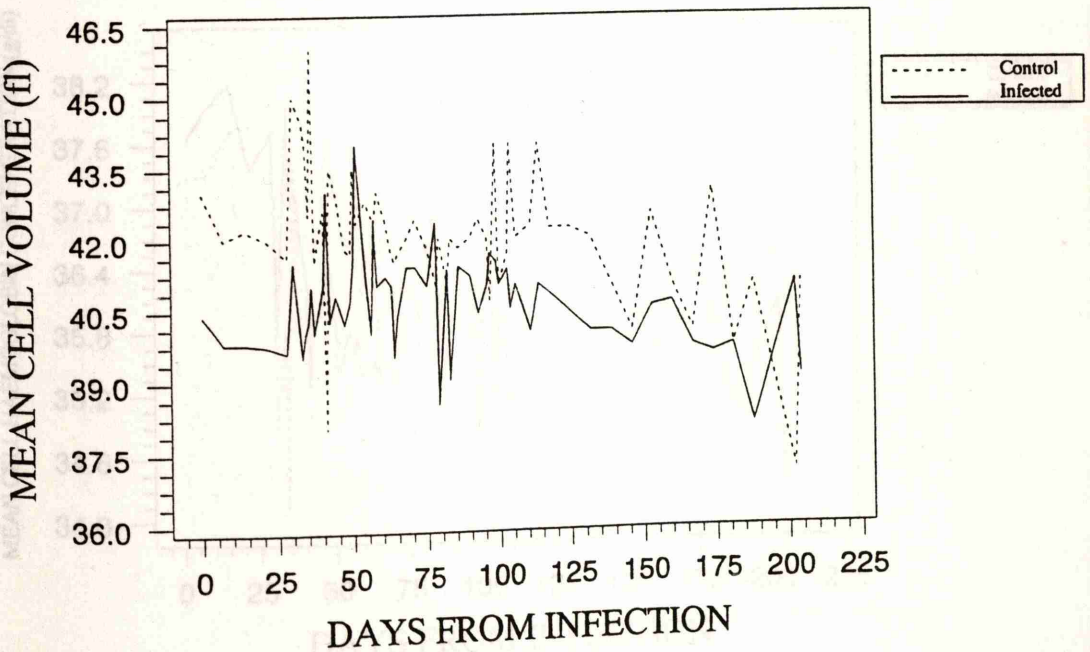


Fig 5.5. Mean Mean Cell Haemoglobin for Control and Infected Groups

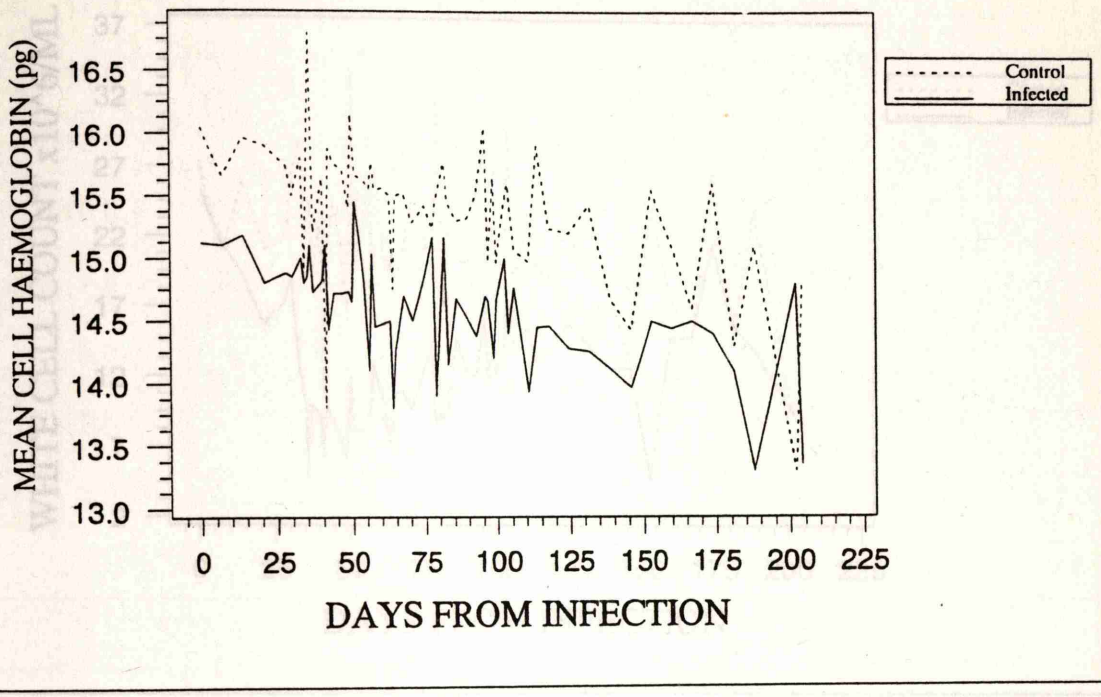


Fig 5.6 Mean Mean Cell Haemoglobin Concentration for Control and Infected Groups

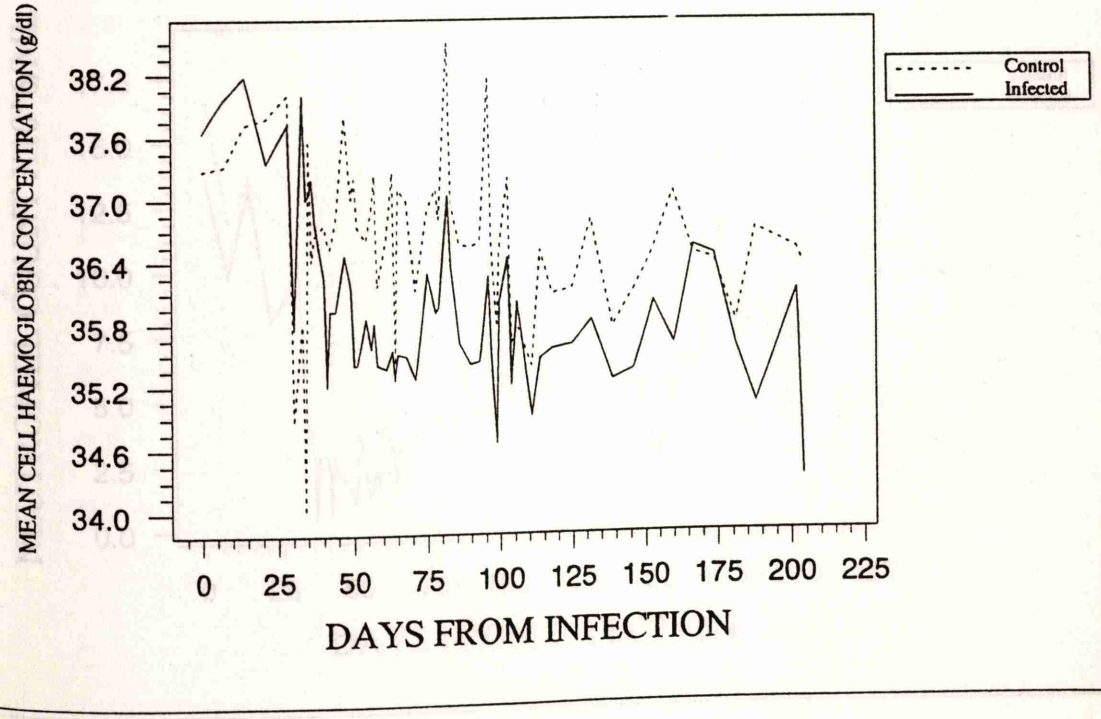


Fig.5.7. Mean White Cell Counts for Control and Infected Groups

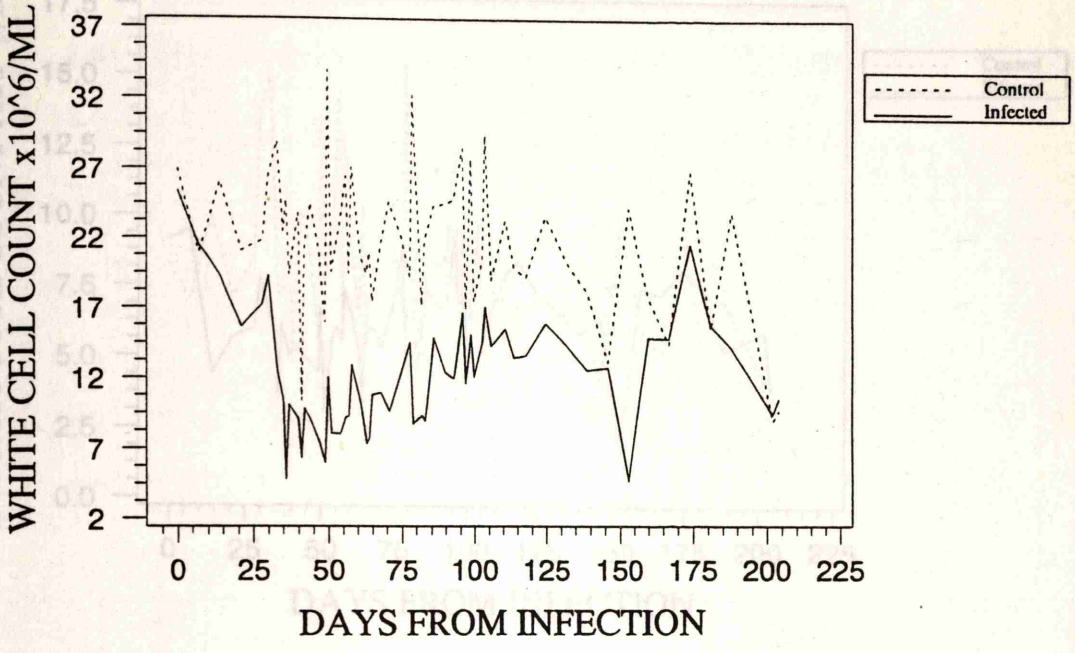


Fig. 5.8. Mean Neutrophil Counts for Control and Infected Groups

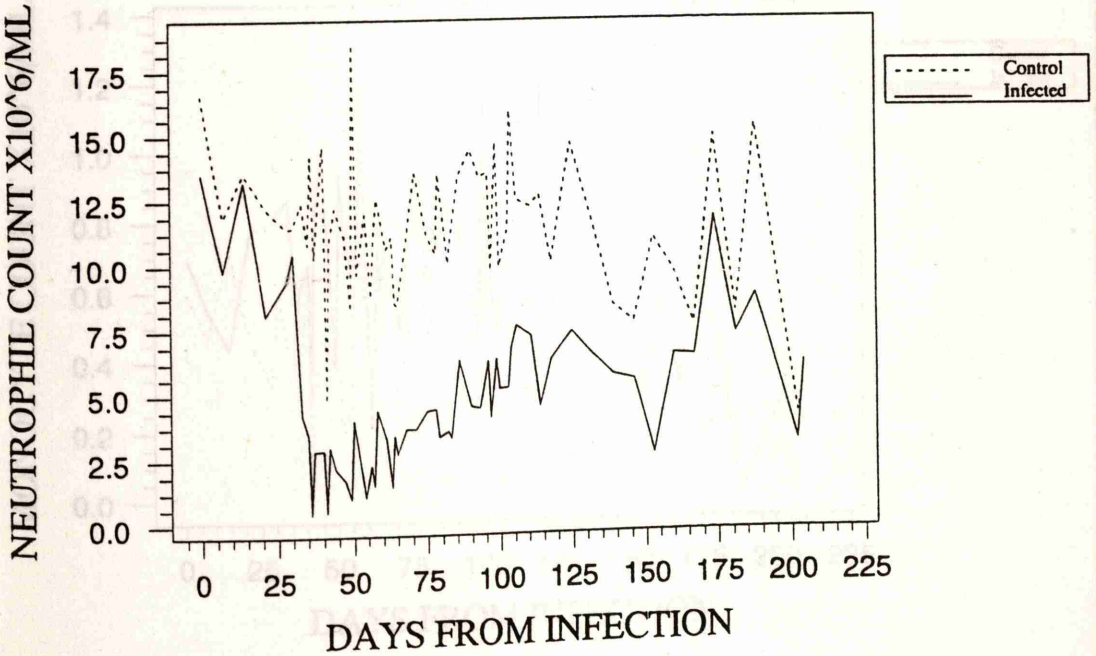


Fig. 5.9. Mean Lymphocyte Counts for Control and Infected Groups

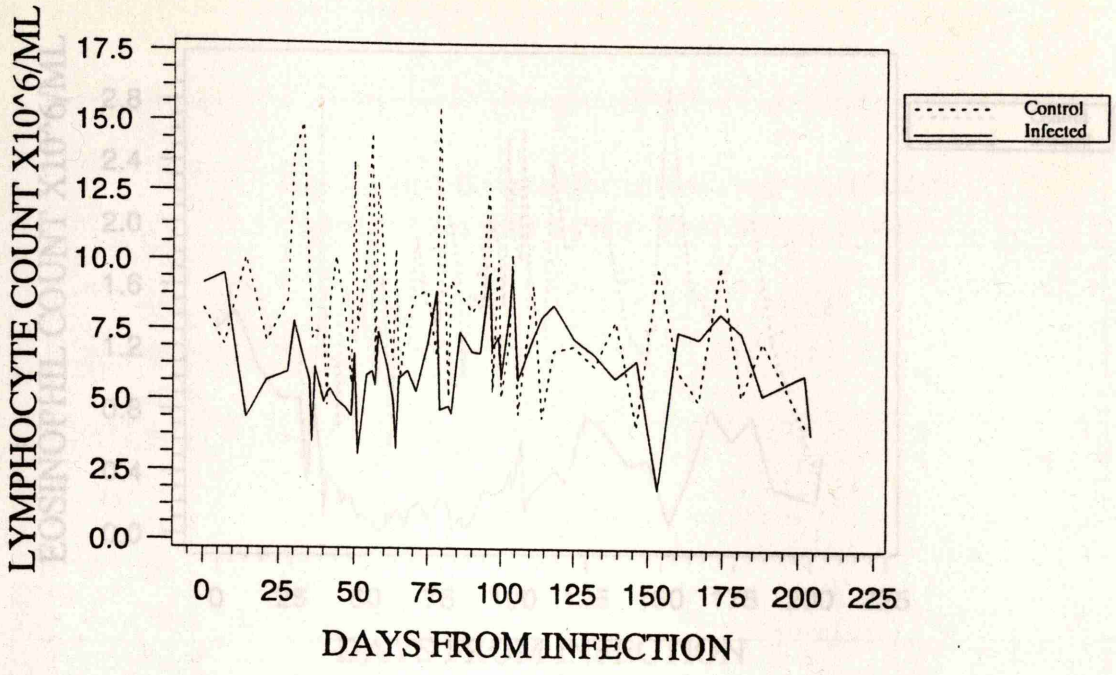


Fig. 5.10. Mean Monocyte Counts for Control and Infected Groups

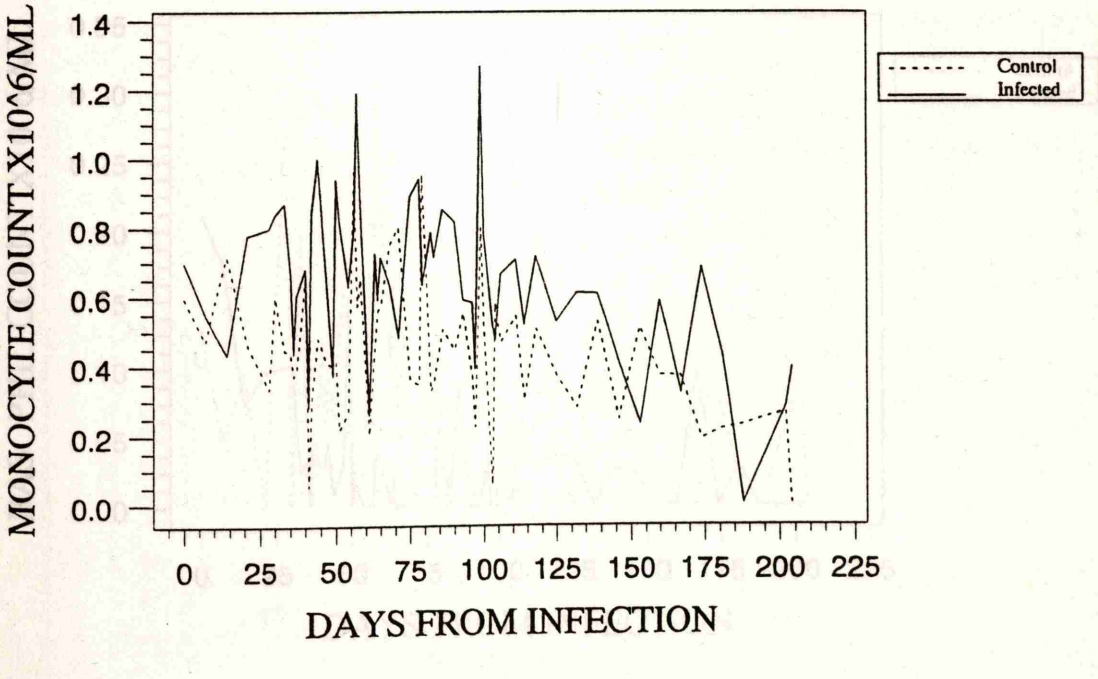


Fig. 5.11. Mean Eosinophil Counts for Control and Infected Groups

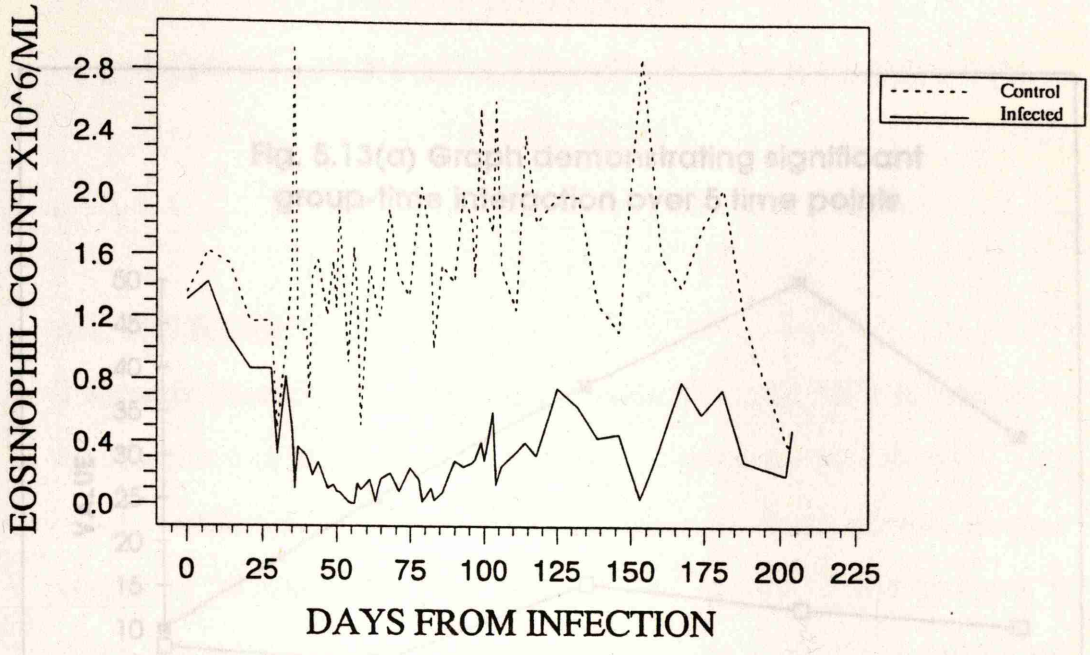


Fig. 5.12. Mean Basophil Counts for Control and Infected Groups

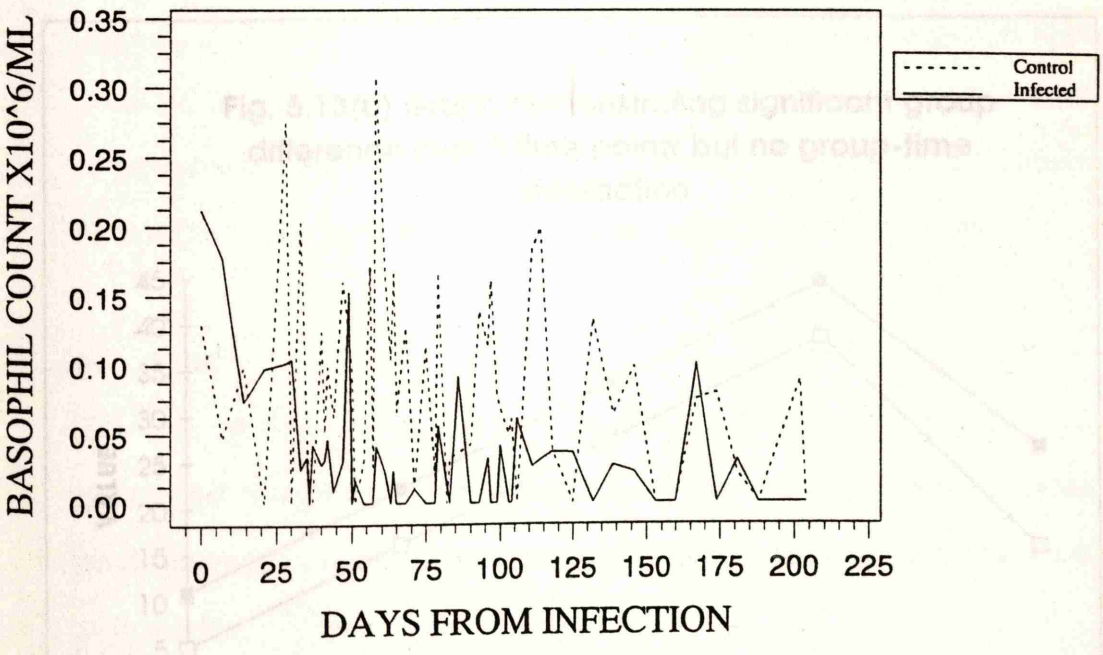


Fig. 5.13(a) Graph demonstrating significant group-time interaction over 5 time points

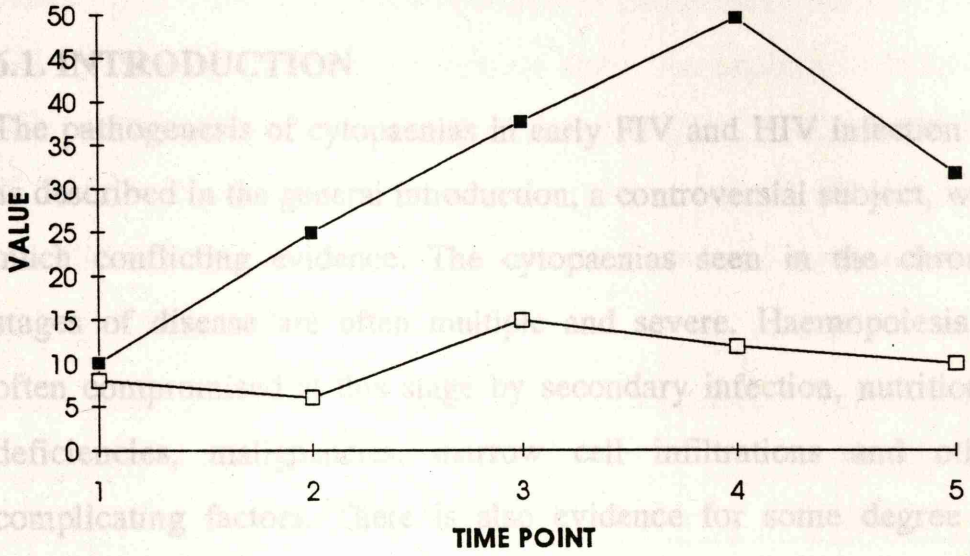
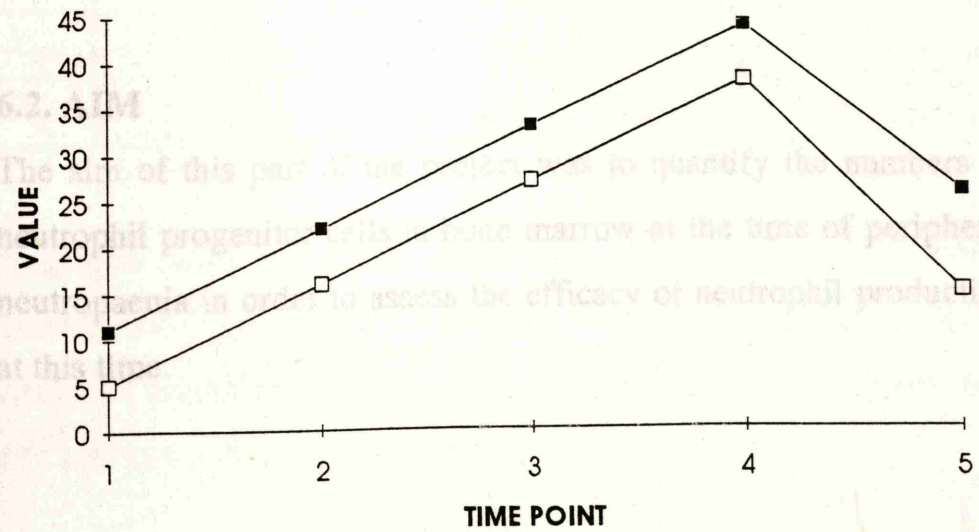


Fig. 5.13(b) Graph demonstrating significant group difference over 5 time points but no group-time interaction



CHAPTER 6

INVESTIGATION OF HAEMOPOIESIS IN EARLY FIV INFECTION

6.1. INTRODUCTION

The pathogenesis of cytopaenias in early FIV and HIV infection is, as described in the general introduction, a controversial subject, with much conflicting evidence. The cytopaenias seen in the chronic stages of disease are often multiple and severe. Haemopoiesis is often compromised at this stage by secondary infection, nutritional deficiencies, malignancies, marrow cell infiltrations and other complicating factors. There is also evidence for some degree of peripheral cell destruction or sequestration. However, in early FIV infection in SPF cats, marrow investigation is not affected by these factors. Therefore, by studying marrow events in early infection it is possible to establish the efficacy of neutrophil production at the time of peripheral neutropaenia, and also to potentially identify the nature of infected cells in marrow at a time of active viral replication.

6.2. AIM

The aim of this part of the project was to quantify the numbers of neutrophil progenitor cells in bone marrow at the time of peripheral neutropaenia in order to assess the efficacy of neutrophil production at this time.

6.3. MATERIALS AND METHODS

Bone marrow aspirates were taken from 5 infected SPF cats (H13 - H17) at three weeks post-infection; at the point peripheral neutropaenia was first observed or 6 weeks p.i.; at 12 and at 20 weeks p.i.. Cells were also obtained at euthanasia (weeks 27-29 p.i.) by flushing cells from a femoral shaft. An aspirate was taken from one of each of the following control cats (H11, H12, H18, H19, H21) each time one was taken from an infected animal. Parallel GM and Mix colony assays were set up from infected and control cats at each time point as follows:

Tube	Cell Concentration	Growth Factor	Feeder Cells
1	5 x 10^4/ml	G-CSF	None
2	5 x 10^4/ml	GM-CSF	None
3	5 x 10^4/ml	IL-3	None
4	5 x 10^4/ml	None	None
5	2.5 x 10^5/ml	None	None
6	5 x 10^4/ml	None	1 x10^5/ml Infected cat
7	5 x 10^4/ml	None	1 x 10^5/ml Control cat
8	None	None	1 x 10^5/ml Infected cat
9	None	None	1 x 10^5/ml Control cat

Progenitor cells were stimulated as shown above, with either recombinant human growth factors, autologous or heterologous irradiated feeder cells. GM assays were incubated for 8 days and mix assays for 14 days before colonies were counted.

The colony numbers for each animal were log transformed (to eliminate the tail seen on the distribution of this type of colony data, personal communication G.Gettinby) and analysis of variance was used to test for significant differences between the two groups, both over the course of the experiment and at single time points. This analysis was performed on the Minitab statistical software package (Minitab for Windows, Minitab Inc., State College, PA, 16001-3008, USA). Groups were considered to be significantly different for $p < 0.05$.

6.4. RESULTS

The numbers of each colony type grown from each cat at each time point are represented in tables 6.1 to 6.10. Figures 6.1 to 6.5 show the GM-CFU results from methylcellulose plates represented on boxplots. The number of GM-CFU colonies grown on each plate from infected cat marrow were entered into a Minitab (Minitab for Windows, Minitab Inc., State College, PA, 16001-3008, USA) database i.e. 3 results for each animal at each time point. Boxplots (as in chapter 3) were created for each marrow sampling. Similar boxplots were created for control cats. Thus, the median, 1st & 3rd quartiles, "whiskers" and outliers were calculated from the pooled results of the 5 infected or control cats respectively.

There was much variability in the numbers of colonies grown in assays from one marrow sample to the next (see tables 6.1 to 6.5 and figures 6.1 to 6.5), as is common for this type of assay. There was also great variability between individual animals, so that the emphasis had to be placed on group statistics. In the case of cat H19 at the second sampling there was no colony growth at all (see table 6.2). This may have been due to poor preparation of the cells before

plating. Cat H13 at the same time had poor cell numbers in the marrow aspirate, so the high concentration plates could not be set up (table 6.2). In some instances it was impossible to count the numbers of colonies in the plates with cells at high concentration due to monolayer formation or overgrowth of colonies. This was the case with cat H18 at week 3 (see table 6.1). In all assays, growth was exuberant and far above expected limits. The cat was haematologically normal, so the results obtained may have occurred as a result of a plating error i.e. cells were plated at a higher concentration than had been calculated.

Colonies grown from infected cats at the point of neutropaenia tended to be smaller, consisting of fewer cells than their counterparts in control animals or from infected animals at other times (figs. 6.6 and 6.7).

Overall growth was poorer in assays from the later time points (see table 6.4). This is probably due to poorer quality marrow samples as a result of marrow fibrosis from previous aspirates.

The results of the statistical analysis are shown in tables 6.11 to 6.14. Significant group-time interactions indicate differences in the pattern of response between the two groups between time points. i.e. a lack of parallel response. Group differences signify a difference in the mean values between the two groups over the course of the experiment. These possible outcomes are illustrated in figure 5.13. Table 6.11 consists of group-time interactions and comparisons between control and infected groups. Tables 6.12 to 6.14 show the analysis of variance results for infected and control groups at individual time points.

6.4.1. Statistical results comparing infected and control group GM-CFU numbers over time

The analysis of variance demonstrated that there was only a significant group-time interaction ($p < 0.000$) in GM-CFU numbers with 2.5×10^5 cells/ml in methylcellulose (see table 6.11). The results of the group-time interaction were, however, affected by the variability in assay quality from time point to time point. The poor numbers of colonies grown at week 20, for example, meant that colony numbers in both groups dropped at that time point, and similarly, the improvement in quality at week 27-29 allowed the mean values in both groups to recover. This trend masked any significant group-time interaction.

There were, however, significant differences ($p < 0.05$) between the mean values for the two groups (see p-values for group interactions, table 6.11). This was true of GM-CFU grown in both agar and methylcellulose, and of all growth factors and plating concentrations except autologous cells grown at 5×10^4/ml in methylcellulose. In all cases, the mean values for the infected group were significantly less than those of the control cats (see figures 6.1 to 6.5).

6.4.2. Statistical results comparing infected and control group GM-CFU numbers at 5 individual time points

Results from comparison of numbers of GM-CFU grown from infected and control cat groups at individual time points are shown in tables 6.12 and 6.13. According to results from the agar colonies (see table 6.12), the only significant differences observed were in

GM-CSF at week 27-29 ($p = 0.041$) and at high plating concentration (2.5×10^5/ml) at week 12 ($p < 0.000$).

In methylcellulose (see table 6.13), there are significant differences in both G-CSF and GM-CSF stimulated colonies ($p < 0.05$) at the point of neutropaenia (5-6 weeks post-infection). There are also significantly less colonies grown at high plating efficiency 2.5×10^5/ml at week 12, as for the agar assay.

6.4.3. Statistical results comparing infected and control group BFU-E numbers over time and at 5 individual time points

There were no significant group-time differences for BFU-E in these animals (see table 6.11). There were significant differences in mean values between the two groups in G-CSF and at high plating concentrations ($p = 0.027$ and 0.018 respectively). There were no significant differences at individual time points (table 6.14).

6.5. DISCUSSION

The colony assay results indicated that in the early stages of FIV infection there were significantly less committed granulocyte-macrophage precursor cells in FIV infected cats than control animals (see table 6.11 to 6.13). This difference was tested over the first 6 months of infection. Analysis of variance tests over this period indicated that there were significantly fewer GM-CFU grown from infected cat bone marrow than control animals (table 6.11). This was true of all growth factors and of both agar and methylcellulose colonies, except autologous cells plated at 5×10^4/ml in methylcellulose. The results represented for these conditions in tables 6.1 to 6.5 demonstrate mean values for infected animals were

lower than those for controls. The difference was not large enough to reach the level of statistical significance.

The significant differences between colonies grown in methylcellulose in infected and control animals stimulated with both G-CSF and GM-CSF (table 6.13) were concurrent with the peripheral neutropaenia (weeks 5-6 post-infection), suggesting that this was a critical time. There were fewer colonies grown in agar from infected cats at this time also, although the difference did not reach statistical significance (see table 6.2 and figures 6.1 to 6.5). It was observed that colonies grown at this stage were smaller than those of the control animals, further reducing cell output. A reduction in bone marrow production at this time would explain the peripheral neutropaenia.

Analysis of variance also indicated that there were significant differences between colonies grown in agar stimulated with GM-CSF in control and infected cats at weeks 27-29 (table 6.12). No difference was recorded using any other growth factor nor in GM-CSF stimulated GM-CFU grown in methylcellulose (tables 6.12 and 6.13). Given the number of statistical tests performed, at the 0.05 significance level 5% of these tests may by chance give a misleading result. This may have been the case for this result.

The high colony numbers grown when cells were plated at 2.5 x 10^5/ml were as a result of higher progenitor numbers, but were also due to high levels of endogenous growth factor production from increased numbers of stromal cells. The significant difference between infected and control cats seen at high plating concentration - 2.5 x 10^5/ml - at week 12 (tables 6.12 and 6.13) therefore suggested an inhibition of endogenous growth factor production, or

failure to respond to these factors. However, the results obtained from the addition of irradiated feeder cells producing growth factor (table 6.8) indicated growth factor production was adequate in infected stromal cells. In these experiments the addition of autologous infected stromal cells or heterologous uninfected stromal cells resulted in a similar number of colonies. This was true of both infected and control precursor cells.

This suggested that adequate quantities of growth factors were being produced by infected stromal cells, as these were able to support similar numbers of colonies as uninfected feeders. The failure of infected precursors to respond to non-infected irradiated feeders would support the failure to respond to growth factor hypothesis.

The GM-CFU colonies grown in methylcellulose differ from those grown in agar in that they are derived from slightly more primitive progenitors. Maximal colony growth in methylcellulose takes 14 days, whilst in agar counts are made at day 8. However, the results obtained from the agar and methylcellulose assays were similar, suggesting that both progenitor types were equally affected. The most notable difference was the absence of significant differences in colony numbers at the point of neutropaenia in the agar assay (see tables 6.12 and 6.13). However, it can be seen from figures 6.1 to 6.5 that colony numbers in infected cats at this time were, in fact, lower than those of controls, but that the differences did not quite reach statistical significance. The underlying trend was, nevertheless, similar.

Overall erythroid growth was poor throughout the course of the study. Analysis of variance suggested that there were significant

differences between control and infected cat BFU-E at week 3 and at weeks 27-29 (see table 6.11). Due to the low numbers of erythroid colonies grown, it was difficult to assess the significance of these results. However, the absence of significant differences at individual time points in the erythroid colony populations between infected and control cats served as a useful control to indicate that the differences seen in the myeloid progenitors were not due to any inconsistency in the assays or in the aspirates obtained from the cats.

Other direct experimental evidence of the pathogenesis of neutropaenia in the FIV-infected cat is sparse. Linenberger *et al* (1995) infected a number of SPF cats with FIV and observed a significant increase in CFU-GM, BFU-E and CFU-E at 1.5 to 3 weeks post-infection. The proportion of these cells in the synthesis phase of the DNA cycle was comparable to control animals. This was not observed in this experiment, but would have been missed by the first marrow sampling time at 3 weeks. By weeks 6 to 12 in Linenberger's experiment, the numbers of colonies grown had dropped in infected cats to significantly below control levels. These findings are in agreement with those of this experiment. The proportion of the remaining cells in DNA synthesis was increased. Linenberger suggested that this was a compensatory increase in response to the fall in progenitor numbers, or possibly a response to virally-induced changes in growth factor and cytokine production. In the same study it was noted that from weeks 4 to 12, autologous serum inhibited growth of CFU-GM in infected animals and failed to support maximal growth of BFU-E. The same serum did not inhibit growth of normal progenitors, but failed to promote maximal growth of CFU-GM. This loss in growth promoting activity was unlikely to

be antibody-mediated due to the failure to depress normal progenitor growth. The authors hypothesised that in acute viraemia, an inhibitory substance in serum acts upon a subset of progenitor cells susceptible to growth inhibition during this period.

Donahue *et al* (1987) argued that similar serum inhibition from HIV-positive patients was antibody-mediated. He hypothesised that antibody was attaching to virus-infected cells expressing HIV-antigen, hence the serum did not inhibit progenitors from uninfected subjects. The nature of the virus-infected cells was not established.

Linenberger *et al* (1991) assessed the number of bone marrow precursor cells of both naturally and experimentally infected cats during the asymptomatic phase and found that the numbers of granulocyte-macrophage precursors (GM-CFU) and erythroid precursors (BFU-E and CFU-E) were similar to that of control animals. There was also no difference in the cell-cycle kinetics, response to growth factors or in the relative growth-promoting activity of serum from infected or uninfected cats. No haematological abnormalities were recorded in these cats at the time of sampling.

Shelton *et al* (1989) performed marrow culture on a single, naturally infected asymptomatic cat. The cat had a mild lymphopaenia and mild neutropaenia at the time of sampling, but the bone marrow was histologically normal. The numbers of CFU-GM, BFU-E and CFU-E were comparable to that of control marrow. However, growth was inhibited in the presence of serum from the infected cat. Marrow cells from normal cats had slightly lower CFU-GM numbers when cultured with the infected cat serum. Incubation of normal progenitors with infected serum, and then complement did not result

in any reduction in growth, suggesting that the inhibition was not antibody-mediated, at least to progenitor cells at that stage.

The total experimental evidence suggests that there is a transient drop in committed GM-CFU in FIV-infected cats at approximately 6 weeks post-infection, and that this is accompanied by a peripheral neutropaenia. Thereafter, colony numbers recover. During the asymptomatic phase of infection, there are no cytopaenias and no marrow progenitor deficits. In late-stage infection, when clinical signs and cytopaenias are present, falls in progenitor cell numbers can once again be detected, as is the case in HIV-infected people (Leiderman *et al* 1987, Steinberg *et al* 1991, Calenda & Chermann 1992, Louache *et al* 1992, Potts *et al* 1992, Zauli *et al* 1992a, 1992b, 1992c, De Luca *et al* 1993, Re *et al* 1993).

There is clearly a dynamic relationship between active viral infection and inhibition of bone marrow productivity. In order to further investigate the mechanism of inhibition of haemopoiesis, it was decided to assess marrow progenitor cells for evidence of direct viral infection.

GM ASSAY WEEK 3	GM-CFU	H13	H14	H15	H16	H17	MEAN		H11	H12	H18	H19	H21	MEAN
G		38.3	49.7	20.3	30.7	37.7	**35.3**		33.7	50.0	517.3	21.7	78.7	**140.3**
GM		37.0	18.0	16.7	24.0	48.0	**28.7**		26.3	34.3	448.0	12.0	75.7	**119.3**
IL-3		31.0	10.7	11.0	22.3	27.0	**20.4**		24.3	26.7	379.7	11.3	57.7	**99.9**
5*10^4		35.7	9.0	15.3	15.0	26.0	**20.2**		18.7	22.3	641.3	12.3	82.7	**155.5**
2.5*10^5		194.7	92.0	112.7	148.7	205.7	**150.8**		118.7	297.3	N/O	76.3	580.0	**268.1**
MIX ASSAY WEEK 3	GM-CFU													
G		26.0	23.3	9.3	11.7	10.3	**16.1**		9.0	28.0	100.0	11.3	46.7	**39.0**
GM		28.3	22.0	15.7	10.3	12.3	**17.7**		18.7	26.0	123.0	13.7	69.3	**50.1**
IL-3		20.7	19.0	11.3	7.3	12.3	**14.1**		10.0	34.3	91.7	8.3	50.3	**38.9**
5*10^4		22.3	28.7	12.0	10.3	10.0	**16.7**		13.0	35.0	119.7	12.0	69.7	**49.9**
2.5*10^5		47.0	38.0	25.3	23.3	18.7	**30.5**		25.0	64.3	N/O	32.3	68.7	**47.6**
MIX ASSAY WEEK 3	BFU-E													
G		5.0	9.0	9.3	6.7	4.7	**6.9**		0.7	5.0	14.0	0.3	7.0	**5.4**
GM		4.7	6.3	4.3	2.7	6.3	**4.9**		0.7	6.5	14.3	1.3	6.3	**5.8**
IL-3		1.3	6.7	4.3	4.7	5.7	**4.5**		0.7	6.7	14.7	1.0	3.7	**5.4**
5*10^4		3.0	10.0	5.7	8.0	4.0	**6.1**		1.7	8.3	19.7	0.0	10.0	**7.9**
2.5*10^5		6.7	36.7	21.7	12.0	6.0	**16.6**		2.0	10.7	N/O	11.3	7.0	**7.8**

N/O = Not obtained
H13-H17 = Infected group
H11, H12, H18, H19, H21 = Control group

Table 6.1. Mean number of colonies grown per cat at week 3 post-infection

GM ASSAY NEUT	GM-CFU	H13	H14	H15	H16	H17	MEAN		H11	H12	H18	H19	H21	MEAN
G		8.0	7.0	27.3	23.0	14.0	**15.9**		51.0	5.3	58.3	N/O	37.7	**38.1**
GM		5.3	7.3	17.3	14.3	9.0	**10.6**		55.3	2.3	48.3	N/O	51.3	**39.3**
IL-3		4.3	5.3	14.3	21.3	8.0	**10.6**		53.3	3.0	55.7	N/O	43.0	**38.8**
5*10^4		1.7	5.3	12.7	24.7	4.0	**9.7**		49.7	3.0	44.7	N/O	51.0	**37.1**
2.5*10^5		N/O	38.3	181.7	26.3	42.0	**72.1**		373.3	19.0	420.0	N/O	N/O	**270.8**
MIX ASSAY NEUT	GM-CFU													
G		6.7	11.3	6.3	9.3	4.7	**7.7**		15.0	11.0	15.0	N/O	25.7	**16.7**
GM		6.0	9.0	12.0	15.0	9.3	**10.3**		27.3	10.3	11.3	N/O	25.3	**18.6**
IL-3		5.0	15.3	7.3	7.7	8.0	**8.7**		18.3	4.7	17.3	N/O	34.7	**18.8**
5*10^4		6.0	11.0	11.3	3.7	6.7	**7.7**		21.3	5.0	24.0	N/O	29.7	**20.0**
2.5*10^5		N/O	15.3	11.7	5.3	20.3	**13.2**		11.7	13.7	17.3	N/O	35.7	**19.6**
MIX ASSAY NEUT	BFU-E													
G		0.7	2.7	4.3	4.3	3.0	**3.0**		2.3	0.7	10.3	N/O	2.7	**4.0**
GM		1.0	0.0	2.7	2.3	4.0	**2.0**		1.3	1.3	6.3	N/O	4.7	**3.4**
IL-3		1.0	0.7	4.7	5.3	3.3	**3.0**		3.3	0.7	9.0	N/O	3.3	**4.1**
5*10^4		2.7	1.3	3.0	0.7	4.7	**2.5**		0.0	0.7	10.7	N/O	3.7	**3.8**
2.5*10^5		N/O	7.0	1.3	0.0	22.0	**7.6**		0.7	2.3	4.3	N/O	7.0	**3.6**

N/O = Not obtained
H13-H17 = Infected group
H11, H12, H18, H19, H21 = Control group

Table 6.2. Mean number of colonies grown per cat at weeks 5-6 post-infection (point of neutropaenia)

GM ASSAY WEEK 12	GM-CFU	H13	H14	H15	H16	H17	MEAN		H11	H12	H18	H19	H21	MEAN
G		10.7	72.0	20.3	16.0	15.0	**26.8**		26.7	55.3	33.0	37.3	36.7	**37.8**
GM		10.0	71.0	21.0	14.0	3.0	**23.8**		29.3	41.3	15.3	20.0	25.3	**26.2**
IL-3		12.3	53.3	13.0	10.3	0.0	**17.8**		22.3	29.0	14.0	12.3	45.7	**24.7**
5*10^4		8.3	27.7	17.3	9.3	2.3	**13.0**		16.3	29.0	9.0	8.0	31.0	**18.7**
2.5*10^5		58.5	63.3	53.0	46.7	51.0	**54.5**		166.0	248.0	113.7	78.0	187.7	**158.7**
MIX ASSAY WEEK 12	GM-CFU													
G		16.0	20.3	4.7	11.3	7.0	**11.9**		15.7	34.7	14.7	9.0	24.3	**19.7**
GM		14.5	16.0	6.3	8.7	8.3	**10.8**		13.3	34.0	19.7	11.0	17.0	**19.0**
IL-3		13.7	16.0	7.7	10.0	9.0	**11.3**		13.3	31.3	15.7	7.3	14.3	**16.4**
5*10^4		7.3	17.7	5.7	11.0	5.0	**9.3**		15.7	34.0	11.7	8.7	21.3	**18.3**
2.5*10^5		4.0	16.0	11.3	14.3	19.7	**13.1**		15.7	60.7	34.0	16.7	31.7	**31.8**
MIX ASSAY WEEK 12	BFU-E													
G		0.0	9.3	1.3	2.0	5.0	**3.5**		5.0	6.7	4.3	0.3	0.0	**3.3**
GM		0.0	7.0	1.3	2.7	4.0	**3.0**		0.7	4.7	11.0	1.0	0.7	**3.6**
IL-3		0.0	6.0	0.3	2.7	6.0	**3.0**		1.0	5.7	8.0	1.7	0.3	**3.3**
5*10^4		0.0	4.3	1.3	2.0	6.0	**2.7**		0.7	7.0	4.7	0.7	1.3	**2.9**
2.5*10^5		0.7	0.0	0.3	1.3	11.3	**2.7**		0.7	11.3	2.7	1.3	0.7	**3.3**

N/O = Not obtained
H13-H17 = Infected group
H11, H12, H18, H19, H21 = Control group

Table 6.3. Mean number of colonies grown per cat at week 12 post-infection

GM ASSAY WEEK 20	GM-CFU	H13	H14	H15	H16	H17	MEAN		H11	H12	H18	H19	H21	MEAN
G		7.3	9.0	6.0	1.7	5.7	**5.9**		2.0	25.7	56.7	0.7	10.7	**19.2**
GM		6.7	0.7	4.3	3.0	14.0	**5.7**		1.0	13.3	27.0	0.7	13.3	**11.1**
IL-3		7.0	0.0	2.7	0.0	8.0	**3.5**		1.3	12.3	15.0	0.7	7.7	**7.4**
5*10^4		2.0	0.7	1.3	1.7	56.3	**12.4**		1.3	6.7	12.0	0.3	5.0	**5.1**
2.5*10^5		130.7	28.0	18.7	33.0	78.7	**57.8**		56.3	137.7	116.7	25.3	108.7	**88.9**
MIX ASSAY WEEK 20	GM-CFU													
G		41.3	5.7	11.0	7.0	11.0	**15.2**		6.3	12.7	69.7	11.0	24.3	**24.8**
GM		104.7	8.7	12.3	14.7	9.3	**29.9**		8.3	12.0	128.3	10.7	31.0	**38.1**
IL-3		35.7	13.7	15.0	10.3	6.7	**16.3**		4.7	8.3	64.0	12.0	23.0	**22.4**
5*10^4		44.7	10.7	9.7	6.7	8.3	**16.0**		9.0	9.3	78.0	5.7	22.3	**24.9**
2.5*10^5		159.0	48.0	28.3	31.0	28.7	**59.0**		13.7	29.0	67.3	28.7	40.3	**35.8**
MIX ASSAY WEEK 20	BFU-E													
G		4.0	0.7	0.3	0.7	4.7	**2.1**		0.0	0.7	4.0	0.0	2.3	**1.4**
GM		0.3	3.0	0.0	0.3	4.0	**1.5**		0.0	2.7	5.0	0.0	0.7	**1.7**
IL-3		0.7	3.0	1.0	1.3	2.7	**1.7**		0.3	0.0	6.0	0.0	4.7	**2.2**
5*10^4		1.7	3.0	0.3	1.0	2.7	**1.7**		0.0	2.0	11.0	0.0	3.0	**3.2**
2.5*10^5		12.7	10.3	2.7	3.3	22.0	**10.2**		0.3	7.7	12.3	0.3	6.0	**5.3**

N/O = Not obtained
H13-H17 = Infected group
H11, H12, H18, H19, H21 = Control group

Table 6.4. Mean number of colonies grown per cat at week 20 post-infection

GM ASSAY EUTH	GM-CFU	H13	H14	H15	H16	H17	MEAN		H11	H12	H18	H19	H21	MEAN
G		17.0	29.0	34.5	17.7	6.3	**20.9**		13.3	26.0	71.7	37.7	46.7	**39.1**
GM		14.7	13.0	18.5	11.7	20.0	**15.6**		18.0	18.3	67.0	24.0	35.0	**32.5**
IL-3		12.3	5.7	14.7	3.7	1.0	**7.5**		2.0	6.7	35.7	14.3	51.7	**22.1**
5*10^4		8.7	15.7	11.7	8.7	1.0	**9.2**		1.7	2.3	33.0	0.3	29.3	**13.3**
2.5*10^5		24.0	26.7	137.5	45.0	58.7	**58.4**		74.3	62.3	152.7	50.3	130.7	**94.1**
MIX ASSAY EUTH	GM-CFU													
G		11.7	12.3	2.3	14.0	7.7	**9.6**		10.7	16.3	6.7	18.0	26.7	**15.7**
GM		15.0	17.3	1.0	22.0	11.0	**13.3**		13.7	10.3	13.7	19.3	18.3	**15.1**
IL-3		16.0	9.7	1.3	9.3	8.7	**9.0**		11.7	7.0	5.7	10.3	20.3	**11.0**
5*10^4		16.7	6.0	1.0	10.0	4.7	**7.7**		13.3	2.0	9.3	13.0	18.3	**11.2**
2.5*10^5		53.0	38.0	7.0	199.7	18.0	**27.1**		32.3	15.3	37.3	44.7	52.3	**36.4**
MIX ASSAY EUTH	BFU-E													
G		0.3	2.7	0.3	1.3	0.0	**0.9**		0.0	0.0	0.3	0.0	1.7	**0.4**
GM		0.7	2.7	0.3	1.3	0.0	**1.0**		0.0	0.0	0.7	0.3	1.0	**0.4**
IL-3		1.7	3.7	0.0	0.7	0.3	**1.3**		0.0	0.0	1.0	0.0	0.7	**0.3**
5*10^4		2.0	2.0	0.3	2.7	0.0	**1.4**		0.0	0.0	6.0	0.3	2.0	**1.7**
2.5*10^5		4.0	17.7	4.3	6.7	0.0	**6.5**		0.0	0.0	11.0	1.3	5.0	**3.5**

N/O = Not obtained
H13-H17 = Infected group
H11, H12, H18, H19, H21 = Control group

Table 6.5. Mean number of colonies grown per cat at week 27-29 post-infection

GM ASSAY WEEK 3	GM-CFU		H13/H18	H14/H19	H15/H21	H16/H11	H17/H12	MEAN
CELLS	FEEDERS							
INF	INF		36.3	26.3	19.7	33.7	23.3	**27.9**
INF	CON		29.0	29.3	14.3	25.3	40.3	**27.6**
CON	INF		532.0	15.7	67.0	40.7	15.3	**134.1**
CON	CON		548.0	12.3	76.3	31.3	30.0	**139.6**
MIX ASSAY WEEK 3	GM-CFU							
CELLS	FEEDERS							
INF	INF		30.0	20.3	13.0	8.7	11.3	**16.7**
INF	CON		17.3	23.0	17.7	13.7	10.0	**16.3**
CON	INF		116.7	8.7	46.3	13.0	22.3	**41.4**
CON	CON		104.5	10.7	50.3	17.0	23.7	**41.2**
MIX ASSAY WEEK 3	BFU-E							
CELLS	FEEDERS							
INF	INF		5.7	9.3	5.0	2.3	4.0	**5.3**
INF	CON		3.0	9.7	3.7	5.3	0.7	**4.5**
CON	INF		19.0	0.7	5.7	0.7	4.7	**6.2**
CON	CON		13.5	1.0	7.7	1.0	3.3	**5.3**

INF = Infected
CON = Control

Table 6.6. Mean number of colonies grown per cat according to type of feeder cells at week 3 post-infection

GM ASSAY NEUT	GM-CFU		H13/H19	H14/H21	H15/H18	H16/H11	H17/H12	MEAN
CELLS	FEEDERS							
INF	INF		6.7	11.7	28.3	22.3	5.0	**14.8**
INF	CON		7.0	4.0	25.7	18.3	7.7	**12.5**
CON	INF		0.0	40.3	51.7	32.7	2.7	**25.5**
CON	CON		0.3	43.0	52.3	74.7	2.7	**34.6**
MIX ASSAY NEUT	GM-CFU							
CELLS	FEEDERS		2.7	N/O	2.0	2.0	4.3	**2.8**
INF	INF		6.0	N/O	4.7	6.0	8.3	**6.3**
INF	CON		0.3	16.7	13.7	11.3	8.7	**10.1**
CON	INF		0.3	21.3	13.3	15.0	9.0	**11.8**
CON	CON							
MIX ASSAY NEUT	BFU-E							
CELLS	FEEDERS							
INF	INF		0.0	N/O	0.7	0.0	2.3	**0.8**
INF	CON		2.0	N/O	2.3	1.0	5.3	**2.7**
CON	INF		0.0	7.3	1.7	0.7	1.3	**2.2**
CON	CON		0.0	3.3	5.0	0.0	2.0	**2.1**

INF = Infected
CON = Control

Table 6.7. Mean number of colonies grown per cat according to type of feeder cells at weeks 5-6 post-infection (point of neutropaenia)

GM ASSAY WEEK 12	GM-CFU		H13/21	H14/18	H15/19	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		11.0	44.3	10.3	13.7	3.7	**16.6**
INF	CON		14.0	66.3	12.3	12.0	8.0	**22.5**
CON	INF		29.7	16.3	14.0	27.0	45.0	**26.4**
CON	CON		45.7	24.7	14.0	26.3	39.7	**30.1**
MIX ASSAY WEEK 12	GM-CFU		H13/21	H14/18	H15/19	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		8.3	6.7	8.7	7.7	8.0	**7.9**
INF	CON		8.7	18.0	16.7	8.3	6.0	**11.5**
CON	INF		15.7	13.0	3.3	8.3	21.0	**12.3**
CON	CON		11.7	13.7	3.3	10.7	22.0	**12.3**
MIX ASSAY WEEK 12	BFU-E		H13/21	H14/18	H15/19	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		0.0	3.0	0.7	0.0	4.3	**1.6**
INF	CON		0.3	3.3	1.3	1.3	4.3	**2.1**
CON	INF		0.0	2.0	0.0	0.0	1.7	**0.7**
CON	CON		0.7	6.0	0.3	0.0	4.0	**2.2**

INF = Infected
CON = Control

Table 6.8. Mean number of colonies grown per cat according to type of feeder cells at week 12 post-infection

GM ASSAY WEEK 20	GM-CFU		H13/19	H14/18	H15/21	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		9.3	4.3	3.7	5.3	18.0	**8.1**
INF	CON		14.3	3.3	5.3	13.7	15.0	**10.3**
CON	INF		1.0	28.7	15.0	12.0	17.5	**14.8**
CON	CON		3.0	31.3	9.7	9.3	18.7	**14.4**
MIX ASSAY WEEK 20	GM-CFU		H13/19	H14/18	H15/21	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		65.3	4.7	9.3	9.3	7.3	**19.2**
INF	CON		66.7	6.0	8.7	9.0	10.7	**20.2**
CON	INF		6.7	68.7	27.7	9.0	11.3	**24.7**
CON	CON		9.3	44.7	19.7	7.0	12.7	**18.7**
MIX ASSAY WEEK 20	BFU-E		H13/19	H14/18	H15/21	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		4.7	0.0	0.0	0.0	3.3	**1.6**
INF	CON		3.3	0.3	0.3	0.3	4.0	**1.6**
CON	INF		0.0	4.3	2.0	0.0	1.7	**1.6**
CON	CON		0.0	1.3	0.3	0.0	2.7	**0.9**

INF = Infected
CON = Control

Table 6.9. Mean number of colonies grown per cat according to type of feeder cells at week 20 post-infection

GM ASSAY EUTH	GM-CFU		H13/19	H14/21	H15/18	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		0.3	13.7	26.7	9.7	4.7	**11.0**
INF	CON		36.3	29.7	20.7	11.7	2.0	**20.1**
CON	INF		18.0	42.7	39.3	7.0	10.0	**23.4**
CON	CON		29.3	33.0	41.3	6.3	8.3	**23.6**
MIX ASSAY EUTH	GM-CFU		H13/19	H14/21	H15/18	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		10.7	9.3	5.7	8.0	6.3	**8.0**
INF	CON		13.3	14.0	4.0	12.7	13.0	**11.4**
CON	INF		16.0	22.3	14.7	11.3	7.0	**14.3**
CON	CON		10.3	20.0	11.0	13.3	8.7	**12.7**
MIX ASSAY EUTH	BFU-E		H13/19	H14/21	H15/18	H16/11	H17/12	MEAN
CELLS	FEEDERS							
INF	INF		0.0	4.3	2.0	0.7	0.0	**1.4**
INF	CON		0.0	3.0	0.7	1.0	0.0	**0.9**
CON	INF		0.0	2.3	4.3	0.0	0.0	**1.3**
CON	CON		0.0	2.3	3.0	0.0	0.7	**1.2**

INF = Infected
CON = Control

Table 6.10. Mean number of colonies grown per cat according to type of feeder cells added at weeks 27-29 post-infection

Table 6.11. P-values for analysis of variance comparing infected and control groups over time for various growth factor sources

		Autologous cells + G-CSF	Autologous cells + GM-CSF	Autologous cells + IL-3	Autologous cells @ 5×10^4/ml	Autologous cells @ 2.5×10^5/ml
GM-CFU (AGAR)	GROUP * TIME	0.726	0.909	0.932	0.188	0.719
GM-CFU (AGAR)	GROUP	**0.000**	**0.000**	**0.000**	**0.002**	**0.000**
GM-CFU (MC)	GROUP * TIME	0.917	0.322	0.273	0.287	**0.000**
GM-CFU (MC)	GROUP	**0.000**	**0.000**	**0.000**	0.191	**0.000**
BFU-E	GROUP * TIME	0.752	0.660	0.671	0.956	0.116
BFU-E	GROUP	**0.027**	0.641	0.401	0.844	**0.018**

Table 6.12. P-values for analysis of variance comparing infected and control groups (GM-CFU grown in agar)

	WEEK 3	WEEK 5-6	WEEK 12	WEEK 20	WEEK 27-29
Cells + G-CSF	0.246	> 0.05	0.127	0.606	0.165
Cells + GM-CSF	0.332	> 0.05	0.370	0.630	**0.041**
Cells + IL-3	0.217	> 0.05	0.283	0.498	0.240
Autologous cells @ 5 x 10^4/ml	0.222	> 0.05	0.413	0.797	0.975
Autologous cells @ 2.5 x 10^5/ml	> 0.05	> 0.05	**0.000**	0.260	0.106

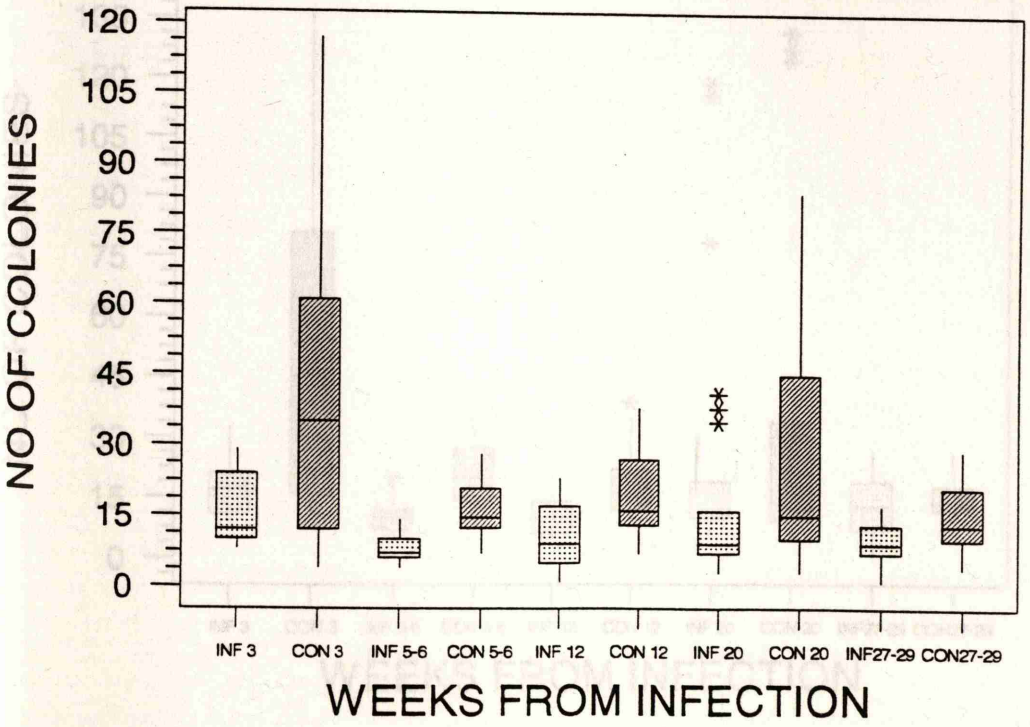
Table 6.13. P-values for analysis of variance comparing infected and control groups (GM-CFU in methylcellulose)

	WEEK 3	WEEK 5-6	WEEK 12	WEEK 20	WEEK 27-29
Cells + G-CSF	0.247	**< 0.05**	0.163	0.485	0.215
Cells + GM-CSF	0.094	**< 0.05**	0.066	0.780	0.434
Cells + IL-3	0.192	> 0.05	0.266	0.890	0.494
Autologous cells @ 5 x 10^4/ml	0.112	0.051	0.076	0.696	0.364
Autologous cells @ 2.5 x 10^5/ml	> 0.05	> 0.05	**0.043**	0.446	0.327

Table 6.14. P-values for analysis of variance comparing infected and control groups (BFU-E in methylcellulose)

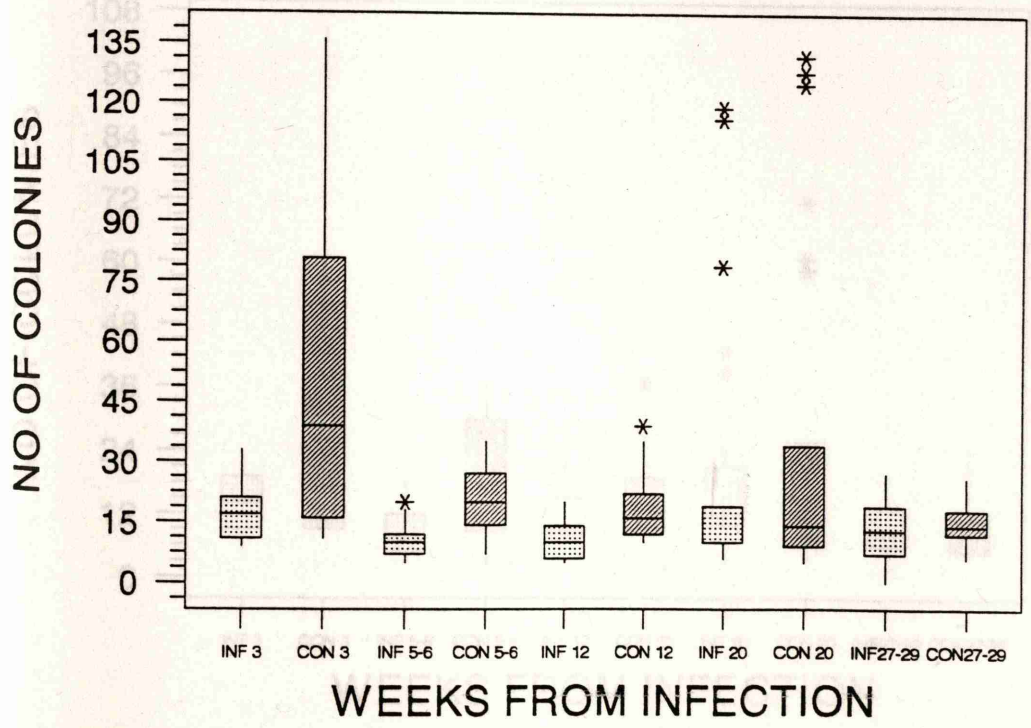
	WEEK 3	WEEK 5-6	WEEK 12	WEEK 20	WEEK 27-29
Cells + G-CSF	0.342	> 0.05	0.908	0.573	0.439
Cells + GM-CSF	0.752	> 0.05	0.939	0.958	0.340
Cells + IL-3	0.815	> 0.05	0.854	0.847	0.201
Autologous cells @ 5 x 10^4/ml	0.856	0.715	0.907	0.933	0.833
Autologous cells @ 2.5 x 10^5/ml	> 0.05	> 0.05	0.614	0.227	0.400

FIG 6.1 BOXPLOT OF COLONIES GROWN (+G-CSF) FOR INFECTED & CONTROL CATS



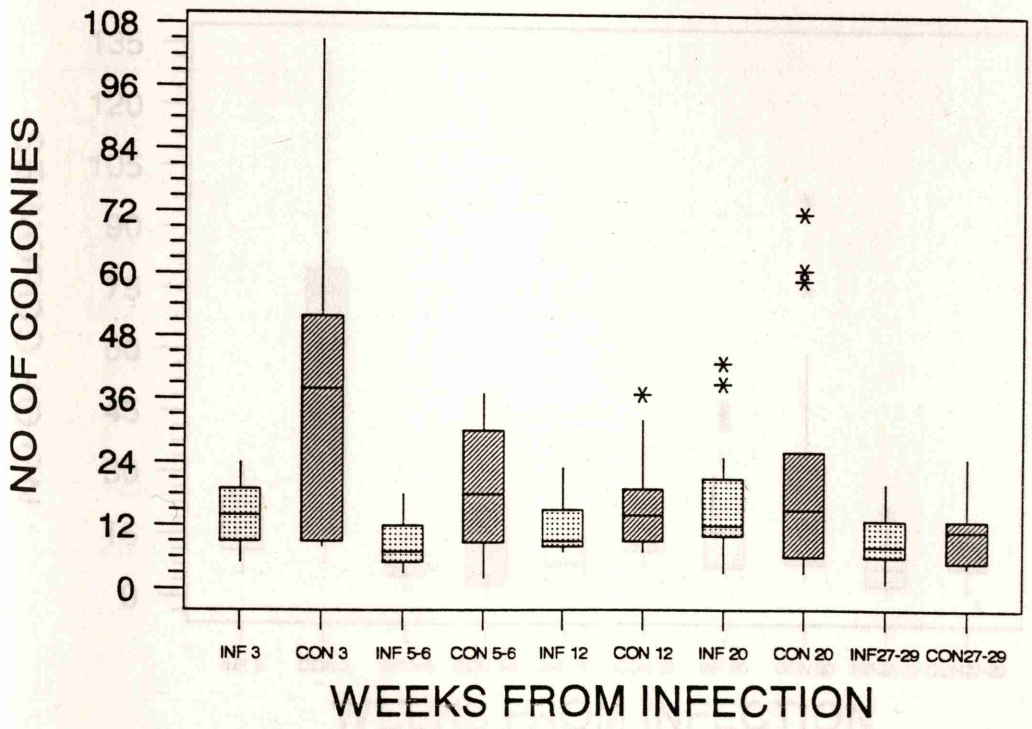
INF = infected
CON = control
3 = 3 weeks p.i.
5-6 = 5-6 weeks p.i.
12 = 12 weeks p.i.
20 = 20 weeks p.i.
27-29 = 27-29 weeks p.i.

IG 6.2 BOXPLOT OF COLONIES GROWN (+GM-CSF) FOR INFECTED & CONTROL CATS



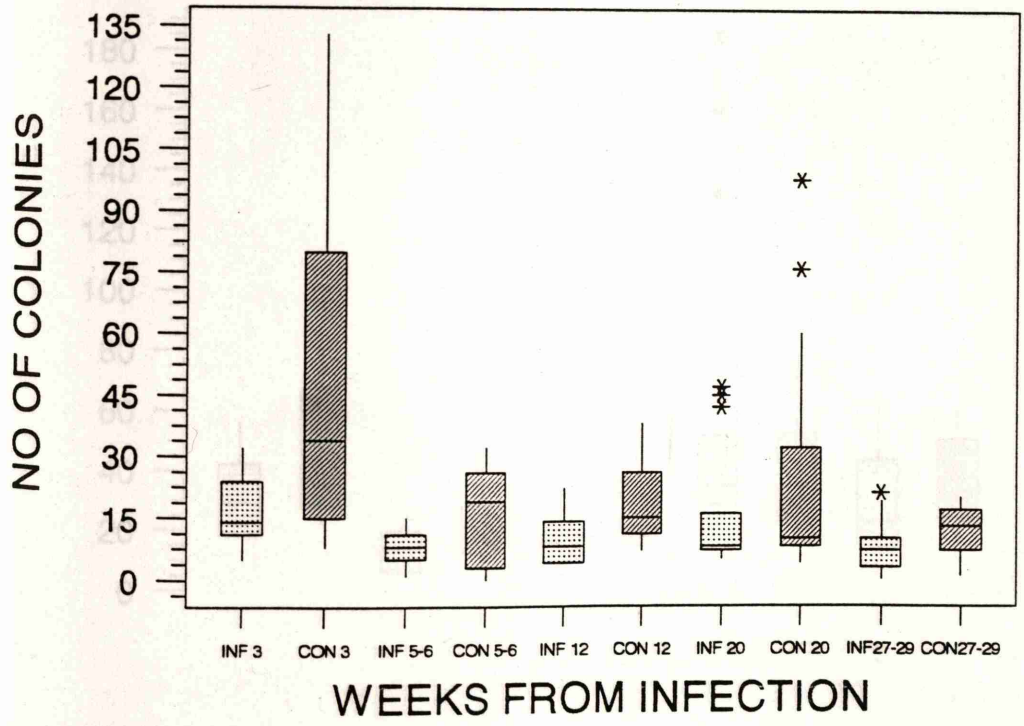
INF = infected
CON = control
3 = 3 weeks p.i.
5-6 = 5-6 weeks p.i.
12 = 12 weeks p.i.
20 = 20 weeks p.i.
27-29 = 27-29 weeks p.i.

FIG 6.3 BOXPLOT OF COLONIES GROWN (+IL-3) FOR INFECTED & CONTROL CATS



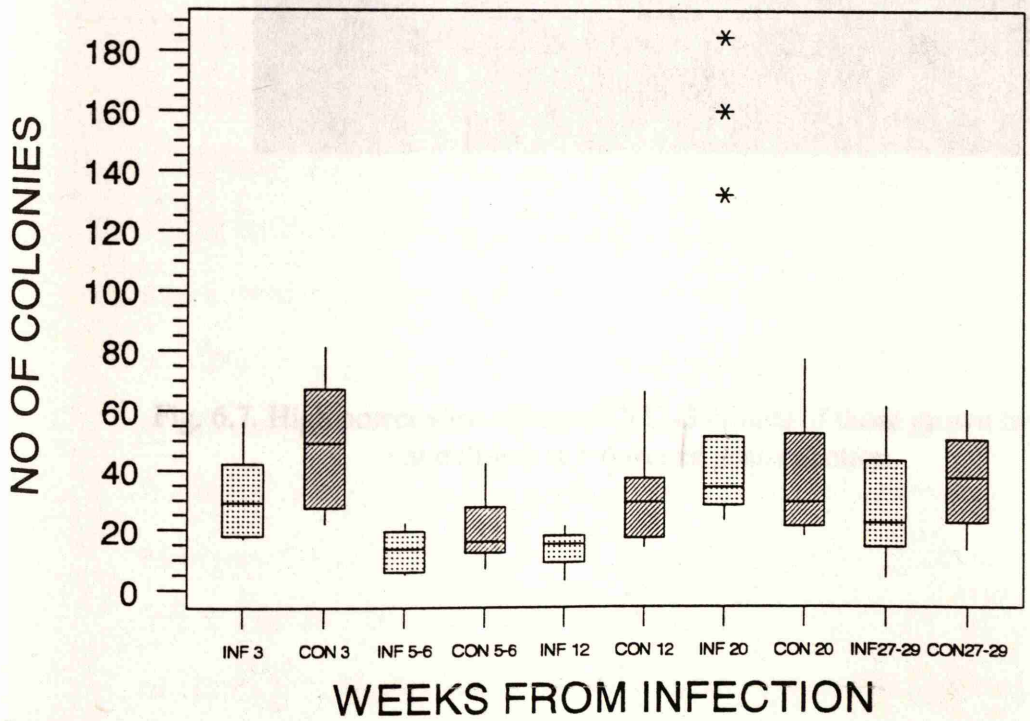
INF = infected
CON = control
3 = 3 weeks p.i.
5-6 = 5-6 weeks p.i.
12 = 12 weeks p.i.
20 = 20 weeks p.i.
27-29 = 27-29 weeks p.i.

FIG 6.4 BOXPLOT OF COLONIES GROWN (5x10^4/ml) FOR INFECTED & CONTROL CATS



INF = infected
CON = control
3 = 3 weeks p.i.
5-6 = 5-6 weeks p.i.
12 = 12 weeks p.i.
20 = 20 weeks p.i.
27-29 = 27-29 weeks p.i.

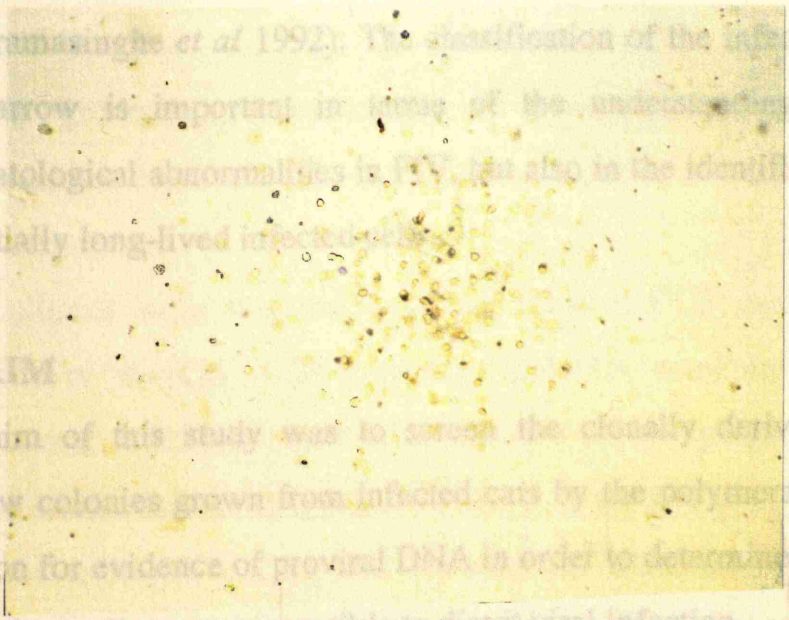
FIG 6.5 BOXPLOT OF COLONIES GROWN (2.5x10^5/ml) FOR INFECTED & CONTROL CATS



INF = infected
CON = control
3 = 3 weeks p.i.
5-6 = 5-6 weeks p.i.
12 = 12 weeks p.i.
20 = 20 weeks p.i.
27-29 = 27-29 weeks p.i.

Fig. 6.6. Low power view of small CFU-G typical of those grown in infected cat cultures at 5-6 weeks post-infection.

Fig. 6.7. High power view of small CFU-G typical of those grown in infected cat cultures at 5-6 weeks post-infection.



CHAPTER 7

DETECTION OF FIV IN BONE MARROW PRECURSOR CELLS BY THE POLYMERASE CHAIN REACTION

7.1. INTRODUCTION

Lentiviruses are known to infect a number of different cell types. FIV and HIV have tropisms for lymphocytes and monocytes, but a number of other cell types have been reported to have been infected. There is considerable debate as to the type of infected cells in bone marrow. Unidentified mononuclear cells, megakaryocytes (Beebe *et al* 1994), and stromal cells have all been positively identified as being infected with virus, suggesting that it is infection of accessory cells which is responsible for haemopoietic dysfunction. The evidence for infection of bone marrow stem and progenitor cells is conflicting, as is described in the general introduction. There has been limited evidence for the infection of immature myeloid cells and nucleated red cells in HIV-infected people (Sun *et al* 1989, Wickramasinghe *et al* 1992). The classification of the infected cells in marrow is important in terms of the understanding of the haematological abnormalities in FIV, but also in the identification of potentially long-lived infected cells.

7.2. AIM

The aim of this study was to screen the clonally derived bone marrow colonies grown from infected cats by the polymerase chain reaction for evidence of proviral DNA in order to determine whether progenitor cells were susceptible to direct viral infection.

7.3. MATERIALS AND METHODS

The sensitivity of the PCR reaction was determined using infected lymphocytes (T3/G8 cells) obtained from Dr. Margaret Hosie in the Department of Veterinary Pathology, Glasgow. Cells were diluted to 3.1 x 10^6/ml, 1 x 10^4/ml, 5 x 10^3/ml, 1 x 10^3/ml, 5 x 10^2/ml and 1 x 10^2/ml. The cell suspensions were boiled in a water bath for 5 minutes to destroy cell membranes and disrupt DNA. Fifteen microlitre aliquots of these cell preparations were then subject to hot-start PCR and Southern blotting as described for the colonies.

Colonies grown in GM and mix assays at each time point were picked from the agar or methylcellulose respectively, washed and resuspended in water. Twelve colonies were picked from each set of GM assays from each infected cat, and 30 from the mix assays from each cat. Four and 6 colonies were picked from uninfected cat GM and mix assays respectively as a control. The colonies picked were of any cell line, the respective proportions of GM-CFU, G-CFU, M-CFU and BFU-E broadly reflected the proportions grown in the plates, and were therefore variable between cats and between time points.

Colonies grown from 2 cats infected for 4 years (Q257, Q258) and an age matched control (Q264) were also subjected to PCR analysis in the same way.

The colonies were then subjected to hot-start PCR analysis for evidence of the FIV LTR sequence. Colonies were screened in random order. PCR products were run down a 4% polyacrylimide gel and electroblotted. A specific FIV LTR probe was then used in a Southern blot to confirm the presence of the viral sequence. Radiographic films were developed over both long and short exposures to ensure maximum accuracy of results.

Following the LTR PCR, a proportion of negative colonies were checked for the presence of adequate quantities of DNA to try to eliminate false negative results.

Cμ primers @ 1μg/μl from a highly homologous region of human, mouse and hamster IgM were obtained from Anne Terry in the Department of Veterinary Pathology for this purpose. The sequences were as follows:

5' end: 5' TGC CTG GTG ACG GGC TTC TCT CC 3'

3' end: 5' ATG CAA CAT CTC ACC C 3'

These primers allowed for amplification of a 410bp fragment. A hot-start PCR was used as for LTR PCR using the same reagents (GeneAmp PCR Reagent Kit and AmpliTaq DNA polymerase, Perkin-Elmer Ltd., Beaconsfield, UK) with wax layers composed as follows:

Lower layer: 1.25μl PCR buffer

1μl dNTP (x4)

0.5μl primers (as above)

5.25μl purified H_2O

Upper layer: 5μl PCR buffer

0.25μl TAQ polymerase

22.25μl purified H_2O

10μl colony sample mix.

The tubes were cycled in the thermal cycler (Omnigene TR3, Hybaid Ltd., Teddington, UK) using the following conditions:

1 min @ 94°C}

1 min @ 60°C} 30 cycles

1 min @ 72°C}

The products were then run on gels and electroblotted in the same way as the LTR PCR products. Southern blots were also performed in the same way, using a ^{32}P labelled probe generated using the above primers on normal feline kidney DNA.

7.4. RESULTS

Sensitivity testing using infected lymphocytes indicated the PCR method could detect sequence in as few as 500 lymphocytes. False positive results in infected cats may have arisen as a result of picking up infected macrophages or lymphocytes along with the colonies from the plates. Great care was taken to try to avoid this, so that any background cells would only have been present in low numbers, well below the detection threshold of the PCR assay. False positives could also have arisen from leakage of PCR product from adjacent wells during the running of the gel, or from shadows on the Southern blots.

Colonies were screened as positive or negative by looking at both exposures and by comparing with gel pictures. It was noted that gels were less sensitive than blots for detecting viral sequences. The agar results are shown in table 7.1 and figure 7.1. None of the control samples from agar were positive for proviral DNA. Colonies from methylcellulose were also screened in random order and the results are shown in tables 7.2 and 7.3 and figure 7.2. One colony, from control cat H19, of a total of 128 colonies tested from control cats tested positive for provirus. This error may have been due to

leakage of PCR product from an adjacent well, or from PCR contamination. All colony types tested were found to be susceptible to virus infection. Relative rates between colony types are represented on figure 7.3. Representative blots are shown in figures 7.4 and 7.5. The relative proportion of positive colonies from the total grown from infected cats in methylcellulose are shown in fig. 7.7.

Results from the colonies from the long-term asymptomatic cats are represented in tables 7.4 and 7.5.

Two bands were present on both gels and Southern blots. The lower band seen in figs 7.4 and 7.5 was the 354bp fragment which was expected to be amplified. The upper band was double the size of this band, and was usually less intense, but was occasionally of equal or greater intensity. The fact the band was also present on the blots indicates that it was a specific product. The most likely explanation is dimer formation of the PCR product.

73 of a total of 343 colonies (21.6%) negative for the FIV LTR sequence were tested for DNA using the Cμ primers to eliminate false negative results. The colonies were from both agar and methylcellulose assays and represented those from infected and control cats. Seventy of seventy-three (95.6%) of these colonies were positive for the Cμ sequence by Southern blot (see figure 7.6), indicating that negative results in the LTR PCR were unlikely to be due to a lack of sufficient DNA in the colony preparation.

7.5. DISCUSSION

Hot start PCR was a very effective and sensitive method of detecting proviral DNA in small numbers of colony cells. The results indicate that FIV was capable of infecting bone marrow

progenitor cells. This confirms the results of several authors who found HIV, albeit at low frequencies, in marrow progenitors (Kojouharoff *et al* 1991, Steinberg *et al* 1991, Kaczmarski *et al* 1992, Chelucci *et al* 1995). However, the results suggested that the infection was not at a constant rate. The pattern of infection was similar in all the infected cats, and for both agar and methylcelluose colonies, indicating all types and stages of colony grown in these assays were equally infectable (table 7.3). The proportion of infected colonies did not appear to be affected by the changing percentages of colony type from each cat at each time point.

At three weeks post-infection, approximately half of colonies were positive for infection (tables 7.1 & 7.2). However, by 5-6 weeks p.i., when the cats were becoming neutropaenic and colony numbers were dropping, the proportion of infected colonies rose to 70-80%. The colonies picked for analysis at this time tended to be small, with fewer cells. However, by week 12, when peripheral neutrophil counts had returned to normal, and colony numbers had recovered, the proportion of infected colonies had risen to approximately 90%. The proportion then fell at 20 weeks, and by 27-29 weeks had fallen back to 60-70%.

The relationship between progenitor cells and FIV is clearly a dynamic one. FIV infection does not appear to be lethal, at least not in all cells, as infected progenitors are surviving and proliferating. All lineages tested in this study had committed progenitors infected by provirus, at equal rates of infection (table 7.3). This suggests that either a more primitive common progenitor was infected, or that virus was capable of infecting each committed progenitor cell line individually. There was no dramatic difference between infection

rates in different cell lines, suggesting that the more likely explanation is that a more primitive common progenitor or stem cell was being infected.

It has recently been suggested that the pathogenesis of HIV consists of continuous viral production in a population of long-lived infected cells (e.g. macrophages), infection of other cells by the virus produced in the long-term infected population and destruction of these cells (Ho *et al* 1995, Wei *et al* 1995). It is likely that a similar mechanism operates in FIV. The proportion of cells infected would therefore depend on the degree of viral replication at a given time. In the initial stages of infection virus is known to be spreading throughout the body and numbers of infected cells are increasing. Virus production in infected cells reaches high levels in the period around 6-8 weeks after infection. This results in the release of large quantities of infectious virus capable of infecting stem cells or their immediate progeny. Simultaneously, there is probably an increased demand for mature neutrophils coinciding with the onset of acute clinical illness. Functional, non-infected committed neutrophil precursors undergo increased cell cycles and supply the demand initially, but become exhausted and die. These cells may not be immediately replaced due to the high numbers of primitive precursors infected, these cells perhaps being functionally impaired. There is therefore a temporary drop in neutrophil production until progenitor numbers are restored. This drop is not seen in other cell lineages as the mature cells are longer lived and turnover rates are not so high. Initially (6-12 weeks) as there is still a great deal of infectious viral production, the replacement cells are infected, but there are enough to satisfy demand. Gradually, as viral replication

falls to basal levels, numbers of uninfected progenitors are restored and neutrophil production returns to within normal limits. Numbers of infected stem and progenitor cells would remain at low levels during the asymptomatic phase, but would rise in association with any increase in infectious virus production. This would make detection of virus in progenitor cells at this stage more difficult.

The results obtained from the long-term infected cats in this study reflect those of cats at 27-29 weeks, that is approximately 50% of colonies positive. This is perhaps higher than would be expected during the asymptomatic phase, given the preceding argument. Alternatively, this may be the basal level of infection in asymptomatic cats. The agar results from the 2 cats give remarkably different proportions of infected cells. This may have been due to the poor colony size and growth in the agar plates from cat Q258. Drying out of the agar, making it more difficult to wash off, and consequently increasing the volume in the PCR reaction may have inhibited the reaction. A larger sample size may have yielded more uniform results. A single colony from the control cat was positive by Southern blot. This is likely to have occurred due to PCR contamination.

Table 7.1. Positive PCR Results for Agar Assay Colonies

CAT NO.	WEEK 3	WEEK 5-7	WEEK 12	WEEK 20	WEEK 27-29		TOTAL
H13	9/13	12/12	11/12	12/12	10/12		54/61
	69.2%	100%	91.7%	100%	83.3%		88.5%
H14	1/12	6/12	10/11	10/12	4/12		31/59
	8.3%	50.0%	90.9%	83.3%	33.3%		52.5%
H15	4/12	12/12	9/12	8/12	10/12		43/60
	33.3%	100%	75.0%	66.6%	83.3%		71.7%
H16	10/12	12/12	11/12	11/12	11/12		55/60
	83.3%	100%	91.7%	91.7%	91.7%		91.7%
H17	8/12	11/12	11/12	12/12	7/12		49/60
	66.6%	91.7%	91.7%	100%	58.3%		81.7%
TOTAL	32/61	53/60	52/59	53/60	42/60		232/300
	52.5%	88.3%	88.1%	88.3%	70.0%		77.3%

Table 7.2. Positive PCR Results from Methylcellulose Colony Assays

	COLONY TYPE	WEEK 3	WEEK 5-6	WEEK 12	WEEK 20	WEEK 27-29	TOTAL	TOTAL %
H13	G	3/7	10/10	8/8	0/2	17/18	38/45	84.4%
	M	2/8	8/11	17/17	0/8	0/0	27/44	61.4%
	GM	0/0	1/2	2/4	1/8	4/11	8/25	32.0%
	BFU-E	6/9	4/5	0/0	3/6	0/1	13/21	61.9%
	?	0/5	2/2	1/1	0/0	0/0	3/8	37.5%
	TOTAL	11/29 37.9%	25/30 83.3%	28/30 93.3%	4/24 16.7%	21/30 70.0%	89/143 62.2%	
H14	G	2/9	6/8	10/10	9/12	4/10	31/49	63.3%
	M	0/7	8/12	13/13	3/10	1/1	25/43	58.1%
	GM	0/0	0/0	1/1	2/2	3/13	6/16	37.5%
	BFU-E	2/10	5/10	6/6	5/6	0/4	18/36	50.0%
	?	2/4	0/0	0/0	0/0	1/2	3/6	50.0%
	TOTAL	6/30 20.0%	19/30 63.3%	30/30 100%	19/30 63.3%	9/30 30.0%	83/150 55.3%	
H15	G	4/7	2/5	8/8	9/14	16/20	39/54	72.2%
	M	1/6	6/12	13/13	5/7	0/1	25/39	64.1%
	GM	0/0	2/2	0/1	0/2	1/1	3/6	50.0%
	BFU-E	4/10	6/10	6/6	2/6	1/3	19/35	54.0%
	?	2/7	0/0	0/0	1/1	1/5	4/13	30.8%
	TOTAL	11/30 36.7%	16/29 55.2%	26/28 96.4%	17/30 56.7%	19/30 63.3%	90/147 61.2%	

H16	**G**	5/6	10/12	12/15	12/24	15/29	**54/86**	**62.8%**
	M	4/10	4/5	4/5	0/0	0/0	**12/20**	**40.0%**
	GM	0/0	1/2	0/0	2/2	0/0	**3/4**	**75.0%**
	BFU-E	7/10	8/10	5/10	0/1	0/1	**20/32**	**62.5%**
	?	1/4	1/1	0/0	2/3	0/0	**4/8**	**50.0%**
	TOTAL	**17/30** **56.7%**	**24/30** **80.0%**	**21/30** **70.0%**	**16/30** **53.3%**	**15/30** **50.0%**	**93/150** **62.0%**	
H17	**G**	3/6	14/19	6/6	7/9	7/21	**37/61**	**60.7%**
	M	7/10	5/5	7/7	2/3	1/1	**22/26**	**84.6%**
	GM	3/3	2/2	6/6	8/10	2/2	**21/23**	**91.3%**
	BFU-E	4/10	4/4	9/10	6/7	5/7	**28/38**	**73.7%**
	?	0/1	0/0	1/1	1/1	0/0	**2/3**	**66.7%**
	TOTAL	**17/30** **56.7%**	**25/30** **83.3%**	**29/30** **96.7%**	**24/30** **80.0%**	**15/30** **50.0%**	**110/150** **73.3%**	
	TOTAL (ALL)	**62/149** **41.6%**	**109/149** **73.2%**	**135/148** **91.2%**	**80/144** **55.5%**	**79/151** **51.6%**	**465/741** **62.3%**	

G = GM-CFU, M = M-CFU, GM = GM-CFU, ? = unknown type

Table 7.3. Total Positive PCR Results by Colony Type from Mix Assays

	WEEK 3	WEEK 5-6	WEEK 12	WEEK 20	WEEK 27-29		TOTAL
G	17/35	42/54	44/47	37/61	59/98		199/295
	48.6%	77.8%	93.6%	60.7%	60.2%		67.5%
M	14/41	31/45	54/55	10/28	2/3		111/172
	34.1%	68.9%	98.2%	35.7%	66.7%		64.5%
GM	3/3	6/8	9/12	13/24	10/27		41/74
	100%	75.0%	75.0%	54.2%	37.0%		55.4%
BFU-E	23/49	27/39	26/32	16/26	6/16		98/162
	46.9%	69.2%	81.3%	61.5%	37.5%		60.5%
?	5/21	3/3	2/2	4/5	2/7		16/38
	23.8%	100%	100%	80.0%	28.6%		42.1%
TOTAL	62/149	109/149	135/148	80/144	79/151		
	41.6%	73.2%	91.2%	55.5%	51.6%		

G = G-CFU, M = M-CFU, GM = GM-CFU, ? = unknown type

Table 7.4. Positive PCR Results from Long-term Infected Cats (Agar)

CAT NO	POSITIVE COLONIES	TOTAL % POSITIVE
Q257	11/12	91.7%
Q258	1/12	8.3%
Q264	0/6	0.0%

Table 7.5. Positive PCR Results from Long-term Infected Cats (Methylcellulose)

	Q257	Q258	TOTAL		Q264
G	9/16 56.3%	8/12 66.7%	17/28 60.7%		1/5 20.0%
M	0/1 0.0%	0/0 0.0%	0/1 0.0%		0/0 0.0%
GM	2/5 40.0%	5/6 83.3%	7/11 63.6%		0/0 0.0%
BFU-E	5/7 71.4%	1/8 12.5%	6/15 40.0%		0/1 0.0%
?	0/1 0.0%	3/4 75.0%	3/5 60.0%		0/0 0.0%
TOTAL	16/30 53.3%	17/30 56.6%	33/60 55.0%		1/6 16.7%

G = G-CFU, M = M-CFU, GM = GM-CFU, ? = unknown type

Fig. 7.1. Graph of percentage colonies positive for FIV LTR by PCR grown in agar for individual cats

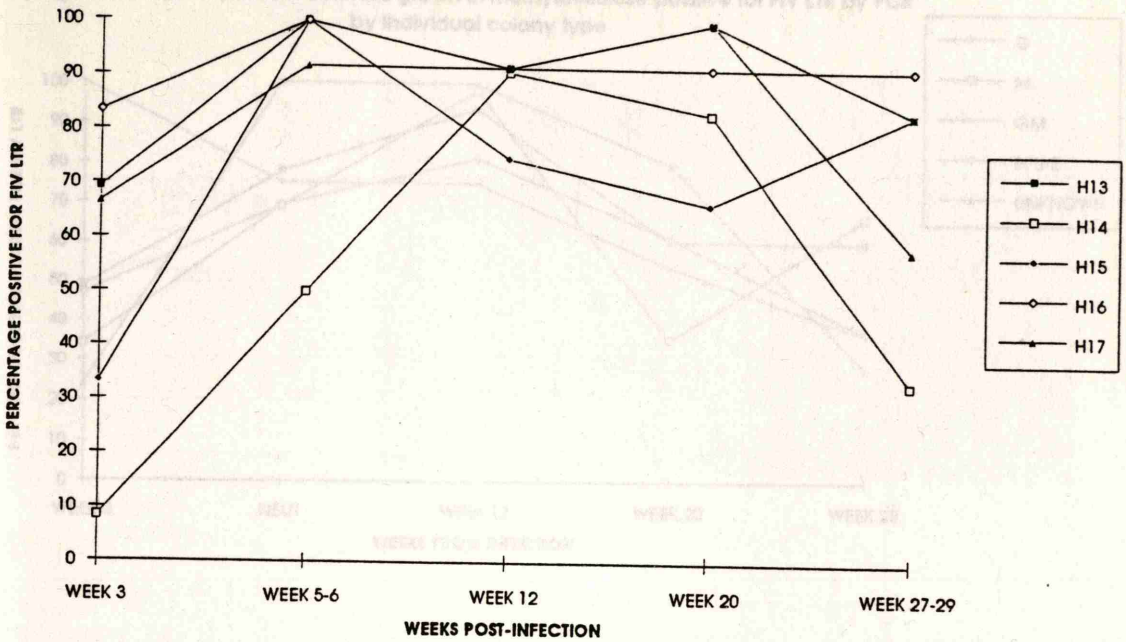


Fig. 7.2. Graph of percent colonies grown in methylcellulose positive for FIV LTR by PCR by individual cats

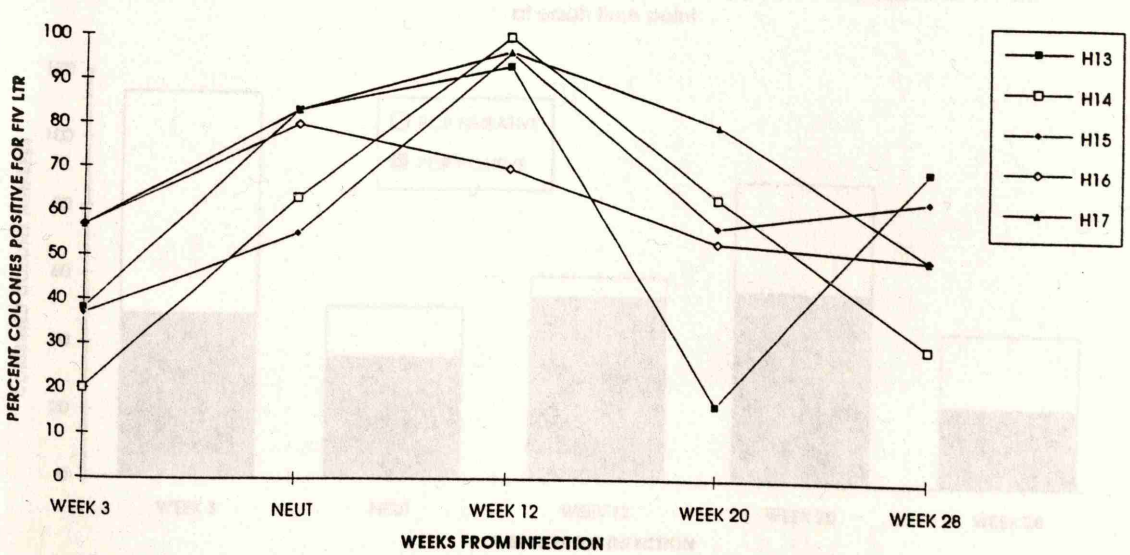


Fig. 7.3. Graph of percent colonies grown in methylcellulose positive for FIV LTR by PCR by individual colony type

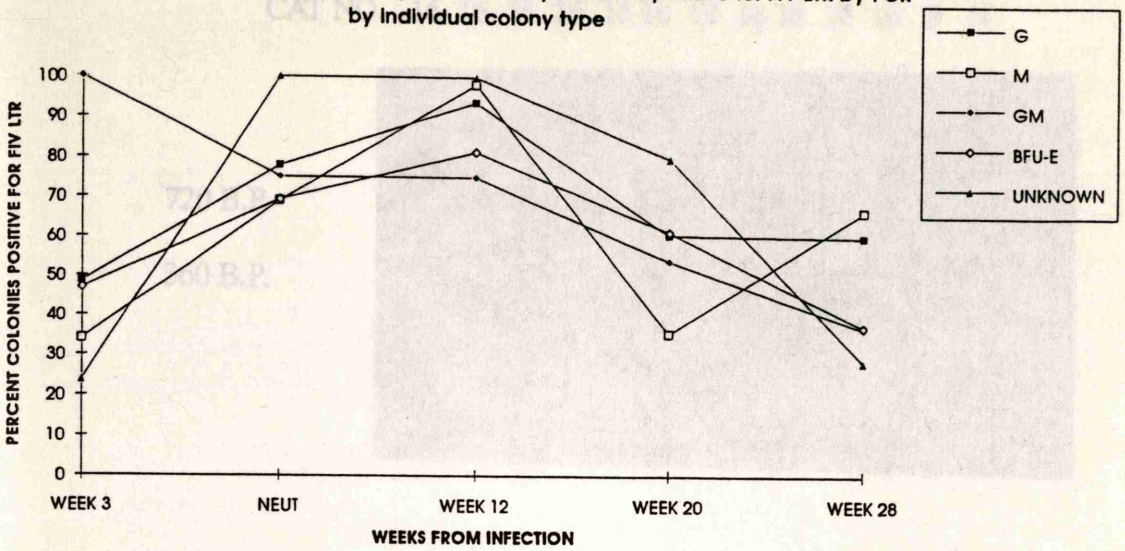
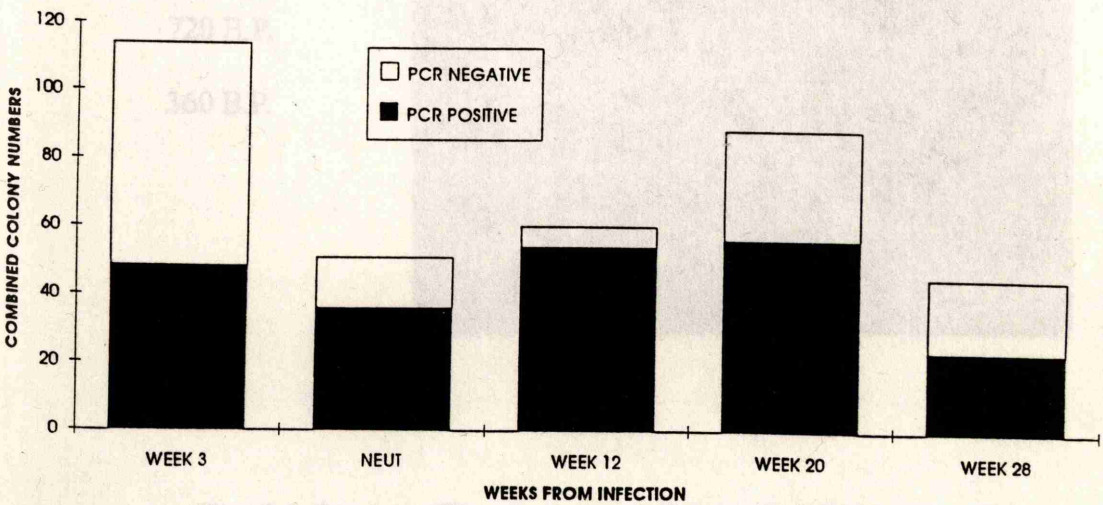


Fig. 7.7. Chart of total number of colonies (infected cats) grown from endogenous growth factors (5 x 10^4/ml) in methylcellulose showing proportion positive for FIV LTR at each time point



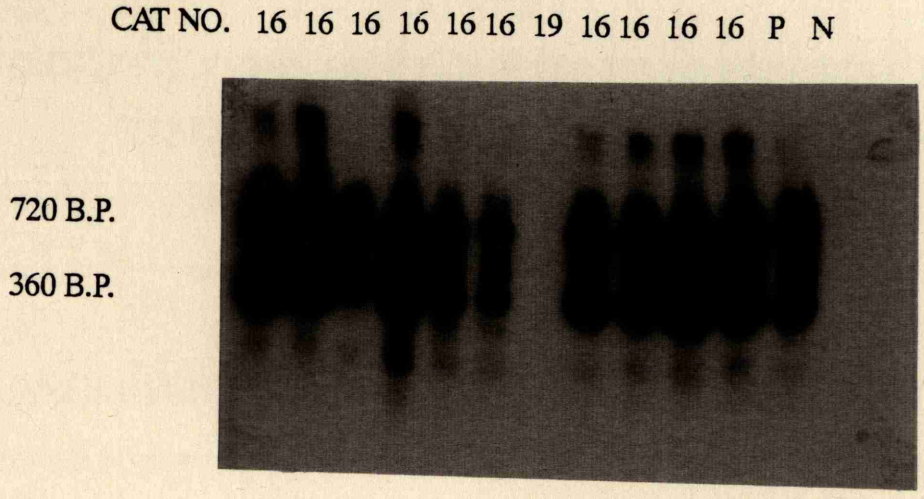


Fig.7.4. Southern Blot Analysis of Colony Cell DNA Grown in Agar and Probed with FIV LTR. All Colonies from Cat H16 (week 12) are Strongly Positive, the Single Colony from Cat H19 is Negative. (Dev. overnight @ R.T.)

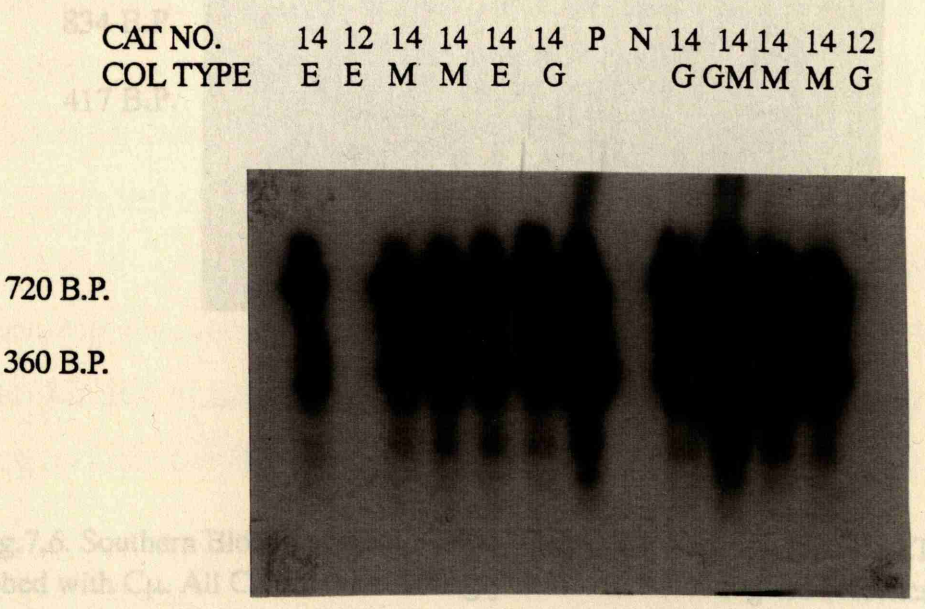


Fig. 7.5. Southern Blot Analysis of Colony Cell DNA Grown in Methylcellulose and Probed with FIV LTR. All Colonies from Cat H14 (week 12) are Strongly Positive, the 2 Colonies from Cat H12 are Negative. (Dev. 2 days @ R.T.)
G: G-CFU **GM**: GM-CFU **M**: M-CFU **E**: BFU-E **P**: Positive Control
N: Negative Control

CAT NO.	15	15	13	15	14	14	13	13	15	13	15	P	N
COL TYPE	G	?	G	M	M	G	GM	?	GM	GM	M		

834 B.P.

417 B.P.

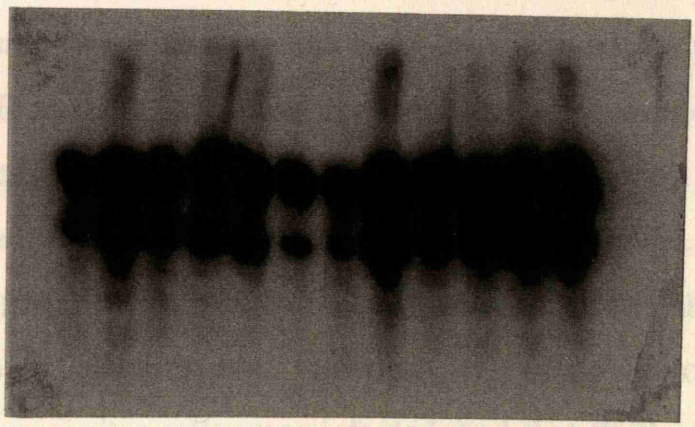


Fig.7.6. Southern Blot Analysis of Colony Cell DNA Negative for FIV LTR Probed with Cμ. All Colonies are Strongly Positive, Indicating the Presence of Sufficient DNA. (Dev. overnight @ -70°C.)
G: G-CFU **GM**: GM-CFU **M**: M-CFU **P**: Positive Control **N**: Negative Control

CHAPTER 8

IDENTIFICATION OF FELINE IMMUNODEFICIENCY VIRUS p24 ANTIGEN IN COLONIES BY IMMUNOHISTOCHEMISTRY

8.1. INTRODUCTION

Although it was possible to determine the presence of FIV DNA in colonies by PCR, the technique used did not distinguish whether the virus was actively replicating or not. In order to determine this, and as a back-up to the PCR analysis, colonies were screened immunohistochemically for the presence of viral protein in cytoplasm. Results were not predicted to be identical to those obtained by PCR analysis, as it was expected that virus would not be equally expressed in all cell lines. However, viral proteins were at least expected to be expressed in macrophage colonies. The demonstration of viral protein in colonies in which all cells should be similar would demonstrate infection of entire colonies, confirming that progenitor cells were infected, and that PCR results were not due to background infected macrophages or lymphocytes being picked up with the colony cells.

8.2. AIMS

The aim of this study was to demonstrate the presence of expressed viral protein in all cells of clonally derived colonies, to confirm FIV infection in these cells as suggested by PCR analysis.

8.3. MATERIALS AND METHODS

Colonies from infected cats were picked from assay plates, washed and spun onto slides, one colony per slide. Six or 8 colonies were picked from agar and 12 from methylcellulose for each cat at each of the 5 time points. Four colonies were picked from GM and Mix assays from non-infected cats as controls. These were prepared in the same way. Colonies were also prepared from long-term infected cats (Q257, Q258) and an age-matched control (Q264). The colonies were then stained with anti-p24 antibody obtained from Professor Niels Pedersen, University of California, Davis USA. Positively stained colonies were identified using the APAAP technique (Dako Ltd., High Wycombe, UK). Infected lymphocytes (FL4 cells) and uninfected lymphocytes (FeTJ cells) were used as positive and negative controls respectively for the staining technique. One slide of each of these preparations was stained along with each batch of experimental slides.

8.4. RESULTS

Transfer of adequate numbers of intact cells from assay plates to slides was difficult. Due to the cells being coated in agar or methylcellulose, they tended to stick to pipette tips and this resulted in a number of cells being lost. During the cytospinning process cells stuck to the funnel, or arrived on the slide with damaged or folded over membranes (fig. 8.10). More cells seemed to survive from the methylcellulose colonies, and macrophages were apparently over represented, suggesting they were more robust.

Interpretation of the staining results proved difficult, with heavy background staining being evident on negative control slides as well as infected cat colonies (fig. 8.6). Cells which had been folded or

damaged during the preparatory process also stained quite heavily, increasing the proportion of false positive results (fig. 8.10). Due to the difficulties in interpretation, a system was devised whereby minimum and maximum numbers of positive slides (colonies) were determined i.e. those which were definitely positive making up the minimum number and those which were probably or possibly positive were added to these to give a maximum number. Macrophages were evaluated separately from other cell types. Results are shown in tables 8.1 and 8.2 and in figures 8.1 to 8.4. Representative slides are shown in figures 8.5 to 8.10.

8.5. DISCUSSION

Immunohistochemistry was able to demonstrate the presence of viral protein in the cytoplasm of colony cells, confirming PCR results in this respect. However, in the early stages of infection (3 weeks) in both methylcellulose and agar plates, very low numbers or no infected cells were detected (tables 8.1 & 8.2 and figures 8.1 & 8.2), even using the maximum figures. Those cells which were positive were macrophages. This is consistent with the knowledge that there are no peripheral cytopaenias at this stage, or any difference in colony numbers between control and infected cats. Numbers of infected cells in the infected animal are generally low at this stage (Beebe *et al* 1994), mainly being confined to the lymphoid tissues.

Colonies taken from cats at the neutropaenic period were only positive in low numbers (tables 8.1 & 8.2 and figures 8.1 & 8.2). More colonies from methylcellulose were positive, but this may have been due to greater numbers of surviving cells from these colonies. The positive cells were again mainly macrophages, but up

to 13.7% of mononuclear cells were positive (table 8.2). Thereafter, numbers of positive mononuclear cells fell to less than 10% maximum. Some of these cells may have been contaminating lymphocytes, or false positives. Assuming that they were genuine colony cells which were truly positive, these made up a very small percentage of the total tested. The proportion of infected macrophages also peaked at 5-6 weeks in most animals (figs. 8.2 & 8.4).

The immunohistochemical results are in contrast to the results obtained by PCR, but this is not entirely unexpected. PCR detected proviral DNA which may have been present in these cells but was not necessarily being expressed. Expression of viral protein is known to occur in cells of the monocyte/macrophage lineage and in lymphocytes, but little is known about haemopoietic cells. Provirus may be present but remain latent. Chelucci *et al* (1995) were able to detect p24 HIV protein in 12% of CFU-GM and rarely in BFU-E infected *in vitro*. This was in contrast to their detection rates of *tat* mRNA of 23% and 17% respectively by RT-PCR. These differences may also reflect a lower sensitivity of the immunohistochemical method of detection.

Immunohistochemistry proved to be a poor method of confirming the PCR results. Transference of cells from semi-solid media to cytospin slides proved to be damaging to many cells, leading to false positive results. Heavy background staining further complicated the interpretation. Reverse transcriptase PCR (RT-PCR) would have been a better method of detecting viral mRNA.

			WEEK 3	%	NEUT	%	WEEK 12	%	WEEK 20	%	EUTH	%	TOTAL	%		*LONG TERM*	%
13	OTHER	MIN	0/5	0	0/4	0	0/8	0	1/8	12.5	0/8	0	1/33	3.0		*0/8*	*0*
		MAX	0/5	0	0/4	0	0/8	0	5/8	62.5	0/8	0	5/33	15.2		*0/8*	*0*
	MAC	MIN	-	-	-	-	-	-	-	-	-	-	-	-		*3/8*	*37.5*
		MAX	-	-	-	-	-	-	-	-	-	-	-	-		*5/8*	*62.5*
14	OTHER	MIN	0/6	0	0/6	0	0/5	0	0/8	0	0/8	0	0/33	0		*0/6*	*0*
		MAX	0/6	0	0/6	0	0/5	0	1/8	12.5	0/8	0	1/33	3.0		*0/6*	*0*
	MAC	MIN	-	-	-	-	-	-	-	-	-	-	-	-			
		MAX	-	-	-	-	-	-	-	-	-	-	-	-			
15	OTHER	MIN	0/6	0	0/5	0	0/6	0	0/8	0	0/7	0	0/32	0			
		MAX	0/6	0	0/5	0	0/6	0	0/8	0	0/7	0	0/32	0			
	MAC	MIN	-	-	-	-	-	-	-	-	-	-	-	-			
		MAX	-	-	-	-	-	-	-	-	-	-	-	-			
16	OTHER	MIN	0/5	0	0/6	0	0/8	0	0/8	0	0/7	0	0/34	0			
		MAX	0/5	0	0/6	0	0/8	0	0/8	0	0/7	0	0/34	0			
	MAC	MIN	-	-	-	-	-	-	-	-	-	-	-	-			
		MAX	-	-	1/6	16.7	-	-	-	-	1/7	14.3	-	-			
17	OTHER	MIN	0/5	0	0/6	0	0/8	0	0/6	0	0/6	0	0/31	0			
		MAX	0/5	0	6/6	100	3/8	37.5	0/6	0	0/6	0	9/31	29.0			
	MAC	MIN	-	-	-	-	-	-	0/6	0	-	-	-	-			
		MAX	-	-	-	-	-	-	1/6	16.7	-	-	-	-			
TOTAL	OTHER	MIN	0/27	0	0/27	0	0/35	0	1/38	2.6	0/36	0	1/163	0.61		*0/14*	*0*
		MAX	0/27	0	6/27	22.2	3/35	8.6	6/38	15.8	0/36	0	15/163	9.2		*0/14*	*0*
	MAC	MIN	-	-	-	-	-	-	-	-	-	-	-	-		*3/14*	*21.4*
		MAX	-	-	1/27	3.7	-	-	1/38	2.6	1/36	2.8	-	-		*5/14*	*35.7*

Table 8.1. Minimum and maximum numbers of positive agar colonies for p24 antigen by immunohistochemistry

			WEEK 3	%	WEEK 5-6	%	WEEK 12	%	WEEK 20	%	WEEK 27-29	%	TOTAL	%		*LONG TERM*	%
H13	OTHER	MIN	0/12	0	0/11	0	0/12	0	0/11	0	0/11	0	0/57	0		*0/12*	*0*
		MAX	0/12	0	0/11	0	1/12	8.3	0/11	0	0/11	0	1/57	1.8		*1/12*	*8.3*
	MAC	MIN	1/12	8.3	7/11	63.6	9/12	75	4/11	36.4	1/11	9.1	22/57	38.6		*2/12*	*16.7*
		MAX	5/12	41.7	8/11	72.7	9/12	75	9/11	81.8	2/11	18.1	33/57	57.9		*3/12*	*25*
H14	OTHER	MIN	0/12	0	0/12	0	0/12	0	0/4	0	1/12	8.3	1/52	1.9		*4/11*	*36.4*
		MAX	0/12	0	0/12	0	0/12	0	0/4	0	3/12	25	3/52	5.8		*4/11*	*36.4*
	MAC	MIN	0/12	0	3/12	25	2/12	16.7	0/4	0	1/12	8.3	6/52	11.5		*0/11*	*0*
		MAX	0/12	0	6/12	50	3/12	25	0/4	0	3/12	25	12/52	23.1		*0/11*	*0*
H15	OTHER	MIN	0/10	0	1/12	8.3	0/4	0	0/11	0	0/9	0	1/46	2.18			
		MAX	0/10	0	1/12	25	0/4	0	0/11	0	1/9	11.1	3/46	6.5			
	MAC	MIN	0/10	0	3/12	25	0/4	0	1/11	9.1	0/9	0	4/46	8.7			
		MAX	0/10	0	3/12	25	0/4	0	2/11	18.1	0/9	0	5/46	10.9			
H16	OTHER	MIN	0/12	0	3/12	25	0/12	0	0/12	0	0/11	0	3/59	5.1			
		MAX	0/12	0	3/12	50	0/12	0	1/12	8.3	0/11	0	7/59	11.9			
	MAC	MIN	0/12	0	7/12	58.3	0/12	0	0/12	0	0/11	0	7/59	11.9			
		MAX	2/12	16.7	7/12	58.3	3/12	25	0/12	0	5/11	45.5	17/59	28.8			
H17	OTHER	MIN	0/12	0	0/12	0	0/12	0	1/12	8.3	0/9	0	1/57	1.8			
		MAX	0/12	0	0/12	0	0/12	0	1/12	8.3	0/9	0	1/57	1.8			
	MAC	MIN	0/12	0	7/12	58.3	0/12	0	0/12	0	1/9	11.1	8/57	14.0			
		MAX	2/12	16.7	8/12	66.7	5/12	41.7	5/12	41.7	2/9	18.1	22/57	38.6			
TOTAL	OTHER	MIN	0/58	0	4/59	6.8	0/52	0	1/50	2	1/52	1.9	6/271	2.2		*4/23*	*17.4*
		MAX	0/58	0	8/59	13.7	1/52	1.9	2/50	4	4/52	7.7	15/271	5.5		*5/23*	*21.7*
	MAC	MIN	1/58	1.7	27/59	45.8	11/52	21.2	5/50	10	3/52	5.8	47/271	17.3		*2/23*	*8.7*
		MAX	9/58	15.5	32/59	54.2	20/52	38.5	16/50	32	12/52	23.0	89/271	32.8		*3/23*	*13*

Table 8.2. Minimum and maximum numbers of methylcellulose colonies positive for p24 antigen by immunohistochemistry

Fig. 8.1. Graph of percent non-macrophage colonies grown in agar positive for p24 antigen by immunohistochemistry (all cats)

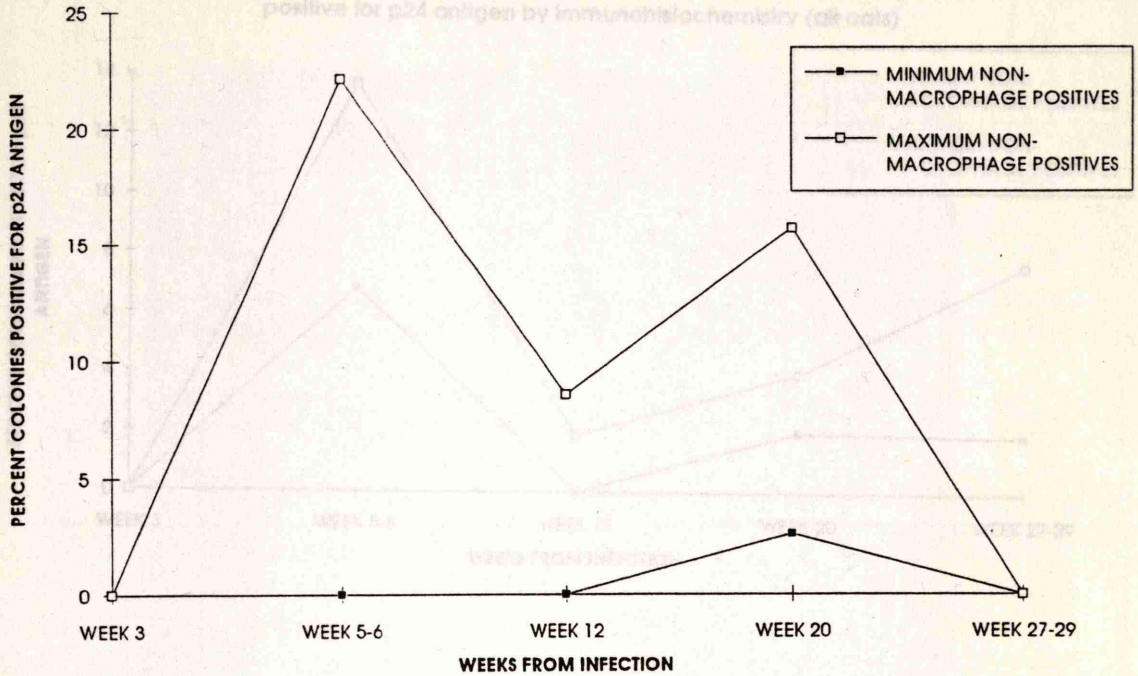


Fig. 8.2. Graph of percent macrophage colonies grown in agar positive for p24 antigen by immunohistochemistry (all cats)

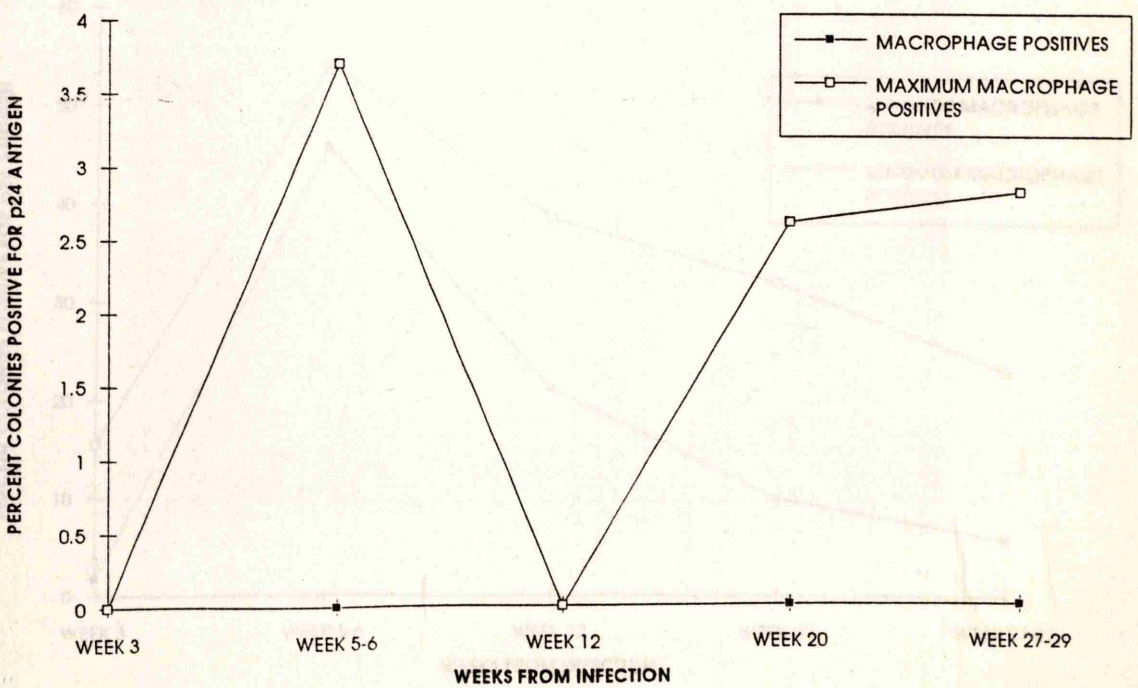


Fig. 8.3. Graph of percent non-macrophage colonies grown in methylcellulose positive for p24 antigen by immunohistochemistry (all cats)

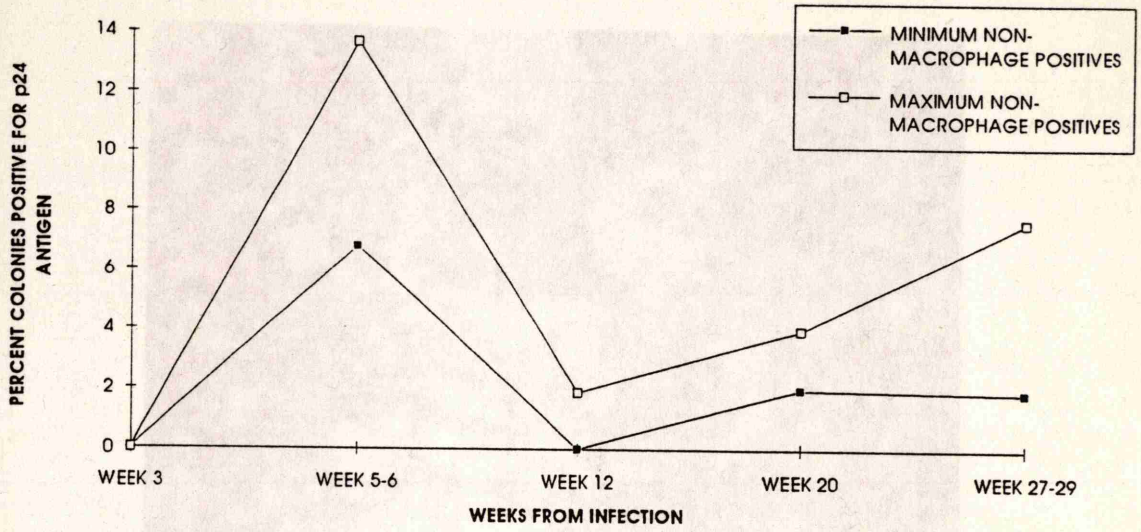


Fig. 8.4. Graph of percent macrophage colonies grown in methylcellulose positive for p24 antigen by immunohistochemistry (all cats)

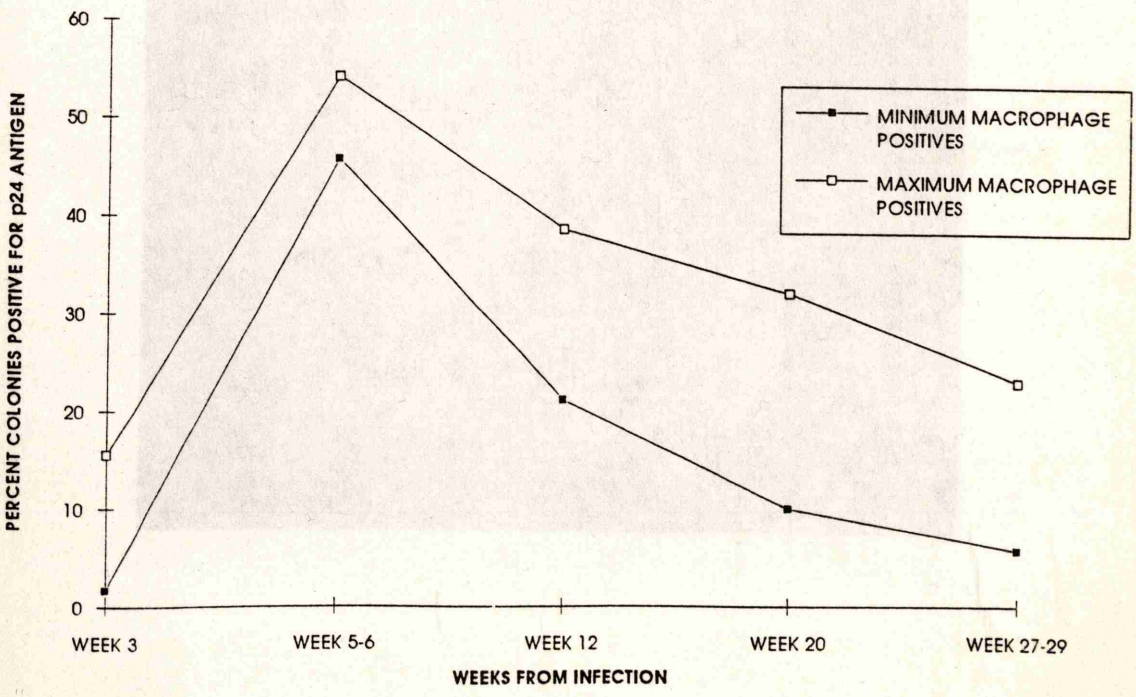


Fig. 8.5. APAAP stained positive control FL4 cells showing bright red cytoplasmic staining in majority of cells (x10)

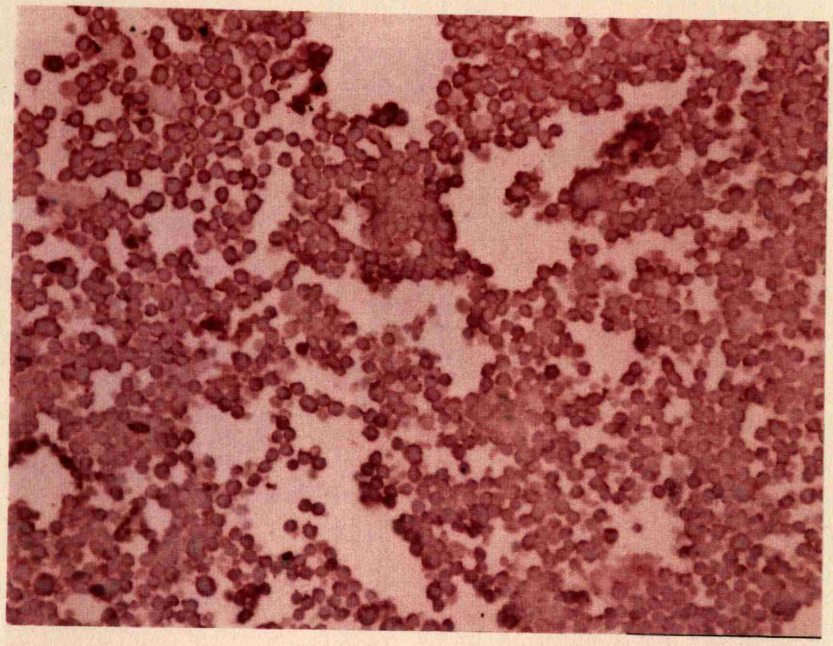


Fig. 8.6. APAAP stained negative control FeTJ cells showing pink background staining in cytoplasm of majority of cells (x10)

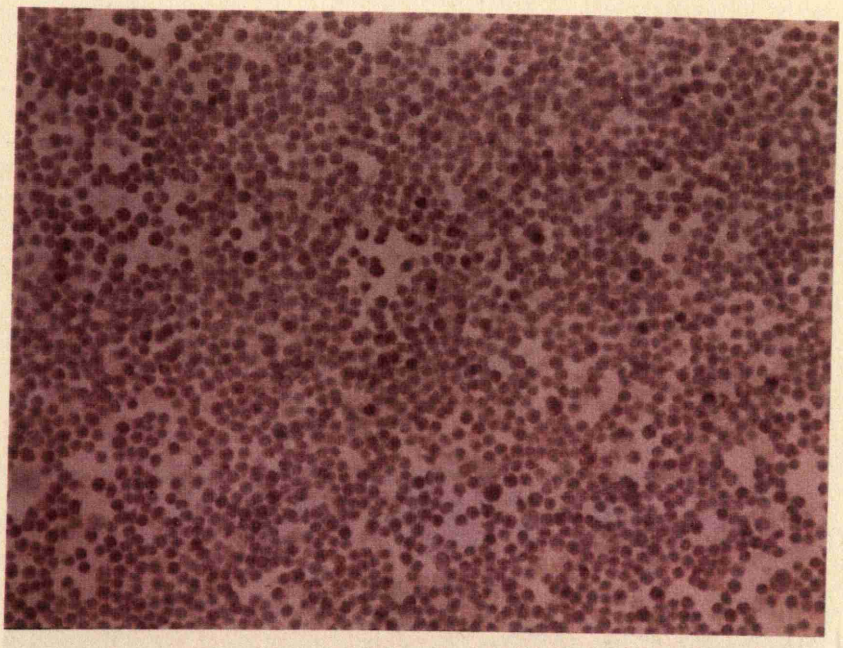


Fig. 8.7. APAAP stained clump of macrophages from FIV positive cat showing positive staining in cytoplasm (H17 from methylcellulose 6 weeks p.i., x20)

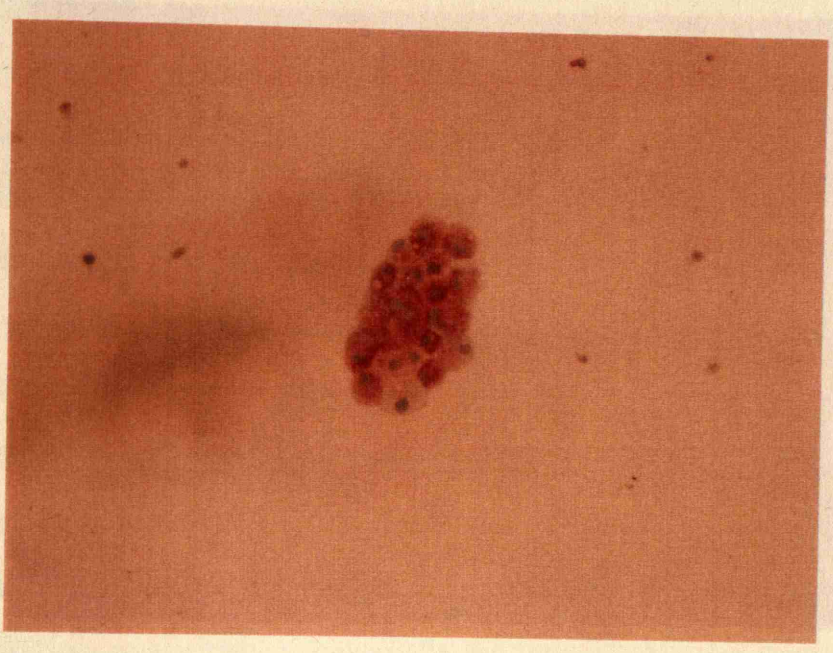


Fig. 8.8. APAAP stained mononuclear cells from FIV positive cat showing positive staining in cytoplasm (H17 from agar 12 weeks p.i., x20)

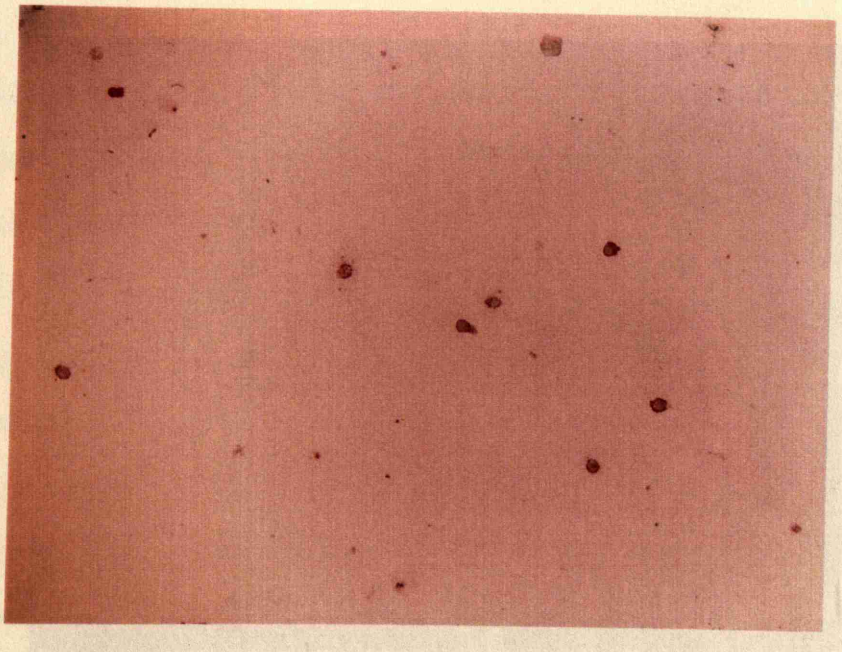


Fig. 8.9. APAAP stained macrophages from FIV negative cat showing only faint pink background staining in cytoplasm (H12 from methylcellulose 12 weeks p.i., x20)

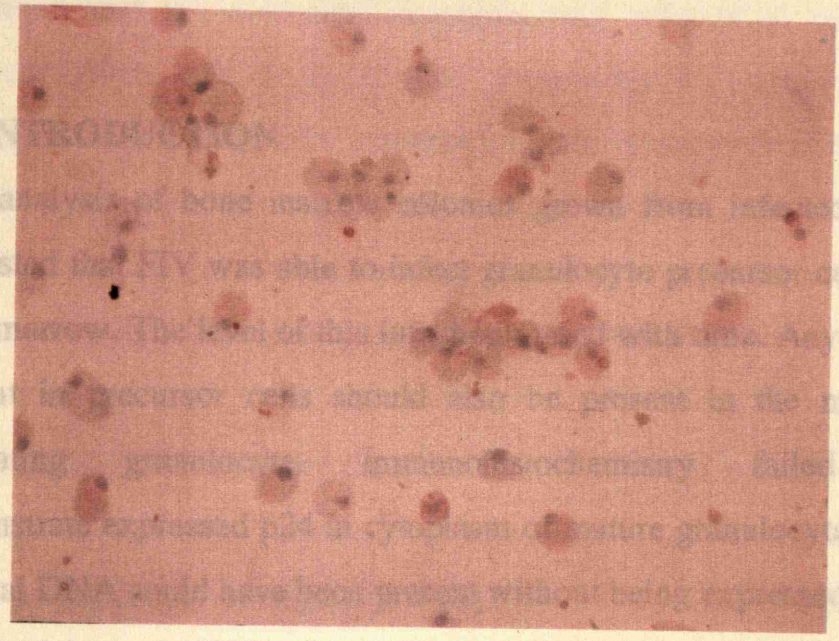
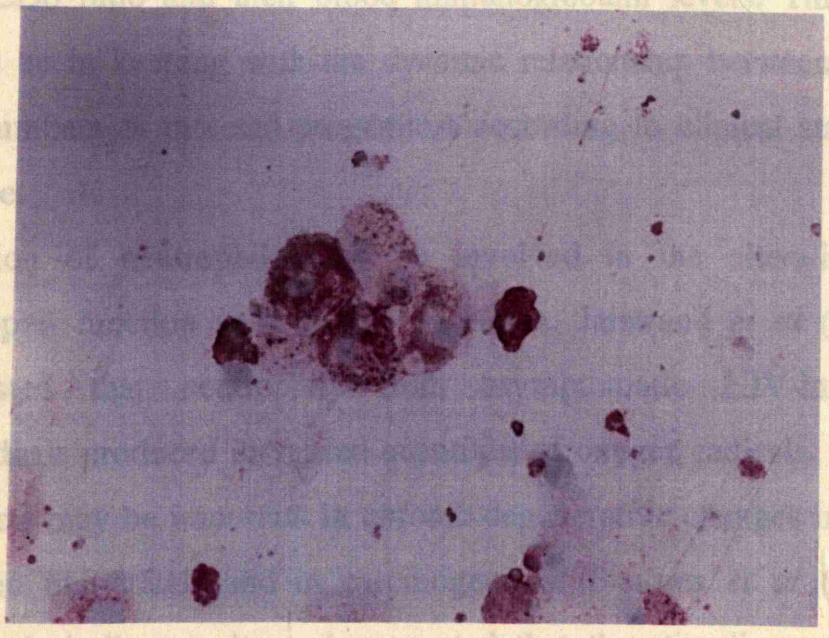


Fig. 8.10. APAAP stained macrophages from FIV negative cat showing false positive staining in shrunken and damaged cells (H21 from methylcellulose 3 weeks p.i., x40)



CHAPTER 9

PCR INVESTIGATION OF PERIPHERAL NEUTROPHILS

9.1. INTRODUCTION

PCR analysis of bone marrow colonies grown from infected cats suggested that FIV was able to infect granulocyte precursor cells in bone marrow. The level of this infection varied with time. Any virus present in precursor cells should also be present in the mature circulating granulocyte. Immunohistochemistry failed to demonstrate expressed p24 in cytoplasm of mature granulocytes but proviral DNA could have been present without being expressed. Gabrilovich *et al* (1993a) were able to demonstrate the presence of the HIV genome in neutrophils of human patients infected with the HIV virus. This is in keeping with the idea that HIV can infect progenitor cells. Levels of detection varied according to the clinical stage of the subject, their peripheral neutrophil count, their CD4:CD8 ratio and their blood immunoglobulin levels. This too, would be in keeping with the dynamic relationship between virus and numbers of infected progenitors according to clinical stage of disease.

Infection of neutrophils may be involved in the alteration in neutrophil function seen in HIV infection. Jarstrand *et al* (1994) suggested that neutrophils from asymptomatic HIV-infected individuals produced increased quantities of oxygen radicals. These products may be important in chronic degenerative changes in HIV infected individuals and in carcinogenesis. Bandres *et al* (1993) obtained similar results and suggested that the stimuli associated with HIV infection enhance the non-specific response of phagocytic

cells to bacterial pathogens. Contrasting results have been obtained by a number of authors, including Chen *et al* (1993). Their results suggested that the neutrophil response was reduced in infected people. Other studies have also demonstrated inhibition of neutrophil migration in HIV positive subjects (Pinegin *et al* 1993). Polymorphonuclear neutrophils also appear to have a role in the modification of the immune response by lymphocytes and monocytes in HIV infection (Gabrilovich *et al* 1993b).

The role of HIV in neutrophil function is clearly controversial, although alteration in efficacy certainly seems to take place. The reasons for these changes are also controversial, with direct infection of cells, cytokine influences, antibody responses and alteration in receptor types and number all having been proposed as significant (Capsoni *et al* 1992, Bandres *et al* 1993, Chen *et al* 1993, Gabrilovich *et al* 1993b, 1993c, Jarstrand *et al* 1993, Pinegin *et al* 1993). The role of the mature neutrophil in the progression of disease may well prove to be very important.

The role of FIV in feline neutrophils has not been investigated, although it may be argued that similar effects to those seen in HIV may be demonstrated. This remains to be elucidated.

9.2. AIM

The aim of this study was to determine whether peripheral neutrophils from FIV-infected cats were positive for proviral DNA.

9.3. MATERIALS AND METHODS

4ml of blood were collected from 4 cats infected with FIV for 4 years (Q252, Q255) and 6 months (A332, A333) respectively. All

cats were asymptomatic and had normal peripheral neutrophil counts. Neutrophils and lymphocytes were separated over a 2-layered Percoll (Pharmacia Ltd., Milton Keynes, UK) gradient, washed, redissolved in red cell lysis buffer and resuspended in PBS (see figs 9.1 and 9.2). The numbers of surviving cells were counted on the ABX Minos Vet automated counter (Roche Products Ltd., Diagnostic Division, Welwyn Garden City, UK).

The cell suspensions were diluted to a concentration of 3.33×10^7 cells/ml so that 30µl would provide DNA from 10^5 lymphocytes or neutrophils. Serial dilutions were then made from 3.33×10^6/ml to 3.33×10^4/ml. The resultant cell suspensions were then boiled for 5 minutes and subjected to PCR analysis in the same way as the colonies had been, using the LTR primers, 30µl of cell suspension and identical conditions. Positive and negative controls were used as for the colony DNA. Southern blots were performed on the PCR products separated by polyacrylimide gel electrophoresis, as for the colony cell PCR.

The most concentrated samples were also checked for DNA by PCR using the Cµ primers, as for the colonies.

9.4. RESULTS

Both by gel electrophoresis and by Southern blotting there was no evidence of the FIV LTR sequence. All suspensions of both neutrophils and lymphocytes in both infected and control cats were negative. Representative blots are shown in fig. 9.3.

Samples checked for DNA were positive by gel electrophoresis.

9.5. DISCUSSION

The lack of evidence for infection of peripheral neutrophils by FIV could be explained in a number of ways. Bloods were taken from these animals at a time when haemopoiesis was normal and circulating cell numbers were within the expected limits. There was also no evidence of clinical illness in any of these animals. Gabrilovich *et al* (1993) in their paper suggested that these conditions may have played an important part in the degree of infection of neutrophils. It may also have been the case that too few cells were used, since the PCR also failed to detect the LTR sequence in lymphocytes from these animals. PCR using the Cμ fragments yielded positive results, indicating that there was sufficient DNA present. Any virus present must have been at very low copy number, below the lower limit of detection.

This evidence lends little to the debate on the role of neutrophils in the pathogenesis of FIV. Investigation of cats in the acute phase of infection would be helpful, as this is the stage at which virus has been most commonly detected in precursors. Function tests would also be indicated at this stage. Investigation of clinically ill cats would also be useful.

Fig. 9.1. Granulocytic cell population resulting from Percoll separation (stained May-Grunwald Giemsa x20)

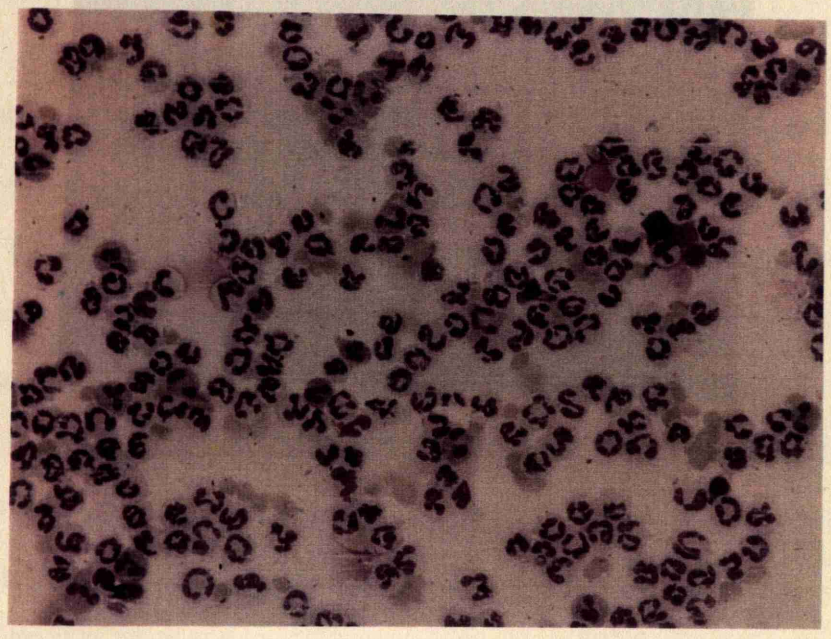
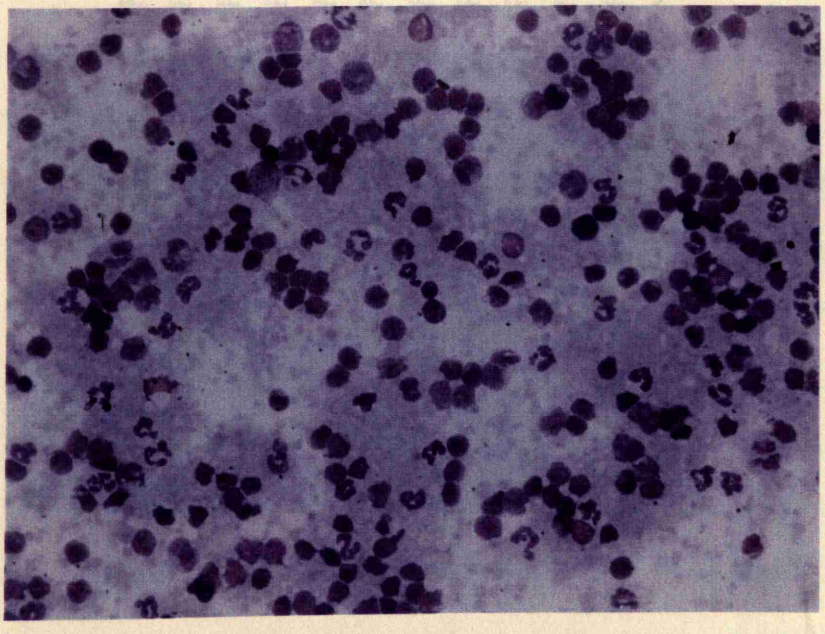
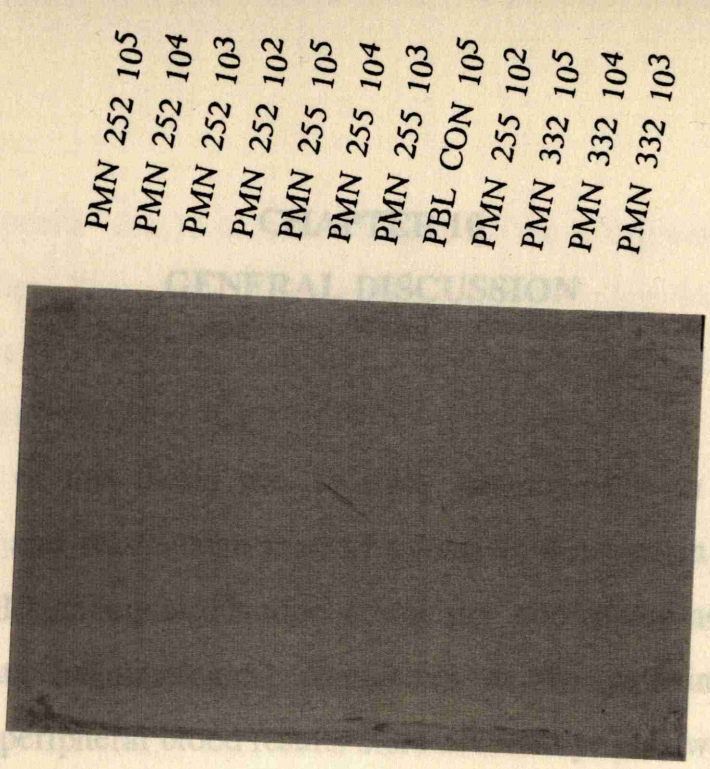


Fig. 9.2. Lymphocytes, occasional granulocytes and platelet rafts resulting from Percoll separation (stained May-Grunwald Giemsa x 20)





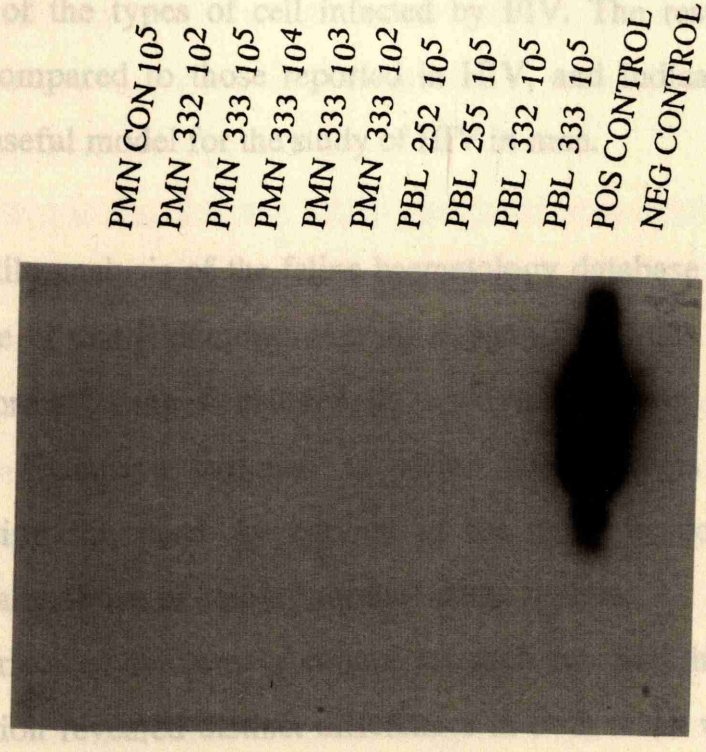


Fig. 9.3. Southern blot analysis of peripheral neutrophils (PMN) and lymphocytes (PBL) from infected (252, 255, 332, 333) and control (CON) cats at various cell numbers showing all negative for the FIV LTR sequence (Dev. 7 days @ -70°C)

CHAPTER 10

GENERAL DISCUSSION

The aim of this thesis was to study haemopoiesis in early FIV infection and relate bone marrow events to changes in peripheral blood cell numbers at this time. It was first considered necessary to re-evaluate haematological parameters in the cat, in order to interpret peripheral blood results more accurately. This was done by database analysis. For the main part of the thesis, haematological data was collected from experimentally infected cats, along with concurrent quantitative assessment of bone marrow activity. Further investigation of marrow cells was then undertaken to determine the nature of the types of cell infected by FIV. The results obtained were compared to those reported in HIV, and indicated that FIV was a useful model for the study of HIV in man.

Percentile analysis of the feline haematology database was a useful exercise in that it demonstrated the superiority of this method over the "normal" ranges traditionally used to interpret haematology results. Extensive variation in white cell numbers was noted, illustrating the need for caution in the interpretation of values marginally above or below "normal" range figures.

Comparison of a colony of control research cats and the general cat population revealed distinct differences in both mean values and in percentile analysis regarding leucocyte numbers. The total white cell count, lymphocyte and eosinophil figures were dramatically increased in comparison to the general cat population. These results were useful in illustrating the danger of using small numbers of so-

called "normal" cats to construct ranges for the interpretation of sick cat samples. These data were also useful in the interpretation of the haematology results derived from the experimentally infected cats used in the rest of this thesis.

Haematological results in experimentally infected cats followed the pattern described by previous authors (Pedersen *et al* 1987, Yamamoto *et al* 1988, Callanan *et al* 1992, Mandell *et al* 1992, George *et al* 1993, Dua *et al* 1994, Linenberger *et al* 1995). Profound neutropaenia was the most dramatic finding, occurring at 5 to 7 weeks post-infection and being maintained for up to 6 weeks in one animal. Those cats with the most profound and lasting neutropaenia were also clinically ill (pyrexia and dullness) at this time. This may have been as a result of their increased susceptibility to secondary infections, or may have been a direct consequence of viral infection and replication.

In addition to the dramatic fall in peripheral neutrophils, there were also significant differences in red cell numbers, haemoglobin, haematocrit, lymphocytes, eosinophils and basophils between the infected and control groups of cats during the course of the haematological monitoring. In all cases, infected cats had significantly fewer cells than their control counterparts. These differences all occurred at intervals between days 28 and 160. The timing of these events and their relation to acute phase clinical illness and neutrophil numbers suggested that the cause of the observed differences may well have been directly related to FIV infection. The compromise in so many of the blood cell lines indicated that a direct infection of a common bone marrow progenitor cell was a possible mechanism. The compromise was

transient, and cell lines had all recovered and stabilised by the end of the study period. The differences in severity of cytopaenia were explained by the life-spans of the various cell lines, short-lived cells being more dramatically affected than red cells, for example.

The pathogenesis of the cytopaenias seen in FIV infection, and indeed in HIV infection, has not yet been elucidated. Cytopaenias occur as a result of either reduced bone marrow production of cells, increased sequestration of cells or premature destruction of mature cells e.g. by immune-mediated mechanisms. Cytopaenias are important in terms of their own morbidity and also in therapy limitation, since many antimicrobial drugs are myelotoxic in their own right. In addition to this, the potential infection of long-lived marrow stem cells could be important in providing a reservoir of virus.

In the terminal stages of FIV and HIV severe and multiple cytopaenias are observed (Hopper *et al* 1989, Yamamoto *et al* 1989, Shelton *et al* 1990, 1991, Ishida *et al* 1992, Sparkes *et al* 1993). The aetiology of these is likely to be multifactorial due to opportunistic and secondary infections, treatments, malignancies and myelophthisis, leading to both reduced production and increased consumption of cells. The distinct and predictable pattern of neutropaenia in early experimental FIV infection presented the opportunity to clarify the role of FIV in peripheral blood cell deficiencies. At this stage, cats are generally free of the complicating factors outlined above seen in the late stage disease. Investigation of bone marrow efficacy and of histopathology at this stage of disease enabled the distinction between reduced production or increased consumption of cells to be made. Callanan (1994)

looked at histopathology of cats euthanased in the early stages of FIV infection and found evidence for sequestration and consumption of neutrophils in peripheral tissues of both cats which were ill and those which were clinically normal. Histopathology of the bone marrow of these animals revealed inconsistent findings, although all showed loss of the reserve pool of mature neutrophils. Myeloid activity, as determined by histopathology, was either normal, hyperplastic or hypoplastic. Thus, although there was evidence for peripheral consumption of cells, marrow results were inconsistent and no firm conclusions could be drawn as to viral activity in bone marrow.

In this investigation, in order to elucidate the events in the bone marrow in early FIV infection, quantitative bone marrow assay techniques were applied at 5 time points to assess progenitor cell activity. There was much variation in the assay quality at different time points, as was expected for this type of assay, illustrating the need for strict controls in this experiment.

The results obtained demonstrated a fall in myeloid cell progenitor cells in early FIV infection. The drop in committed precursors took place at 5-6 weeks after infection, at the same time as the dramatic depletion of peripheral neutrophils. The fall in marrow productivity provides an explanation for the peripheral cytopaenia. Although marrow neutrophil production is not abolished, it is restricted, and this together with a potential increase in peripheral demand for mature neutrophils with a short half-life of 7.4 hours (Prasse *et al* 1973) results in a dramatic deficit of circulating cells.

These results were in keeping with those of Linenberger *et al* (1995), who detected a significant drop in colony numbers at 6-12

weeks post-infection. Linenberger's data was unable to show the fluctuations in colony numbers from individual animals over time as the cats used were serially euthanased.

Having established that marrow progenitor activity was temporarily compromised in early FIV infection, further investigation was then undertaken to try to identify the mechanism of the compromise. Progenitor cell activity could be depressed by direct infection and killing of the progenitors or their precursors by virus; direct infection of these cells with no killing but functional compromise; or by an indirect means whereby infection of stromal or feeder cells dysregulated growth factor and cytokine production. The colony assay work suggested that stromal cell activity was not an important factor, as irradiated stroma from control cats did not improve colony numbers in infected cats compared with infected cat stroma, and likewise infected cat stroma did not inhibit control cat progenitors. Examination of the colonies grown from the assays was undertaken in order to establish whether progenitor cells were directly infected by the virus. Single colonies, each clonally derived from a single progenitor cell, taken from both types of assay were investigated by PCR for the LTR sequence of the FIV genome.

PCR analysis revealed that progenitor cells were in fact infected by virus, and that the numbers of cells infected varied over the first few weeks post-infection. The fact that colonies i.e. the products of multiplying progenitor cells were infected, suggests that direct infection of these cells does not kill the progenitors, nor stop their multiplication. All cell lines investigated were affected, including erythroid progenitors, adding weight to the suggestion that a more primitive precursor was infected. The fact all cell lines were

potentially infectable was significant in providing a clue as to the pathogenesis of the more severe and multiple cytopaenias seen in late-stage FIV.

The relationship between virus and multiplying marrow cells is clearly a dynamic one. Wei *et al* (1995) and Ho *et al* (1995) suggested that, in HIV infection, there is a constant cycle of virus production and *de novo* infection of cells. If this is the case, and is also true of FIV, then the high rate of virus replication in the early stage of infection could provide large quantities of virus to infect susceptible marrow haemopoietic cells. This is reflected in the near 100% infection observed from 5 to 12 weeks post-infection. The proportion of infected cells then falls, probably as a result of reduced virus replication as the initial multiplicative phase passes into the low level production typical of the asymptomatic stage of infection. Infection rates in long-term infected asymptomatic cats were nearer 50%, reflecting a lower rate of virus production at this time.

At the time of the peripheral neutropaenia, there were fewer CFU-G and CFU-GM actively proliferating. The colonies produced at this time also consisted of fewer numbers of progeny cells than those of healthy animals. Most of these colonies were FIV positive, suggesting that there was some functional hindrance associated with viral infection, although multiplication was obviously not abolished. As the viral burden fell, there was compensatory division of more primitive progenitors so healthy CFU-G and CFU-GM were produced to restore peripheral cell numbers.

Donahue *et al* (1987) found that serum from HIV-positive patients inhibited colony growth from virus-positive patients but not from control marrows. He suggested that the effect was antibody-

mediated, the antibody only attaching to cells expressing viral antigen, and thus having no effect on uninfected control cells. Linenberger *et al* (1995) reported that similar inhibition of progenitor growth was recorded using serum and marrow from FIV-infected cats. As the PCR results indicated that committed precursors could be infected by virus, expression of viral protein by these cells and antibody binding is a possible mechanism of inhibition of these cells.

Further to the PCR results, colonies were also examined for expression of the viral p24 protein by immunohistochemistry. Few colonies were found to be positive from agar plates as compared to methylcellulose, suggesting that the agar coating on the cells inhibited the binding of antibody. Cells from the methylcellulose plates stained more efficiently, although many cells were damaged during the washing process. Positive cells were mainly macrophages, but a small number of mononuclear cells did stain positively. This is in keeping with expectation, cells of the monocyte/macrophage lineage being known to express virus. In the case of mononuclear cells, although the p24 antigen was not being expressed in many cells, this does not preclude the existence of the proviral genome as indicated by the PCR results. The proportion of colonies staining positive was maximal at the point of neutropaenia, 5-6 weeks post-infection. This confirmed the pattern of the PCR results.

The examination of peripheral neutrophils for PCR delivered negative results in the limited study done here. However, since the cells were taken from healthy cats in the asymptomatic stage of

infection, this was not surprising. Had the investigation been undertaken on cats at the time of peripheral neutropaenia when maximal numbers of colonies were positive for proviral DNA by PCR, a positive result may have been expected. Previous studies on human neutrophils have indicated that positive PCR on neutrophils occurs at low frequency in sick patients (Gabrilovich *et al* 1993a).

Taken as a whole, the haematology, colony assays, PCR and, to a lesser extent, immunohistochemistry all point toward a similar conclusion as to the role of FIV in bone marrow. The extent of involvement of FIV in haemopoiesis fluctuates according to the degree of viral activity in the body at any time. After initial infection there is a period of intense viral replication. Cats can become clinically ill at this stage, and a peripheral neutropaenia occurs. This lasts for a matter of days in most animals and is followed by a period of recovery when haematological parameters return to within expected limits and the cats are clinically healthy. This phase lasts for months or years.

At the time the cats become clinically ill and suffer the peripheral neutropaenia, there are fewer functional committed neutrophil precursors in bone marrow. There is a concurrent increase in demand for a short-lived mature cell due to intense viral activity and associated tissue damage. Together, these factors result in a temporary fall in circulating mature neutrophils and a depletion of the reserve pool in marrow. As the initial activity of the virus decreases with its establishment in preferred tissues, the numbers of precursors in the marrow recover to levels comparable with control animals and peripheral cell counts become normal. This occurs despite a proportion of progenitors still being virus infected. There

are, however, higher numbers of non-infected progenitors and cell production is maintained at "normal" levels.

It may be the case that virus initially infects the precursor cells immediately preceding the CFU-G, CFU-GM, CFU-M and BFU-E (see fig. 10.1). At the point of neutropaenia, there are fewer progenitors due to compromised production by their immediate predecessors. Those precursors present are infected and themselves functionally impaired. Some non-infected progenitors still remain but these are becoming functionally exhausted due to the increased demand. By 12 weeks post-infection, numbers of progenitors have recovered but most are still infected. However, peripheral demand has dropped and there are sufficient numbers to supply replacement cells for normal turnover. Subsequently numbers of infected progenitors dropped, due to replacement by newly generated uninfected predecessor cells. Thereafter, during the asymptomatic phase numbers of progenitors are maintained within normal limits and haemopoiesis is unperturbed, despite a low level of infected progenitors. Most peripheral neutrophils are free from infection.

This study was limited to following the events in early infection, and to a restricted investigation of marrow progenitors and peripheral neutrophils in asymptomatic cats. The situation in animals suffering from ARC or AIDS was not investigated. It is likely that any increase in viral replication would lead to a similar pattern of reduced precursor numbers and increasing proportions of infected progenitors as in the early stage of infection. Thus, an increase in peripheral cytopaenias would be expected, and all cells lines would be vulnerable. The number and severity of cytopaenias would increase due to viral load, an increasingly weak immune response

and to complicating factors such as opportunistic infections, myelotoxic drugs etc.

The dynamic nature of the relationship between virus and blood cells explains the rather conflicting evidence produced by previous authors in investigating marrow activity and peripheral blood cells (Linenberger *et al* 1991, 1995, Shelton *et al* 1989). The results obtained are dependent on the clinical health of the subject and the degree of viral production at the point of sampling.

In order to further investigate this complex subject, it would be useful to repeat the experimental infection in cats and perform further colony assays. The availability of feline haemopoietic growth factors in the laboratory would be helpful in elucidating the role of growth factors in the period of reduced colony growth. RT-PCR (reverse transcriptase - PCR) may have been more useful than immunohistochemistry to try to confirm PCR colony results. The establishment of the nature of the most primitive infected progenitor cells would elucidate whether long-lived stem cells in bone marrow act as a reservoir of virus.

Peripheral neutrophils from cats in early infection should also be examined for proviral DNA. Comparisons should be made with sick FIV positive cats.

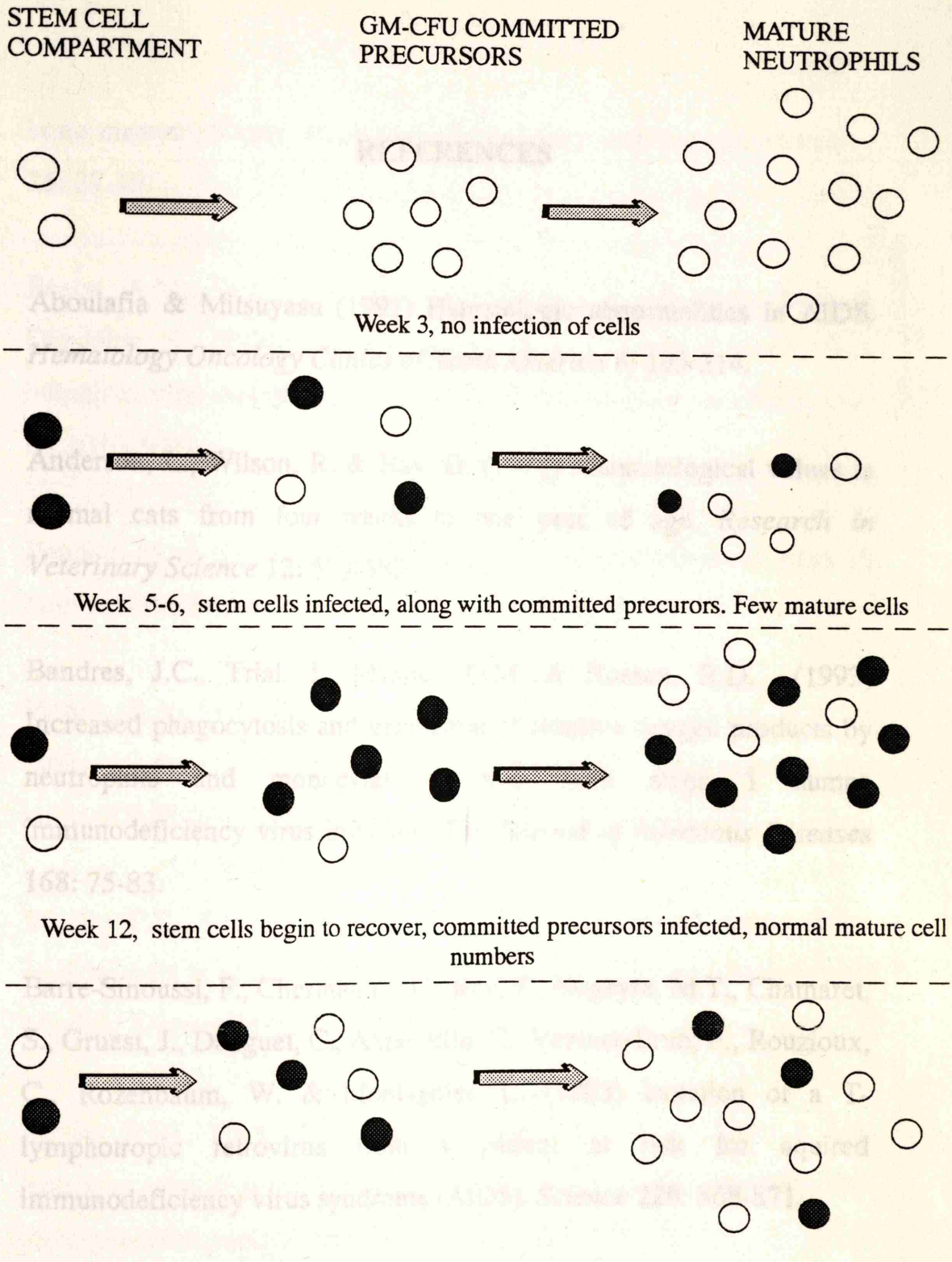


Figure 10.1 Diagrammatic representation of dynamic nature of haemopoiesis in early FIV infection.

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