

**FELINE IL-12 AND IL-18; ADJUVANTS IN FeLV DNA  
VACCINATION STUDIES**

By

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*To mum for her constant support and encouragement and in memory of dad.*

## SUMMARY

Cytokines are glycoproteins produced by many different cell types which have wide ranging effects on the haemopoietic and immune systems and normal homeostatic mechanisms. The advent of recombinant DNA technology and the cloning of human cytokines has facilitated the production of recombinant cytokines in sufficient quantities to allow the characterisation of their biological properties and subsequent use as novel therapeutic agents in the treatment of viral and bacterial infections, cancer and cytopenias. However, the therapeutic use of heterologous cytokines in domestic animals has been of limited success, mainly due to the variable degree of conservation between species. Therefore, the isolation and characterisation of species specific cytokines is desirable in order to facilitate further studies of the role of cytokines in diseases of domestic animals. This thesis describes the approach used to isolate and clone the feline Th1 type cytokines interleukin 12 (IL-12) and interleukin 18 (IL-18), express the recombinant IL-18 protein in a mammalian expression system and investigate the potential of both IL-12 and IL-18 to act as genetic adjuvants in FeLV DNA vaccination studies.

IL-12 is a recently discovered heterodimeric cytokine, which serves as a pivotal regulator of T and NK cell function, stimulating proliferation, cytolytic activity and cytokine induction. IL-18 is produced predominantly by activated macrophages, acts to strongly augment IFN- $\gamma$  production by spleen cells and enhances natural killer cell activity (NK). However, perhaps the most striking property of these cytokines is their marked ability to synergistically increase IFN- $\gamma$  production, a cytokine known to be integral to the development of a functional cellular immune response, in T cells previously exposed to antigen. cDNA clones encoding the p35 and p40 subunits of IL-12 and IL-18 were isolated using RT-PCR and their sequences determined. The p35 IL-12, p40 and IL-18 cDNAs encoded predicted full length proteins of 222, 329 and 192 amino-acids, respectively. All three sequences possessed a high degree of homology with the respective cytokines of other species at both the nucleic acid and protein level. In order to evaluate the potential of IL-12, IL-18 and IFN- $\gamma$  DNA constructs to act as *in vivo* genetic adjuvants in FeLV DNA vaccination studies, these cytokines were cloned

into the mammalian expression vector pCI-neo, and IL-18 protein and mRNA expression were demonstrated in an *in vitro* mammalian expression system using western and Northern blotting techniques.

To assess the potential of a DNA vaccine to protect against feline leukaemia virus (FeLV) infection, a significant pathogen of the domestic cat, a novel FeLV DNA vaccine was constructed, consisting of two separate plasmids, expressing FeLV *gag/pol* and FeLV *env A* genes. The constructs encoding feline interleukin 12 (IL-12), interleukin 18 (IL-18) and interferon gamma (IFN- $\gamma$ ) were coinoculated with the vaccine to establish if protection could be enhanced. Twenty-nine SPF cats were included in the trial. Cats in group A were immunised intramuscularly with the FeLV DNA vaccine alone, cats in groups B, C and D were inoculated with the FeLV DNA vaccine and plasmids expressing IFN- $\gamma$ , IL-12 or IL-12 and IL-18, respectively, and the control cats, in group E, were immunised with empty pCI-neo plasmid. One hundred micrograms of each DNA construct was inoculated at 0, 2 and 4 weeks and intraperitoneal challenge with FeLV-A/Glasgow-1 viral isolate was performed at 7 weeks. No detectable FeLV-specific humoral immune response was elicited following immunisation. Fifteen weeks after challenge, virus isolation (VI) revealed that 2 of the 6 cats in group A, 2 of the 5 cats in group B, 4 of the 6 cats in group C, and 3 of the 6 cats in group E, tested virus isolation positive, while all the cats in group D (vaccine, IL-12 and IL-18) tested virus isolation negative. These findings demonstrate that the combination of IL-12 and IL-18 may act as a potent vaccine adjuvant, markedly enhancing the efficacy of the novel FeLV DNA vaccine.

These studies provide the basis for further investigations of the potential of IL-12 and IL-18 in the treatment of feline disease, particularly as vaccine adjuvants against other feline pathogens, such as FIV, and the examination of their wider clinical potential in immunotherapy and cancer treatment. Ultimately these cytokines may form part of a new array of therapeutic agents to treat feline disease.

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## DECLARATION

The studies described in this thesis were carried out in the Department of Veterinary Pathology at the University of Glasgow Veterinary School between October 1994 and December 1998. The author was personally responsible for all the work described herein except for the following. Haematology analyses described in chapter four were performed by Ronnie Barron and Kenny Williamson in the Department of Veterinary Haematology, University of Glasgow. The FeLV diagnostic tests and bone marrow culturing techniques were performed by Mathew Golder and Mike MacDonald in the Feline Virus Unit, Department of Veterinary Pathology, University of Glasgow. Dr Lesley Nicolson and her colleagues in the EHV laboratory constructed the PsecI plasmid and generated the rabbit anti-equine IL-18 polyclonal antibody. The construction of the pUSE1<sup>+</sup> mammalian expression vector series, FeLV antigen DNA constructs and performance of the transient co-transfections to establish that FeLV antigen constructs were able to produce virions *in vitro* were carried out by Dr Derek Bain. Statistical analyses of results presented in chapter four was performed by Dr Bill Byron. Endotoxin assays described in chapter four were performed by Q1 Biotech Ltd., West of Scotland Science Park, Glasgow.

## **1. CHAPTER ONE; GENERAL INTRODUCTION**

## **1.1 FELINE LEUKAEMIA VIRUS**

### **1.1.1 CLASSIFICATION AND ORIGINS OF FeLV**

Feline leukaemia virus, (FeLV), is a naturally occurring retrovirus (Hayes et al. 1992), prevalent in the world domestic cat population. Retroviruses are single-stranded, diploid RNA viruses whose replication cycles involve reverse transcription into DNA that becomes stably integrated into host genomic DNA as proviruses. The retroviridae consist of three separate subfamilies Spumavirinae, Oncovirinae and Lentivirinae, as reviewed by Teich (Teich, 1984), (Teich, 1985). A member of the Oncovirinae subfamily, FeLV is also classed as a C-type retrovirus. The latter classification system is based on differences in morphology, visualised by electron microscopy (Bernhard, 1958). Separate types of retroviridae are characterised according to differences in core structure and features of the surrounding membrane. FeLV is thought to have originally evolved from an ancestral rodent virus (Benveniste et al. 1975) and is remarkably similar in genetic structure and sequence to the leukaemogenic C-type retroviruses of the laboratory mouse (MuLV) (Neil et al. 1991).

### **1.1.2 DISCOVERY OF FeLV**

FeLV was first reported to be an infectious disease, in 1964 by Jarrett et al (Jarrett et al. 1964). This group demonstrated that when a tumour homogenate from a field case of thymic lymphosarcoma was injected subcutaneously into four newborn kittens, all developed lymphosarcoma. Electron microscopy of the tissues of one kitten subsequently revealed virus-like particles in intracellular vesicles which closely resembled those of the viruses known to cause leukaemia in chickens and mice (Jarrett et al. 1964).

FeLV was rapidly recognised as a significant pathogen of the domestic cat and as a model of contagiously transmitted retroviral disease in a natural, outbred mammalian population (Roy-Burman, 1995), (Neil et al. 1991). Thus FeLV has been of great importance in comparative medicine, influencing the studies which led to the isolation and characterisation of the human retroviruses, human T-cell leukaemia virus (HTLV-1) and human immunodeficiency virus (HIV-1) (Jarrett, 1991).

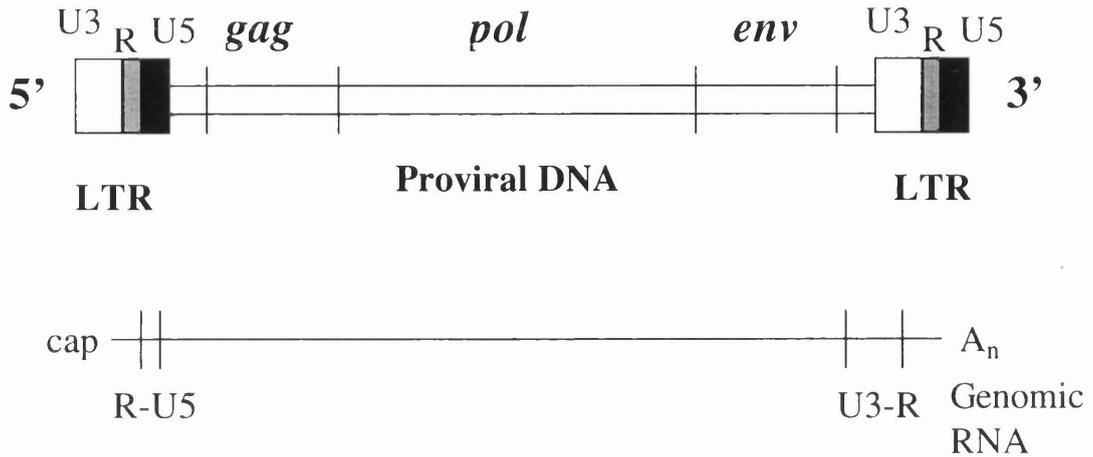
### 1.1.3 STRUCTURE OF FeLV

The genomic organisation of all retroviruses is broadly similar and has been reviewed by Coffin (Coffin, 1984), (Coffin, 1985). The RNA genome of FeLV consists of three genes, *gag*, *pol* and *env*, arranged from the 5' to the 3' end. Figure 1.1. illustrates the genetic organisation of FeLV. The *gag* gene (group-specific antigen gene) codes for the polyprotein precursors of the internal non-glycosylated core proteins, with molecular weights of 15,000 (p15), 12,000 (p12), 27,000 (p27), and 10,000 (p10) Daltons. These proteins are similar in all FeLV isolates. The *env* gene encodes the polyprotein precursor of the envelope proteins which is cleaved to yield a glycoprotein of 70,000 Daltons (gp70) and an non-glycosylated protein of 15,000 Daltons (p15(E)). Variations in the nucleotide sequences of the *env* genes determines the subgroup specificity of each individual isolate. The *pol* (polymerase) region codes for the protease, integrase and reverse transcriptase gene products.

The FeLV genome also consists of 5' and 3' U3, U5 and R regions. These are copied to form the proviral LTR. The LTRs contain sequence which is capable of initiating transcription, as well as the polyadenylation of viral RNA. The U3 region of the LTR is known to contain enhancer elements which promote viral transcription.

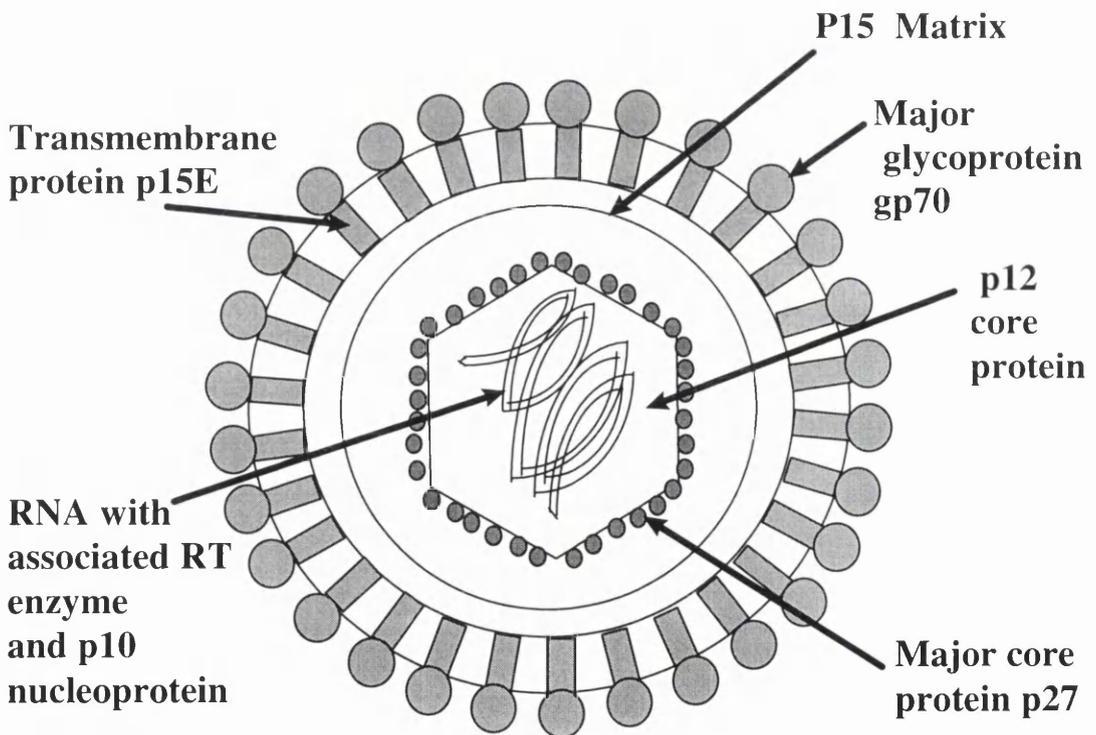
In a virus particle, the viral RNA and the reverse transcriptase are contained in a protective protein core, consisting of p27, p12 and p10. Surrounding this core is the p15 protein, which forms an inner protein coat. The viral envelope is the outermost structure. This is a phospholipid membrane into which the gp70 and p15(E) proteins are inserted. The gp70 protein forms spikes, displayed on the outside surface of the

FeLV particle, and these are anchored into the membrane by the hydrophobic p15(E) protein. Figure 1.2. illustrates the structure of a FeLV particle, and highlights the important antigens.



**Figure 1.1. FeLV genome structure**

**LTR**, long terminal repeat; **A<sub>n</sub>**, polyadenylation; **cap**, the capped nucleotide at the 5' end of the viral RNA; **RT**, reverse transcriptase enzyme (see below).



**Figure 1.2. FeLV particle and important antigens**

#### 1.1.4 REPLICATION OF FeLV

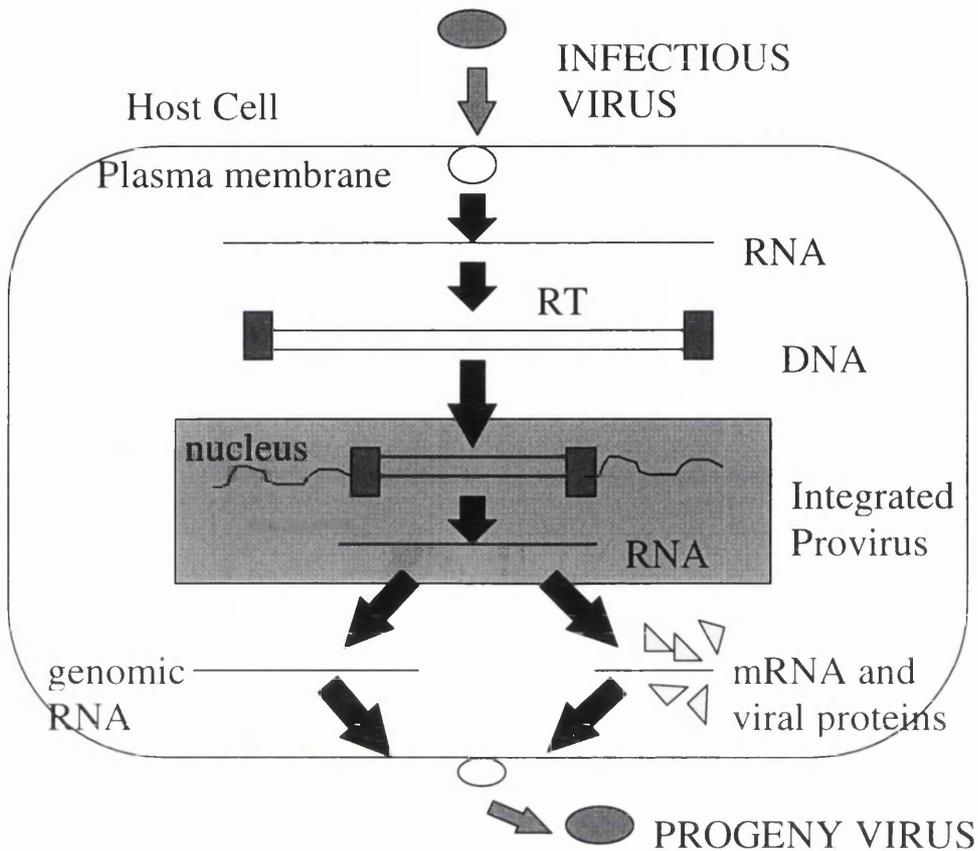
A specific interaction of the viral surface glycoprotein with a host cellular receptor, initiates the infectious cycle of the virus (Rigby, 1989). Subsequent fusion between viral and cellular membranes allows entry of the virus into the host cell. The viral envelope is then shed, to allow the release of viral RNA into the cytoplasm. A single stranded DNA copy is synthesised from the RNA, by the viral reverse transcriptase (RT). This single stranded DNA, in turn, acts as a template for the formation of the double stranded DNA provirus. The provirus integrates into the host cell chromosome, facilitated by the virally encoded enzyme integrase, and in this way becomes part of the cellular genome.

Once integrated into the genome, the provirus utilises the host cell's machinery for transcription and processing of mRNA. Two separate mRNAs are transcribed from proviral DNA; the whole genome, *gag-pol-env*, from which the proteins *gag* and *pol* are translated; and *env*, which is transcribed independently. The constituents of the viral envelope travel to the cell surface and are inserted into the plasma membrane, to form a cup-like structure called the viral bud (Bolognesi et al. 1978). The *gag* polyprotein precursor is then transported to this site. One end of this polyprotein is connected to the viral RNA, while the other is linked to the viral envelope (Bolognesi et al. 1978).

This polyprotein precursor is then cleaved to release the individual core proteins, p15, p12, p27 and p10, which align to create the internal structure of the virus. The *env* precursor, meanwhile, is glycosylated and cleaved to yield gp70 and p15(E) moieties (Bolognesi et al. 1978). The latter is further processed to produce p12(E) (Neil et al. 1980). When assembly and maturation are complete, new virus particles are released through the host cell membrane by budding, without damaging the cell. This results in a viral envelope consisting of host cell membrane and viral glycoproteins. This release of virus by infected cells, characteristic of the Retroviridae, occurs only during mitosis (Jarrett, 1994). This is an important feature of FeLV pathogenesis, since the main sites

of viral replication are in actively dividing tissues, such as the bone marrow. A schematic overview of the life cycle of FeLV is illustrated in Figure 1.3.

There are multiple copies of endogenous FeLV-related sequences, known as enFeLV, in the genome of the domestic cat (Benveniste et al. 1975). Although even the full length enFeLV proviruses do not appear to encode infectious virus (Neil et al. 1991), they are believed to contribute to FeLV pathogenesis by recombining with exogenous FeLV A isolates to generate FeLV B variants (Neil et al. 1987), (Stewart et al. 1986). A truncated *env* protein, expressed by defective endogenous FeLVs may have a role in mediating resistance to infection with FeLV-B isolates (in the absence of FeLV-A isolates) by means of receptor blockade (McDougall et al. 1994). The subgroups of FeLV are described more fully in section 1.1.5.



**Figure 1.3. Schematic representation of the life-cycle of FeLV**

**RT**, reverse transcriptase enzyme.

### 1.1.5 FeLV SUBGROUPS

Exogenous FeLV isolates are classified into three distinct subgroups, A, B and C, on the basis of viral interference with superinfection (Sarma and Log, 1973). These subgroups most likely define envelope subtypes that use different cellular receptor molecules for viral entry (Rohn et al. 1997). The occurrence of the different subgroups in the field has been described in detail by Jarrett (Jarrett, 1980). FeLV-A viruses are ecotropic; that is, capable of infecting only feline cells. They are present in all field isolates (Jarrett and Russell, 1978), (Stewart et al. 1986), are readily transmissible and are highly conserved. This subgroup represents the dominant form of FeLV in nature.

By contrast FeLV-B viruses are polytropic, infecting human cells in addition to feline cells and are estimated to occur in 30-60% of field isolates (Neil et al. 1991). This subgroup, and subgroup C, are always isolated together with FeLV-A viruses. Indeed, subgroup A viruses may facilitate transmission of B and C subgroup isolates as pseudotypes *in vivo* (Jarrett and Russell, 1978). The replication and transmission of B and C isolates is less efficient, and therefore their ability to establish persistent viraemia is significantly reduced. Concurrent infection with an FeLV-A subgroup isolate, however, may result in phenotypic mixing, whereby a FeLV-B or FeLV-C genome can be packaged in a FeLV-A envelope, thus allowing productive infection of cells not normally available to the FeLV-B and C subgroups (Jarrett et al. 1984), (Jarrett et al. 1973), (Rigby et al. 1992). FeLV-B isolates are thought to arise *de novo*, from recombination events between exogenous FeLV-A *env* sequences and endogenous FeLV (enFeLV) *env* elements (Stewart et al. 1986), (Tsatsanis et al. 1994), as described in section 1.1.4.

The FeLV-B subgroup is generally associated with a higher rate of malignancies and immunosuppressive disorders, and can, in some cases, act to accelerate disease induced by weakly oncogenic FeLV-A isolates (Tzavaras et al. 1990). FeLV-B is overrepresented in viraemic cats with lymphosarcomas, relative to infected but otherwise healthy animals (Jarrett and Russell, 1978), (Jarrett et al. 1978). Moreover, although experimental infection with subgroup B viruses is inefficient, the occurrence of lymphoid tumours in animals inoculated with subgroup B virus alone has been documented (Jarrett et al. 1978). This demonstrates the existence of subgroup specific differences in viral pathogenicity and virulence.

FeLV-C subgroup isolates are polytropic and occur in only approximately 1% of infected cats. Viruses of this subgroup are always isolated with FeLV-A or a combination of FeLV-A and FeLV-B (Jarrett et al. 1978), again inferring that FeLV-A viruses are essential for the replication of subgroup C isolates *in vivo*. FeLV-C isolates are thought to arise *de novo* by mutation in the *env* gene of FeLV-A (Neil et al. 1991) and are not transmitted in nature. They are uniquely associated with the development of pure red cell aplasia, one of the most acute and aggressive degenerative retroviral diseases known (Onions et al. 1982), (Mackey et al. 1975).

## **1.1.6 FeLV INFECTION**

### **1.1.6.1 Source and Transmission of FeLV**

Persistently viraemic cats are the main source of FeLV infection (Hardy et al. 1973). Virus is secreted continuously in the saliva of viraemic cats (Francis et al. 1977) and, therefore, is transmitted to susceptible animals during intimate social exchange. Although large amounts of infective virus are excreted in saliva, FeLV is extremely labile and virus survival in the environment, under conditions of desiccation, is less than two hours (Francis et al. 1977).

The generation of long distance aerosols or fomites in the environment are not important factors in the transmission of FeLV (Francis et al. 1977), and although the virus does replicate in the mucosal epithelium of the intestine and the urinary bladder, FeLV is poorly preserved in urine and faeces (Hoover et al. 1977). Similarly, although venereal transmission of the virus is possible, as virus has been detected in semen and vaginal fluids, the concurrent exposure to FeLV in saliva probably represents the greatest risk to the uninfected cat (Hoover and Mullins, 1991). Efficient transmission of FeLV, then, necessitates either direct transfer of virus in salivary or nasal secretions exchanged during intimate contact such as licking, or the prolonged use by infected and non-infected cats of common feed and water sources (Francis et al. 1977).

FeLV can also be transmitted congenitally, from an infected queen to her kittens. The virus may cross the placenta, infecting the developing embryos, and foetal resorption or abortion may occur (Cotter et al. 1975). Indeed, FeLV is the single most common infectious cause of infertility in queens (Jarrett, 1994). Less commonly, the pregnancy may be carried to term and congenitally infected kittens may be born (Hoover and Mullins, 1991). These kittens are predisposed to neonatal mortality, and if they survive, they will generally succumb to an FeLV-related disease within two years. Interestingly, the transmission of FeLV from a latently infected non-viraemic queen to her kittens, via milk, has also been documented (Pacitti et al. 1986). This resulted in persistent

FeLV infection of several litters, who were then able to transmit virus to other susceptible kittens by contact. Latent FeLV infection will be described in more detail in section 1.2.2.3.

#### **1.1.6.2 *In vivo* spread of FeLV**

FeLV infection usually results from oronasal exposure to the virus. The virus enters and replicates in the tonsils and other regional lymphoid tissue, in the first two to four days following exposure (Loar, 1993). This initial phase results in the replication of virus within a small number of circulating monocytes and lymphocytes (Rojko et al. 1979b). Dissemination of these virus-infected lymphocytes then occurs, and by fourteen days post-exposure, viral colonisation and replication is evident in the spleen, visceral lymph nodes, thymus and gut associated lymphoid tissue (Loar, 1993).

Crucially, in the next two weeks (14 to 28 days), the virus may spread to infect the bone marrow and intestinal epithelium. At this stage, viral antigen may be detected in increasing numbers of bone marrow cells, in particular the myelomonocytic precursors. As granulocytes mature, the quantity of antigen present increases and soon circulating neutrophils contain considerable amounts of cytoplasmic viral antigen. The release of free viral particles in the blood coincides with the appearance of these antigen-containing neutrophils and the growth of virus in the bone marrow (Hoover et al. 1977).

Standard diagnostic tests can typically identify viraemia within 14 to 28 days after viral exposure. However, certain FeLV infected cats may require an incubation period of up to eight weeks before diagnostic tests produce a positive result. Finally, normally within days of the onset of viraemia (between 28 and 56 days post-exposure), the virus spreads to non-haemopoietic tissues. The virus replicates in and is excreted from various epithelial sites, particularly the salivary glands, bladder, oropharynx, pancreas and intestine (Loar, 1993), and in this way is transmitted to susceptible cats.

### **1.1.6.3 Diseases associated with FeLV infection**

#### *1.1.6.3.1 Introduction*

A large number of diseases are associated with FeLV infection. These have been described in detail by Jarrett (Jarrett, 1984) and are listed in Table 1.1. Broadly, these can be divided into two categories; firstly, malignant or neoplastic diseases and secondly, non-malignant or degenerative diseases. The diseases occur most commonly in young adult cats, between two and four years of age (Hosie et al. 1989), although there are some notable exceptions, such as alimentary lymphosarcoma, which occurs in older animals. Almost 80% of persistently infected cats die from FeLV-associated diseases, with only 20% of these dying from neoplasia.

### **Malignant haemopoietic diseases**

Lymphosarcoma	thymic lymphosarcoma, multicentric lymphosarcoma, alimentary lymphosarcoma.
Leukaemias	lymphoid leukaemia, myeloid leukaemia, erythroleukaemia.

### **Non-malignant haemopoietic diseases**

Anaemias	haemolytic anaemia, erythroid hypoplasia.
Immunodeficiency	
Marrow aplasia	

### **Non-malignant non-haemopoietic diseases**

Reproductive failure	
FeLV-related enteropathy	
Neurological syndromes	

**Table 1.1. Diseases associated with FeLV infection**

#### *1.1.6.3.2 Malignant disease associated with FeLV*

Malignant disease associated with FeLV include lymphosarcomas (thymic, multicentric and alimentary) and leukaemias (lymphoid, myeloid and erythroleukaemia). Lymphosarcoma is the most common feline malignant tumour and, in fact, represents approximately one third of all tumours (Jarrett, 1994). It is invariably fatal and, therefore, is a major cause of death in the adult cat population (Jarrett, 1994). However, FeLV is not isolated from all cats with lymphosarcoma (Hardy et al. 1980). While 80% of cats with thymic lymphosarcoma and 60% of cats with multicentric or lymphatic lymphsarcoma are viraemic, only 30% of cats with alimentary lymphosarcoma are viraemic (Jarrett, 1994).

There is some evidence to suggest, however, that FeLV is involved in the pathogenesis of these virus negative lymphosarcomas, perhaps by initiating the neoplastic process, before being attacked and removed by the immune system. In fact, an epidemiological association in multi-cat households (in which FeLV is enzootic) has been documented, between FeLV exposure and infection, and the occurrence of cases of virus-negative lymphosarcomas, as well as virus positive tumours (Hardy et al. 1980). Lymphoid cells are involved in approximately 90% of feline haemopoietic tumours. Tumours of the non-lymphoid elements of the haemopoietic system, myeloid and erythroid cell leukaemias, meanwhile, are much less commonly diagnosed (Jarrett, 1994), but are thought to occur more frequently than is generally realised (Jarrett, 1994).

#### *1.1.6.3.3 Non-malignant disease associated with FeLV*

Non-malignant or degenerative haemopoietic diseases include anaemias, such as haemolytic anaemia, (which is rare in the cat), and erythroid hypoplasia, bone marrow aplasia and immunodeficiency (Jarrett, 1994). FeLV-associated anaemia may develop as a consequence of lymphoid or myeloid leukaemia, or it may arise due to a direct effect of the virus, as in erythroid hypoplasia. FeLV-associated immunodeficiency can occur in both kittens and adult cats. In adults, immunosuppression is the most common cause of death associated with FeLV. In young kittens the thymus may atrophy, leading to T lymphocyte depletion and a defective cell-mediated immune response (Anderson et al. 1971). The mechanism by which FeLV produces a state of immunodeficiency is still not fully understood. The strain of the virus may be an important causal factor, as some FeLV isolates have been documented to kill T lymphocytes *in vitro* (Hoover et al. 1987). Another hypothesis is that the viral protein, p15(E), is directly cytotoxic to lymphocytes (Mathes et al. 1979). Regardless of the mechanism, viraemic immunocompromised cats are susceptible to infections with opportunistic, microbial agents, manifested clinically by respiratory tract and enteric infections, gingivitis, abscesses and septicaemia.

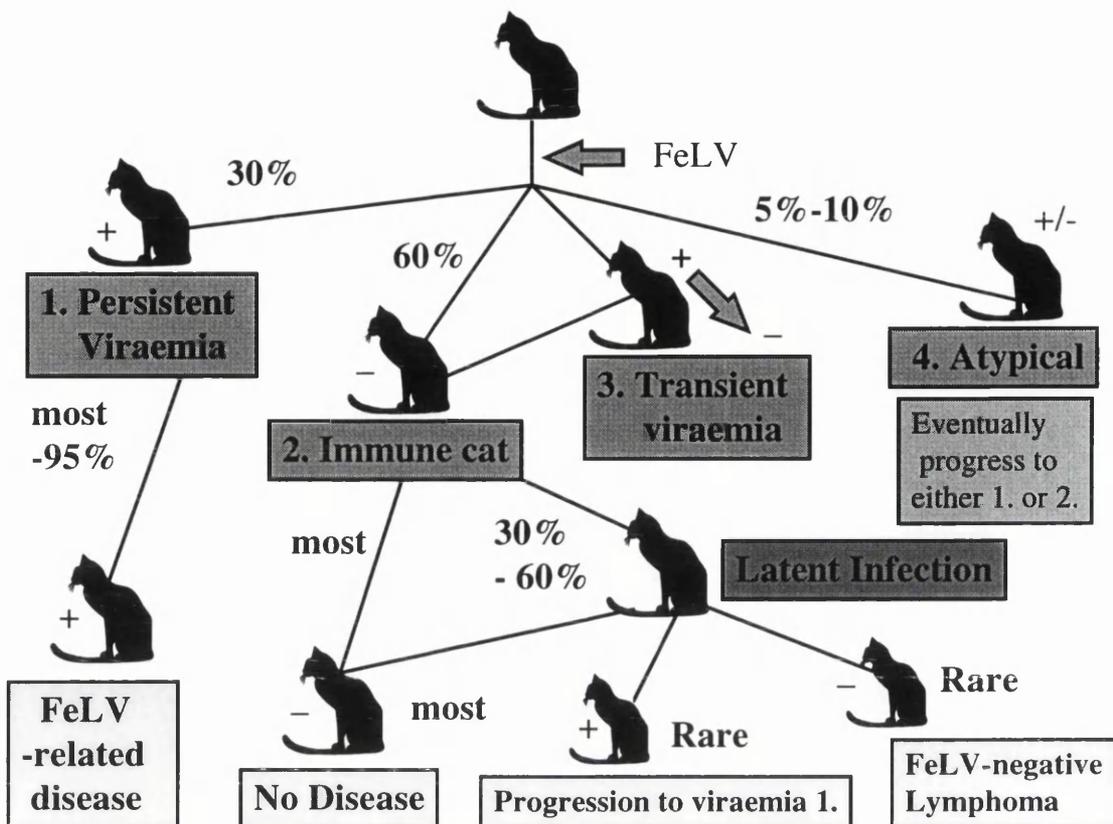
Less commonly, FeLV may be associated with non-malignant, non-haemopoietic disease, such as reproductive failure (mentioned in section 1.1.6.1), neurological

syndromes and FeLV-related enteropathy, a chronic wasting/diarrhoeal syndrome. FeLV-related enteropathy is often associated with concurrent immunodeficiency syndromes or may be due to primary infection of the germinal (crypt) cells of the intestinal mucosa. Therefore, the origins of this condition are still to be fully understood (Hoover and Mullins, 1991).

## **1.1.7 OUTCOMES OF FeLV INFECTION**

### **1.1.7.1 Factors which influence the outcome of FeLV infection**

In the first weeks following viral exposure, interactions between the host's immune system and the virus itself determine the eventual long-term outcome of infection; i.e., whether persistent productive infection is established, whether a latent infection develops or whether the virus is completely cleared (a self-limiting infection), and immunity is established (Hoover and Mullins, 1991). Rarely, an atypical infection develops, as explained in section 1.1.7.4., although these cases are likely to progress to either extinguished infection or persistent viraemia, over time (Rojko and Hardy, 1994). Figure 1.4. provides a schematic overview of the possible outcomes of FeLV infection.



**Figure 1.4. Possible outcomes of FeLV infection in a multi-cat household**

The above figure illustrates the four possible outcomes of FeLV infection, described in section 1.1.7: persistent viraemia, transient viraemia, atypical infection, and immunity, and the estimated percentages of cats in each category.

A number of factors are important in determining the eventual outcome of viral exposure (Sparkes, 1997). Probably the most important factor is the age at which the cat is first exposed to FeLV. Hoover et al. found that the susceptibility of cats to FeLV infection decreased with age (Hoover et al. 1976). When virus was inoculated experimentally, 100% of newborn kittens became persistently viraemic, as compared to only 15% of cats aged between four months and one year becoming persistently infected. The remaining cats in this group cleared the virus and became immune.

Other significant factors which influence the outcome of FeLV infection are the dose of the virus received, the frequency of exposure to the virus and the strain and pathogenicity of the viral isolate. In nature FeLV infection is more prevalent in multi-cat households or breeding colonies, where prolonged, close, social contact ensures that uninfected individuals are constantly exposed to high doses of virus from shedding, infected cats. In contrast, the cycle of viral transmission in free-ranging outdoor cats involves the excretion of small amounts of virus, for short periods, by transiently viraemic cats. There is widespread dissemination of virus throughout the cat population, and the majority of cats are therefore exposed. However, the dose of virus is small and, consequently, most cats recover. The nature of an individual cat's immune response is also important. Cats which are immunosuppressed naturally, due to concurrent disease, or experimentally, due to the administration of high or prolonged doses of corticosteroids, have an increased risk of becoming persistently viraemic. In fact, natural age resistance to FeLV infection can be overcome by the administration of high doses of exogenous corticosteroids (Rojko et al. 1979a). It has also been suggested that the genotype of an individual animal is important in determining innate immunity and resistance to FeLV infection (Hoover et al. 1980).

### **1.1.7.2 Persistent infection**

Bone marrow infection is generally established within two to six weeks of exposure to FeLV. If the immune response fails to contain viral replication at this crucial stage, there is the potential for large quantities of virus to be produced, which may overwhelm the immune system and establish persistent viraemia (Hoover and Mullins, 1991). The persistently infected state is characterised by the presence of free infectious virus and viral antigen in the blood, and virus excretion (Hoover and Mullins, 1991). The *in vivo* spread of virus in a persistently infected cat is described in section 1.1.6.2.

Although persistently infected cats may remain asymptomatic for a period of months to years, the long term prognosis is very poor; most die within three years of infection, most commonly from non-neoplastic disease (Hardy, 1980). The majority of free-ranging, outdoor cats are exposed to the virus, but due to natural age resistance to FeLV infection, and the low virus doses transmitted, most healthy adults are able to

resist persistent infection and develop naturally acquired protective immunity (Pedersen et al. 1977). In contrast, a significant proportion of adult cats that are immunocompromised, or are subjected to high doses of virus (such as breeding colony cats), (Hardy et al. 1973) and kittens, who are immunologically immature, become persistently infected (Hoover et al. 1976). Overall, persistent viraemia, (progressive infection), develops in approximately 30% of unvaccinated exposed cats in multi-cat households (Hardy et al. 1976).

### **1.1.7.3 Recovery from FeLV infection; immune cats**

#### *1.1.7.3.1 Introduction*

Approximately 60% of cats exposed to FeLV develop a self-limiting infection and produce adequate cellular and humoral immune responses in the early stages of lymphoid tissue viral replication (Hoover and Mullins, 1991). Thus, viral replication and expression are effectively contained within four to eight weeks of FeLV infection, and immunity is established. In most cats, immunity is established before or during bone marrow infection, so a marrow-associated viraemia does not develop. In a fraction of cats, however, protective immunity does not develop until shortly after bone marrow-origin viraemia is established (Jarrett et al. 1982). In these cats (between 30 and 40% of all recovered cats), transient viraemia and/or antigenaemia is observed for a short period, usually lasting only days to weeks, before the virus is cleared and the animal ostensibly recovers from FeLV infection (Hoover and Mullins, 1991). However, the development of transient viraemia, which usually remains undetected, may increase the likelihood that latent bone marrow infection will develop (Sparkes, 1997), (section 1.1.7.5).

#### 1.1.7.3.2 *Virus neutralising antibodies*

Recovered, immune cats are those that have apparently eliminated all virus and virus infected cells from their bodies, and are immune to subsequent FeLV exposure. They consistently test negative for the presence of virus or viral p27 antigen in the blood, and usually develop a significant virus-neutralising (VN) antibody titre (Hoover and Mullins, 1991). Virus-neutralising antibodies inactivate virus and, by fixing complement, can destroy virus-expressing cells, *in vitro* (Grant et al. 1983). These antibodies are assumed to have a similar protective effect *in vivo* and are often detected in cats that have eliminated a transient viraemia (Jarrett et al. 1973); they are, in fact, often associated with recovery from FeLV infection. Moreover, small amounts of virus-neutralising antibodies have been shown to protect kittens from viraemia, when they were challenged with large doses of virus (Jarrett et al. 1977).

#### 1.1.7.3.3 *Anti-FOCMA antibodies*

A second type of antibody elicited in infected cats, are anti-FOCMA antibodies. These are raised against a cell membrane antigen, feline oncornavirus-associated cell membrane antigen, FOCMA, which is expressed on the cell surface of FeLV-transformed cells (Vedbrat et al. 1983). Originally, FOCMA was defined as an antigen present on the surface of the FL74 feline lymphosarcoma cell line, established by Theilen (Theilen et al. 1969), which reacted with sera from cats which had recovered from feline sarcoma virus infection (Essex et al. 1971), or exposure to FeLV (Riggs, 1971).

However, the exact origin of FOCMA has not been completely established but is most likely to be through transcription of endogenous FeLV related sequences, (enFeLV), in FeLV infected cells (Rojko and Kociba, 1991). Anti-FOCMA antibodies in sera are very common, especially amongst free-ranging urban cats. Rogerson in 1975 (Rogerson et al. 1975) found that 50% of urban cats had identifiable levels of these antibodies in their sera, whereas in a study by Russell and Jarrett in 1978 (Russell and Jarrett, 1978), only 4% of a similar population possessed a detectable titre of virus neutralising antibody. Thus, it appears that the presence of FOCMA antibodies in sera is a sensitive indicator of exposure to FeLV.

Anti-FOCMA antibodies are not protective against persistent FeLV infection, as even persistently viraemic cats may often possess low antibody titres. However, the presence of these antibodies has been found to correlate with protection against the development of FeLV-related neoplasia (Rojko and Hardy, 1994), (Grant et al. 1980), as first suggested by Essex in 1971 (Essex et al. 1971). Low or non-existent antibody titres are frequently associated with the development of leukaemias or lymphomas, while high titres are often associated with protection from the development of neoplasia. Complement-mediated (antibody-dependent) lysis of the transformed cells is thought to be the mechanism by which anti-FOCMA antibodies achieve this protection (Grant et al. 1979).

Two of the five commercially available vaccines claim to incorporate FOCMA. However, if a vaccine protects against FeLV infection, it will also protect against the development of FeLV-related neoplasia, regardless of whether FOCMA is present in the preparation or not. There is no evidence to suggest that FOCMA affords any significant degree of protection from FeLV infection (Loar, 1993). Thus, the inclusion of FOCMA in a vaccine formulation is probably of no benefit.

#### *1.1.7.3.4 Latency*

A substantial proportion of ostensibly immune, non-viraemic/non-antigenaemic cats harbour a latent FeLV infection (Rojko et al. 1982). The latent state is discussed in more detail in section 1.1.7.5.

#### **1.1.7.4 Atypical FeLV infection**

Atypical infections develop in a small proportion of FeLV-infected cats, perhaps between five and ten percent (Hoover and Mullins, 1991). A sequestered source of FeLV p27 antigen or virus producing cells persist somewhere in the body (Jarrett et al. 1991), but a partially protective immune response is thought to prevent replication and widespread viral dissemination. Viral p27 antigen produced by infected cells may reach the blood, but, presumably due to protective levels of circulating virus

neutralising antibody, infective virus cannot. Therefore, these cats are frequently discordant (Hayes et al. 1992); they exhibit intermittent antigenaemia but are usually non-viraemic, over a prolonged period.

Atypical, localised FeLV infections have been described in the mammary gland of a lactating queen (Pacitti et al. 1986), in the spleen and intestinal crypt epithelia of a non-viraemic cat (Hayes et al. 1989) and in spleen, lymph nodes, bone marrow and small intestine of five non-viraemic animals, in a survey to determine the incidence of localised FeLV infection (Hayes et al. 1992). The full significance of these type of infections in the pathogenesis and epidemiology of FeLV is yet to be elucidated, although they are likely to progress to either extinguished infection or persistent viraemia, over time (Rojko and Hardy, 1994).

#### **1.1.7.5 Latent FeLV infection**

Latent infection of bone marrow occurs in a significant proportion (between 30 and 60%) of ostensibly immune cats, following recent recovery from FeLV infection and viraemia (Pedersen et al. 1984). The marrow is thought to be one of the last reservoirs of FeLV, during recovery (Pedersen et al. 1984). Latent infection, is characterised by the presence of integrated provirus, within the genome of myelomonocytic precursor cells in the bone marrow (Rojko et al. 1982), and the generation of an immune response to the virus, identified by the presence of FeLV virus neutralising antibodies (Madewell and Jarrett, 1983). The phenomenon of viral persistence in the presence of an immune response is not unique to FeLV; it has been described in other retroviral diseases, such as enzootic bovine leukosis (Piper et al. 1979), equine infectious anaemia (Crawford et al. 1978), visna virus infection in sheep (Narayan et al. 1977) and human immunodeficiency virus (Maniatis et al. 1982).

Infectious virus released from latently infected cells is killed by the protective humoral immune response, maintaining the virus in its quiescent, integrated form and ensuring that virus does not reach the peripheral circulation. A study by Pacitti demonstrated that virus neutralising antibody prevented the spread of virus from latently infected cells *in vitro* (Pacitti et al. 1986). However, reactivation of productive infection and

reversion to viraemia can occur, when host immunological control is breached. This can be attained by culturing bone marrow cells *in vitro*, or by immunosuppression *in vivo*; achieved experimentally by the administration of corticosteroids or naturally, due to concurrent illness, stress or changes in endogenous hormone levels (Rojko et al. 1982). Latent infection, however, is usually a temporary state {Pedersen, Meric, et al. 1984 ID: 1566}, (Pacitti and Jarrett, 1985), and by six to eight months post-viraemia, most cats have completely eliminated the virus (Pedersen et al. 1984). However, a small proportion of animals may remain latently infected for longer periods (Pacitti and Jarrett, 1985) or even indefinitely (Hoover and Mullins, 1991).

The significance of the latent carrier state in the pathogenesis and epidemiology of FeLV infection is still to be completely defined, in terms of whether reversion to viraemia, productive infection and virus excretion can occur. Reactivation of latent FeLV infection in the post recovery period, due to substantial impairment of immune defences, has been observed in a small number of cats, (Rojko et al. 1982), (Hoover and Mullins, 1991). However, most animals mount an adequate immune response, produce significant amounts of virus neutralising antibody and eliminate virus following a latent infection. Therefore, reversion to the viraemic state is rare (Rojko and Hardy, 1994).

Several researchers have also considered whether the development of latency predisposes to the development of FeLV-related diseases, even in the absence of viraemia. Proviral integration into the host genome may result in insertional mutagenesis; that is, activation of oncogenes or suppression of tumour suppressor genes, which may eventually culminate in neoplasia. In a study by Rojko, FeLV was isolated from cultured bone marrow cells derived from two cats with FeLV negative lymphosarcoma (Rojko et al. 1982). Moreover, a study by Pedersen of 400 FeLV recovered cats documented a higher than expected incidence of virus negative neoplasia (of the type usually associated with active FeLV infection), although the number of latently infected cats was not stated (Pedersen et al. 1984). These findings suggest that cats with lymphosarcoma which test negative for virus may host latent viral infections which may predispose to the development of disease.

A study by Jarrett, however, provided contrasting results; marrow cells collected and cultured from two cats with FeLV negative neoplasia, did not yield infectious virus (Madewell and Jarrett, 1983). More cases of FeLV-free lymphosarcomas and leukaemias must be investigated, and cats with latent infections examined, to further elucidate the role of latency in FeLV related disease. Interestingly, *in vitro* studies have identified a persistent neutrophil dysfunction, in the form of impaired cytotoxic function, in persistently and latently infected cats, (Lafrado et al. 1989). This may result in an immunosuppression, which renders the latently infected animal susceptible to the same opportunistic pathogens and disease syndromes associated with chronic persistent FeLV infection. The above may represent a mechanism by which latent FeLV infection establishes disease and, in wider terms, illustrates that persistent retroviraemia is not essential for the establishment of immunosuppression.

In terms of virus excretion, it appears that most latently infected cats do not shed virus, and therefore do not transmit virus to susceptible animals (Madewell and Jarrett, 1983). However, transmission of FeLV via the milk to the kittens of a latently infected nonviraemic queen has been demonstrated (Pacitti et al. 1986). This demonstrates that in a breeding colony, at least, latent FeLV infection may represent a potential source of infective virus.

## **1.1.8 CONTROL OF FeLV INFECTION**

### **1.1.8.1 Test and removal schemes**

Before the advent of efficacious vaccines, test and removal programmes were adopted in an attempt to limit and eradicate FeLV infection, especially in multi-cat households. Hardy described an effective control programme (Hardy et al. 1976) which basically involved testing all cats in the household for FeLV, identifying the cats that tested positive, then separating positive and negative animals. All cats were then retested after 12 weeks, and those which tested positive again were considered persistently viraemic, and were removed from the cohort (or isolated). Those which tested negative

on second sampling were kept in isolation until they tested positive on two consecutive occasions and those that tested negative twice were considered truly non-viraemic. Frequent retesting, every six to twelve months was then recommended. This programme was indeed successful; in a study of multi-cat households, after removal of viraemic cats, only three out of 657 animals, (0.46%), became viraemic, compared to 55 out of 284, (19.3%), which became persistently infected when viraemic cats were not removed (Hardy et al. 1976).

## **1.2 FELINE LEUKAEMIA VIRUS VACCINES**

### **1.2.1 EARLY HISTORY OF FeLV VACCINATION**

Several observations led to the belief that the production of an effective vaccine against FeLV was possible. Firstly, many cats developed natural protective immunity to FeLV. Thus, recovered cats generated virus neutralising antibodies (Jarrett et al. 1973) and became resistant to re-infection with the virus. Secondly, maternally derived antibody was found to protect kittens from viral challenge (Jarrett et al. 1977), and thirdly, as the virus was transmitted through contact and not genetically (Hardy et al. 1973), the cycle of infection could potentially be broken.

Many different strategies for the development of FeLV vaccines have been employed, and advantages and disadvantages of these approaches are outlined in table 1.2. Jarrett reported the first attempt to produce a FeLV vaccine in 1974 (Jarrett et al. 1974), (Jarrett et al. 1975). Two cellularly derived vaccines were produced, which consisted of either live or paraformaldehyde-inactivated lymphoblastoid cells of the FL74 line, established by Theilen (Theilen et al. 1969). Both vaccines induced anti-FOCMA antibodies and it was found that the live cell vaccine protected cats against viral challenge. However, the cats vaccinated with the inactivated cell preparation were not challenged, so the efficacy of this vaccine, in terms of its ability to protect against infection, was never evaluated.

A major drawback to this work, however, was that the live FeLV-infected tumour cells used in the vaccine could generate infectious virus and therefore could potentially cause disease in some animals. The use of a live retroviral vaccine was also considered, at that time, to be a potential public health hazard. Consequently, attention was focused on the development of inactivated cell vaccines, using formalin, heat and ultraviolet light to kill live virus. Although these vaccines were able to elicit anti-FOCMA and occasionally virus neutralising antibodies, post-challenge protection from

viraemia was uncommon. For example, in a study by Pedersen et al, an inactivated whole FL74 cell vaccine did not elicit a protective antibody response or confer protection against persistent infection, whereas a live virus vaccine was found to be highly efficacious in preventing the development of persistent infection (Pedersen et al. 1979).

Moreover, when killed FeLV virus was added to inactivated cell preparations, in an attempt to improve vaccine efficacy, this actually appeared to increase the prevalence of FeLV associated disease in vaccinates (Olsen et al. 1977). Initially, this problem was attributed to the FeLV envelope protein p15E, (Mathes et al. 1979), as this protein had reportedly caused immunosuppression *in vivo* and *in vitro*. Now, however, this is not a widely held belief. One study found that antibodies raised against p15E may play a role in FeLV neutralisation (Francis et al. 1977) while others have suggested that the presence of the p15E protein may actually improve vaccine efficacy, perhaps by enhancing the potency of cell-mediated immune responses (Hoover et al. 1991). In fact, data began to accumulate in the early 1980s, which suggested that inactivated whole virus vaccines could protect cats from persistent viraemia (Pedersen et al. 1979), (Pedersen et al. 1986).

Type of vaccine	Advantages	Disadvantages
Live attenuated virus vaccines.	Both cellular and humoral immunity generated. Provide good protection.	Not safe; may revert to virulence and cause disease in some animals
Inactivated whole virus vaccines.	All B and T cell epitopes presented.	Questionable safety. No CTL response. Expensive production. Adjuvants needed.
Viral subunit vaccines (from native virus, recombinant proteins, or peptides).	Only selected epitopes presented. Safe.	Adjuvants needed. No CTL response. (exception: ISCOM)
Live viral vector vaccines.	B and T cell responses.	Repeated boosters not possible? Limited antigenicity.

**Table 1.2. Approaches for the development of FeLV vaccines; advantages and disadvantages**

CTL, Cytotoxic T lymphocyte; ISCOM, immunostimulating complex vaccine

### **1.2.2 SUBUNIT PRODUCTS; THE FIRST COMMERCIAL FeLV VACCINE**

In the late 1970s it was demonstrated that the manipulation of the tissue culture medium used to grow FL74 cells (FeLV infected tumour cells) allowed the generation and expression of large amounts of soluble FeLV antigens, including gp70 and FOCMA, without the production of large quantities of live infectious virus (Wolff et al. 1979). These viral antigens were later harvested, purified, and combined with an adjuvant, to produce a vaccine which Olsen and colleagues found to be protective against viral challenge (Lewis et al. 1981). A commercial vaccine based on this

approach, originally manufactured by Norden Laboratories, (Lincoln, Nebraska, USA), became available in 1985. This vaccine, Leukocell, a subunit vaccine (Sharpee et al. 1986), was the first commercial FeLV vaccine, and required the administration of three doses for initial immunisation. In 1988, modification of the original vaccine led to the licensing of its successor, Leukocell 2, which required the administration of only two immunisations in the initial vaccination course.

Several studies reported the efficacy of Leukocell and Leukocell 2 (Sharpee et al. 1986), (Haffer et al. 1990), (Lafrado, 1994), (Pollock and Scarlett, 1990). Pollock conducted a long-term study with Leukocell, using natural exposure challenge in an infected multi-cat environment and found that the incidence of persistent viraemia, after challenge, was approximately three times greater among the non-vaccinates than among the vaccinates (Pollock and Scarlett, 1990). However, several recent studies have questioned the efficacy of Leukocell (Legendre et al. 1990), (Hoover et al. 1996), (Pedersen and Johnson, 1991).

A study conducted by Legendre, using natural exposure challenge, found that 64% of unvaccinated kittens and 70% of vaccinated kittens became infected after viral challenge, while 43% of unvaccinated and 39% of vaccinated kittens died (Legendre et al. 1990). These results indicated that the Leukocell vaccine provided no significant protection against infection with FeLV. Hoover, meanwhile, demonstrated a preventable fraction (PF) of only 12%, when testing the efficacy of Leukocell (Hoover et al. 1996). The PF is defined as the proportion of cats protected by vaccination in excess of that protected by natural innate resistance (Loar, 1993) so that the fact that often considerably less than 100% of control cats develop persistent viraemia is taken into account in the calculation. PF is calculated using the following formula.

$$\text{PF (\%)} = \frac{\% \text{ controls with persistent viraemia (PV)} - \% \text{ vaccinates with PV}}{\% \text{ controls with PV}}$$

However, other factors are important in assessing vaccine efficacy. An evaluative study of Leukocell 2, conducted by Lafrado, illustrates this point (Lafrado, 1994).

Twenty-six eight week old SPF kittens were vaccinated subcutaneously with 2 doses of the FeLV vaccine, and 26 age-matched specific pathogen-free cats were similarly vaccinated with a placebo vaccine containing the same adjuvant as the FeLV vaccine. Cats then were randomly assigned to 2 groups of 26 cats and each group was housed with 5 viraemic cats, previously inoculated with FeLV. All cats were tested biweekly for the next 26 weeks for evidence of FeLV antigenemia and the PF at the end of the trial was calculated as 100%. However this result reflected the development of persistent viraemia in only one of 26 control cats, and none of the 26 vaccinates. The fact that a very small percentage (4%) of the unvaccinated controls became persistently viraemic suggests that viral challenge was not vigorous enough. Therefore, taking into account the inadequacy of the challenge, it is impossible to evaluate the efficacy of Leukocell 2 from the results produced by this study. To date, the efficacy of these widely used vaccines, Leukocell and Leukocell 2, are still in question.

### **1.2.3 WHOLE VIRUS PRODUCTS**

Although vaccination with attenuated but non-inactivated virus was found to be highly efficacious in protecting cats against FeLV challenge, the safety risks involved with the use of replicating live virus led many researchers to develop FeLV vaccines using distinct strains of inactivated virus, with or without adjuvants, (Hines et al. 1991), (Sebring et al. 1991), (York and York, 1991). These vaccines, Fevaxyn FeLV (Solvay-Dulphar/Fort Dodge), Fel-o-vax (Fort Dodge) and Leucat or VacSYN (Rhone Merieux/Synbiotics), respectively, were found to be highly efficacious by the researchers involved in their development. Chronologically, by the date of licensing, the vaccines became available in the US in the following years: Covenant (Haver/Diamond Scientific) in 1988, withdrawn in 1989, VacSYN (Rhone Merieux/Synbiotics) in 1989, Fel-o-Vax (Fort Dodge) in 1989 and Fevaxyn (Solvay-Dulphar/Fort Dodge) in 1991. Of these vaccines, only Fevaxyn FeLV is available in the United Kingdom.

However, the efficacy of several of the above vaccines and Leukocell has been questioned by many researchers (Osterhaus et al. 1989), (Pedersen and Johnson,

1991), (Legendre et al. 1991), (Jarrett and Ganiere, 1996). Disadvantages associated with these vaccines were their inability to induce cytotoxic T lymphocyte (CTL) activity and their inherent lack of immunogenicity; powerful adjuvant systems were needed to induce an adequate immune response in vaccinates. In 1991, Legendre evaluated Fel-o-Vax, Leukocell 2 and VacSYN (Legendre et al. 1991) and found that although Fel-o-vax completely protected all vaccinates from viral challenge, a PF of only 34% was obtained with Leukocell 2 and a PF of 21% with VacSYN. A study by Pedersen, reported similar results (Pedersen and Johnson, 1991). A PF of only 17% was obtained after vaccination with Leukocell 2, and a PF of 39% after vaccination with VacSYN.

These results contrast dramatically with those of the developers of the vaccines (York and York, 1991), (Pollock and Haffer, 1991) and in-house studies performed by the vaccine manufacturers. However, due to differences in challenge methods, numbers of animals used, vaccine strains and doses, post-challenge monitoring protocols and the researchers' definition of persistent viraemia, it is often extremely difficult to interpret and then to compare results to those obtained in other studies. Another problem is that the majority of FeLV vaccine trials have been performed or supported by vaccine manufacturers or distributors; fully independent trials are obviously preferable. However, experimental evidence does suggest that many conventional inactivated viral vaccines simply do not generate sufficient immunity to completely protect against the development of persistent (Jarrett and Ganiere, 1996) or transient viraemia, thus increasing the likelihood that latent bone marrow infection will develop (Sparkes, 1997).

Two inactivated whole viral vaccines, which were demonstrated to be highly efficacious in protecting cats against persistent viraemia by several investigators were Fel-O-Vax, (Fort Dodge), (Hoover et al. 1996), (Legendre et al. 1991) and Fevaxyn FeLV, (Solvay-Dulphar/Fort Dodge), (Pedersen, 1993), (Hines et al. 1991). Although Fel-O-Vax is extremely efficient in protecting against the development of persistent viraemia, one study demonstrated that almost one half of the non-viraemic, protected cats were transiently viraemic during the trial (Legendre et al. 1991) and almost half were latently infected, 23 weeks after viral exposure.

Fevaxyn FeLV meanwhile, may be superior to Fel-O-Vax in terms of protecting against the development of transient viraemia and latent FeLV infection. However, in one trial using this vaccine (Hines et al. 1991), a significant number of cats were persistently viraemic at the end of the trial (12 out of 144 animals) or transiently viraemic during the trial (10 cats from the 132 protected cats). Also, five of the transiently viraemic cats were found to be latently infected at the termination of the experiment. Another study involving Fevaxyn FeLV, found that this vaccine completely protected all vaccinates (10/10), from latent infection (Pedersen, 1993). However, the strain of FeLV used for challenge, CT600, does not produce a high proportion of latent infections, compared with other strains, such as Rickard-FeLV (Pedersen et al. 1984). It is, therefore, difficult to judge how effective this particular vaccine is in preventing the development of latency. In summary, although Fevaxyn FeLV and Fel-O-Vax are efficacious inactivated whole virus vaccines, neither are able to completely prevent the development of transient or latent FeLV infection.

As well as the serious questions raised relating to the efficacy of the inactivated virus vaccines, there were concerns regarding the expense of their production and misgivings regarding the safety of administering the vaccines to cats (Loar, 1993). Firstly, there was the concern, albeit controversial, that the p15E protein present in whole virus and some subunit products, could exert an immunosuppressive effect (Mathes et al. 1979). Secondly, there was the fear that inoculation with an impure preparation, containing extraneous proteins to which cats had been sensitised to previously, perhaps through being vaccinated against other feline diseases, might increase the likelihood of initiating an allergic reaction (Kensil et al. 1991). A vaccine which contained serum proteins or feline cellular antigens could also potentially induce serum sickness or autoimmune disease. Lastly, and probably most importantly, there was the fear that residual, live virus could survive the inactivation process, contaminate the vaccine and replicate and establish FeLV infection in the vaccinate. All the aforementioned problems associated with inactivated whole virus vaccines led to attempts to produce novel vaccines using recombinant DNA technology.

## **1.2.4 GENETICALLY ENGINEERED VACCINES**

### **1.2.4.1 Introduction**

Recombinant DNA technology allows for the consistent production of large quantities of pure, stable antigen of predictable quality. A major advantage that genetically engineered vaccines have over inactivated whole virus formulations is that specific, protective amino acid sequences may be selected for inclusion in the vaccine, while non-protective or potentially immunosuppressive sequences are deleted. Moreover, because live virus is not required for the production of the antigen, there is absolutely no chance that residual virus may survive the inactivation process. The manufacture of pure recombinant antigen also ensures that extraneous proteins and residual media components, which may initiate allergic reactions, or protease contaminants, which may destroy important antigenic proteins, are not present in the vaccine.

In the mid 1980s, much of FeLV vaccine research was focused on developing genetically engineered viral subunit protein vaccines. Among the various structural proteins of FeLV, the gp70 envelope glycoprotein was considered by many to be crucial to the development of protective immunity in cats (Salerno et al. 1978). Pedersen, aware of the growing interest in recombinant subunit FeLV vaccines, realised that the ability to protect against viral challenge and immunogenicity of native viral protein subunits had to be determined first. Therefore he produced a purified subunit FeLV vaccine, consisting only of viral gp70, which had been isolated from purified virions by affinity chromatography (Pedersen et al. 1986). This vaccine, however, proved to be ineffective and may actually have enhanced infection, as a greater proportion of vaccinated kittens became persistently viraemic after viral challenge compared to non-vaccinates (Pedersen et al. 1986).

### **1.2.4.2 Viral vector vaccines; vaccinia virus**

Since then, several live viral vector systems, expressing one or more FeLV antigens in infected cells, have been considered as candidate vaccines (Willemse et al. 1996), (Wardley et al. 1992), (Tartaglia et al. 1993), (Gilbert et al. 1987). These vaccines

were expected to elicit potent humoral and cellular immune responses, through the expression of endogenously processed antigen. However, a potential hazard associated with this type of vaccination is that booster immunisations may initiate an inappropriate immune response to the delivery vector which, in turn, may interfere with the immune response directed to the antigenic component of the vaccine (Ramsay et al. 1997). An attempt to use vaccinia virus to express the FeLV gp70 antigen protein was unsuccessful (Gilbert et al. 1987). Although cats raised a typical virus neutralising antibody response to the vaccinia virus vector, no antibodies elicited against FeLV gp70 were detected in any vaccinates. Moreover, a subsequent “booster” vaccination with killed FeLV demonstrated that immunologic priming had not been elicited by the recombinant vaccine. A personal communication from the above authors, cited in another paper, (Tartaglia et al. 1993), reported that this vaccine did not protect cats against viral challenge.

#### **1.2.4.3 Viral vector vaccines; canarypox virus**

In contrast, a canarypox vector, (ALVAC), vaccine, expressing both *gag* and *env* FeLV proteins, was successful (Tartaglia et al. 1993). This vaccine protected all of six cats against persistent viraemia, in the absence of detectable virus neutralising antibodies at the time of challenge. However, all protected cats developed virus neutralising antibody activity between nine and twelve weeks post-challenge. This suggests that protection was associated with the ability to raise a neutralising antibody response after challenge but does not rule out the possibility that other immune mechanisms such as the induction of cytotoxic lymphocyte activity, were also involved. This vaccine is in development for commercial use (Jarrett, 1996).

#### **1.2.4.4 Viral vector vaccines; feline herpesvirus**

Feline herpesvirus type 1 (FHV-1) had been considered a good candidate vector for expression of FeLV proteins, mainly because its primary sites of replication, the mucosae of the cat’s oral and nasal cavities, are also the primary targets of FeLV infection (Willemse et al. 1996). The first attempt to vaccinate with FHV-1, expressing only FeLV *env* protein, did not protect cats against viral challenge (Wardley

et al. 1992). Meanwhile, attempts to vaccinate with herpesvirus expressing both FeLV *gag* and *env* proteins were also unsuccessful until a booster vaccination with baculovirus expressed FeLV *gag* and *env* proteins was administered (Wardley et al. 1992). A later attempt, using recombinant FHV-1 alone, expressing *env*, produced better results (Willemse et al. 1996). The construct used here differed from the previous study in that the insertion site and the promoter driving expression of the FeLV *env* gene were altered. The different insertion site, the thymidine kinase gene rather than the ORF2 locus, may have influenced local replication of the recombinant. Although this vaccine did afford some degree of protection against persistent infection in that 3/4 vaccinates were protected while 5/6 controls became persistently infected after challenge, all but one of the vaccinates was found to have latent FeLV infection in bone marrow (Willemse et al. 1996). Moreover, the small number of animals involved in this trial makes it difficult to accurately evaluate the efficacy of this vaccine.

#### **1.2.4.5 Recombinant subunit vaccine; Leucogen**

##### *1.2.4.5.1 Introduction*

Since the importance of the gp70 protein in protection from FeLV infection was identified, several attempts have been made to produce efficacious FeLV vaccines using this glycoprotein (Salerno et al. 1978), (Pedersen et al. 1986). These attempts were not successful, as described earlier in this section, either in terms of serological response or protection from viral challenge. However, one vaccine based on pure, recombinant FeLV gp70 antigen has proved to be efficacious and is now in widespread use (Kensil et al. 1991), (Marciani et al. 1991), (Clark et al. 1991).

##### *1.2.4.5.2 Production of Leucogen*

A portion of the envelope gene of a FeLV subgroup-A clone, encoding the entire exterior envelope protein gp70 and the first 34 amino acids of the transmembrane protein p15E, was cloned into a prokaryotic expression vector (Kensil et al. 1991). The recombinant antigen was then expressed by *E. Coli* and purified, by extracting

from bacteria with urea and precipitating on to aluminium hydroxide. Finally, the antigenic protein component of this vaccine, the non-glycosylated form of gp70, termed p45 *env*, was used in conjunction with a novel purified saponin adjuvant, QS-21 (Marciani et al. 1991). Thus, the use of recombinant DNA technology allowed the production of large quantities of purified recombinant protein which could then be combined with a highly effective adjuvant. This resulted in the production of the efficacious commercial vaccine, Leucogen (Virbac), which is perhaps the most widely used FeLV vaccine in the United Kingdom.

#### *1.2.4.5.3 Antigenic component of Leucogen*

The question as to why Leucogen was able to elicit a protective immune response against FeLV challenge while other gp70 protein based vaccines did not (Salerno et al. 1978), (Pedersen et al. 1986), (Gilbert et al. 1987) was explored. The development of an effective subunit vaccine must take both the antigenic and the adjuvant components into consideration. In terms of the antigenic component, a contributing factor to the lack of efficacy of previous subunit vaccines was thought to be the subgroup of the virus used for gp70 antigen isolation. A strong correlation between the generation of neutralising antibodies to FeLV A, but not subgroup B or C viruses, and protection against leukaemia, has been reported. Meanwhile, the production of many subunit vaccines have involved a mixture of viral subgroups, A, B and C, with subgroup B viruses as the immunodominant component. In contrast, the Leucogen vaccine contains only FeLV subgroup-A gp70 recombinant protein (Marciani et al. 1991).

Another interesting observation is that the gp70 protein does not need to be in its native form, in terms of glycosylation and conformation, to elicit a protective immune response to FeLV (Marciani et al. 1991). The recombinant gp70 protein used in the Leucogen vaccine was heavily denatured in production so that it was unable to fold towards a native conformation (Marciani et al. 1991). In contrast to the protective immune response elicited by this recombinant vaccine, immunisation with native FeLV gp70 showed an enhancement of FeLV infection (Pedersen et al. 1986).

#### *1.2.4.5.4 Adjuvant component of Leucogen*

An adjuvant may act as a potent immunostimulator and immunomodulator and/or as a carrier of the antigenic component of a vaccine. Addition of a potent adjuvant was considered crucial in overcoming the poor immunogenicity of the FeLV antigenic component of Leucogen (Marciani et al. 1991). The developers of the vaccine found that formulation of the same antigen with other commonly used adjuvants, as opposed to the novel saponin adjuvant present in Leucogen, generated a poor protective immune response in cats. The potency of the humoral immune response was found to be dependent on the adjuvant used (Marciani et al. 1991). Alum and oil emulsion adjuvants were found to induce a poor neutralising antibody response, in accordance with other reports (Salerno et al. 1978), (Pedersen et al. 1986). Therefore, the efficacy of the Leucogen recombinant subunit vaccine was probably due, in part, to the inclusion of a potent and effective adjuvant. Indeed, Pedersen suggested that the failure of their native viral subunit vaccines could be due to the adjuvant component (Pedersen et al. 1986).

#### *1.2.4.5.5 Efficacy of Leucogen*

In terms of efficacy, preliminary trials involving Leucogen, demonstrated that 85% of vaccinates (seventeen out of twenty), resisted persistent FeLV infection, following stringent challenge exposure (Clark et al. 1991). This translates to a preventable fraction of 78.6% when taking into account the percentage of control cats persistently viraemic at the end of the trial. Similarly, Lehmann reported a preventable fraction of 93.3% in a study involving FIV positive and negative cats in both control and vaccinated groups; only 1/8 cats was persistently viraemic on completion of the study (Lehmann et al. 1991). Interestingly, FIV status did not affect the protective immune response generated by the vaccine, although decreases in CD4+ lymphocyte populations have been observed in FIV infected cats as early as two months after infection. This result illustrates that in the early phase of FIV infection the immune system is not appreciably compromised and cats may be successfully vaccinated against FeLV (Lehmann et al. 1991).

However, a recent independent study comparing the efficacy of Leucogen with two commercial FeLV inactivated virus vaccines demonstrated a level of protection significantly less than had been previously reported (Jarrett and Ganiere, 1996). Although superior to the two other vaccines, Leukocell 2 and Leucat, in protecting against FeLV-A challenge, 5/12 cats vaccinated with Leucogen were persistently viraemic at the termination of the study (Jarrett and Ganiere, 1996). This translated to a preventable fraction of only 52.4%. This study demonstrated, however, that although Leucogen was representative of only one FeLV subgroup, FeLV A, it could protect against challenge from all three subgroups, A, B and C.

Also of note regarding the efficacy of Leucogen, was the significant proportion of vaccinates which were transiently viraemic, post-challenge, in the trial conducted by Clark (Clark et al. 1991). Forty percent of cats (8/20) were transiently viraemic in the twelve weeks following viral challenge. The development of transient viraemia, which usually remains undetected in the field, has been reported to increase the probability that latent bone marrow infection will develop (Sparkes, 1997), (section 1.1.7.5). In conclusion, the Leucogen vaccine is not capable of providing complete protection against either transient (Clark et al. 1991) or, more importantly, persistent viraemia (Jarrett and Ganiere, 1996).

### **1.2.5 FeLV ISCOM VACCINE**

This type of FeLV vaccine was first described by Osterhaus in 1985 (Osterhaus et al. 1985), and later in 1989 (Osterhaus et al. 1989) and 1991 (Osterhaus et al. 1991). The immunostimulating complex, ISCOM, was a novel structure for the antigenic presentation of membrane proteins from enveloped viruses (Morein et al. 1984). The matrix of the ISCOM was composed of the glycoside Quil A which in micelle form has regions accessible for hydrophobic interaction with the membrane proteins. In this way complexes of antigen were formed. The conformation of the FeLV envelope antigens, presented on the surface of the ISCOM matrix, appeared to greatly augment the host immune response, both cellular and humoral. Indeed, ISCOM preparations generated in this way were found to be highly immunogenic. They were able to elicit an antibody

response to even poorly antigenic membrane proteins (Osterhaus et al. 1985), without the attendant risks of immunisation with killed or live attenuated virus vaccines.

The construction of the FeLV ISCOM vaccine was reported initially by Osterhaus (Osterhaus et al. 1985). Virus was obtained from the cell culture supernatant of a persistently infected feline T cell lymphoma cell line, F422, or the FL74 tumour cell line. The virus was processed and the gp70/85 antigen-containing fraction was collected, pooled and complexed with matrix to generate ISCOMs. In the initial experiments, six kittens were vaccinated with the F422 derived preparation, and these and the six controls were challenged oronasally, with FeLV-A. Ten weeks after challenge none of the vaccinated cats was found to be persistently infected with FeLV, while three of the unvaccinated controls became persistently viraemic (Osterhaus et al. 1985).

In a subsequent trial, involving 137 FeLV-negative household cats, the potential of the ISCOM preparation to induce anti-FeLV and virus neutralising, (VN), antibodies, was compared to that of Leukocell, a commercial inactivated FeLV vaccine, and a negative control group (Osterhaus et al. 1989). The cats were tested for seropositivity towards FeLV, before the trial began and were defined as seropositive or seronegative. In contrast to the animals in the two other groups, almost all cats vaccinated with the ISCOM preparation responded by seroconverting, or developing an increased antibody titre, two weeks after the last of three immunisations. It should be noted that viral challenge was not performed, so serological changes occurred in response to vaccination and not to challenge. Only 2 of 35 seronegative cats vaccinated with Leukocell developed a VN antibody titre, as opposed to 28 of 35 seronegative cats vaccinated with the ISCOM preparation. Similarly, only two of 12 seropositive cats vaccinated with Leukocell demonstrated a rise in VN antibody titre, in contrast to six of nine seropositive cats vaccinated with the ISCOM preparation. None of the cats in the negative control group developed a neutralising antibody response (Osterhaus et al. 1989). This result is significant, as very few experimental or commercial FeLV vaccines are able to induce significant titres of virus neutralising antibodies, after vaccination (Jarrett, 1996).

The authors reported, however, that the ISCOM preparations used in these studies were not highly purified, and they elicited antibodies to a variety of FeLV antigens other than gp70/85 (Osterhaus et al. 1985), (Osterhaus et al. 1989). Therefore, the protective effect of this vaccine may not have been solely due to gp70/85. This point was raised by Pedersen, when reporting the efficacy of a native gp70 protein subunit vaccine, which enhanced rather than protected against infection (Pedersen et al. 1986). In order to prove that the envelope proteins alone were responsible for the protective immunity generated by the ISCOM gp70/85 vaccines, ISCOM vaccines containing only highly purified gp70/85 antigen should be administered to cats.

ISCOM preparations have been shown to be highly effective in eliciting B-cell responses resulting in the generation of biologically active anti-viral antibodies, and T helper cell responses (Morein, 1988). Most viral subunit protein preparations are unable to elicit CD8+ MHC class I restricted CTL activity. In contrast to most other non-replicating immunogens, however, the ISCOM system is also capable of eliciting this type of cellular immune response. A single subcutaneous immunization with ISCOM preparations, containing either purified intact gp160 envelope glycoprotein of the human immunodeficiency virus (HIV-1) or influenza haemagglutinin, has been shown to induce reproducible and long-lasting priming of HIV specific or influenza specific CD8+, MHC class I restricted CTL, in mice (Takahashi et al. 1990). ISCOM vaccines have also been reported to protect against infection with viruses belonging to virtually all membrane virus families (Morein, 1988). For the above reasons, ISCOM formulations have been developed as candidate vaccines against retroviral disease, such as FeLV, FIV and HIV (Osterhaus et al. 1985), (Tijhaar et al. 1997), (Sjolander et al. 1996).

### **1.2.6 COMMERCIALY AVAILABLE FeLV VACCINES**

At present there are five commercial FeLV vaccines marketed in Europe and the USA (Sparkes, 1997), although some are marketed by different companies, and under various labels, in different countries. These vaccines have been reviewed earlier in this

section; none consistently provide 100% protection against persistent infection or generate sufficient mucosal immunity, to routinely prevent transient viraemia, after viral exposure (Sparkes, 1997). Table 1.3. provides details of the various vaccine preparations.

<b>Vaccine</b>	<b>Type of vaccine</b>	<b>Manufacturer or distributor</b>	<b>FeLV subgroups included</b>	<b>Inclusion of FOCMA</b>	<b>Availability in the UK</b>
Fevaxyn	Inactivated, adjuvanted, whole virus	Solvay-Dulphar/ Fort Dodge	A & B	No	Yes
Leucat/ VacSYN	Inactivated, non-adjuvanted, whole virus	Rhone Merieux/ Synbiotics	A, B & C	Yes	No
Fel-O-Vax	Inactivated, adjuvanted, whole virus	Fort Dodge	A & B	No	No
Leucogen/ Genetivac/ Nobivac FeLV	Purified, adjuvanted, recombinant, non-glycosylated form of gp70	Virbac/ Mallinckrodt/ Intervet	A	No	Yes
Leukocell 2	Inactivated, adjuvanted, mixed sub-unit from FeLV-infected tissue culture filtrate	Pfizer	A, B & C	Yes	Yes

**Table 1.3. FeLV vaccines commercially available within and outside the UK.**

## 1.2.7 CONCLUSION

Many different types of FeLV vaccines have been constructed, with varying success. From the limited data available, regarding the commercial products, the whole virus vaccines, Fel-o-vax, (Fort Dodge) and Fevaxyn FeLV, (Solvay-Dulphar/Fort Dodge), appeared to demonstrate the most consistent protection against FeLV challenge. However, as reviewed in section 1.2.3. neither vaccine was able to completely prevent the development of transient or latent FeLV infection. Moreover inactivated virus vaccines have inherent disadvantages, regarding the quality and range of the immune response they induce, attendant risks, regarding safety, and production problems, regarding expense. Several of the experimental vaccines, such as the canarypox viral vector vaccine and the ISCOM preparations, proved to be efficacious in experimental trials; however, these vaccines are not without their shortcomings. The data reviewed here highlights the need to produce an FeLV vaccine capable of protecting against persistent and transient viraemia, and the consequent development of latent infection, and FeLV-related disease (Rojko and Hardy, 1994). For all these reasons, the development of a novel FeLV DNA vaccine was considered.

## **1.3 DNA VACCINATION**

### **1.3.1 INTRODUCTION**

Naked DNA vaccination presents a new approach in vaccine development. DNA vaccine constructs are created by the insertion of DNA encoding a desired antigen into an eukaryotic plasmid expression vector (Robinson and Torres, 1997). The purified plasmid DNA is inoculated directly into the host where it can transfect cells, following delivery by a number of different routes. The immunising protein is then expressed in transfected cells *in vivo* under the control of the plasmid expression vector promoter. Consequently, an immune response is elicited to the expressed antigen. As the host cells' transcriptional machinery is utilised, the protein is expressed directly in the cells with the appropriate post-transcriptional modifications and tertiary structure to produce conformationally specific antigens. Also, through presentation via the endogenous pathway, cellular immune responses may be induced (Donnelly et al. 1997b). This unique approach to immunisation may allow the development of safe and efficacious prophylactic and therapeutic vaccines and may be useful as a research tool, for example, in the production of monoclonal antibodies (Tang et al. 1992). Indeed, numerous animal models for DNA vaccines against viral, bacterial and parasitic diseases have now been described (Donnelly et al. 1997b) and, more recently, human clinical trials have been undertaken (MacGregor et al. 1998).

### **1.3.2 EARLY HISTORY OF DNA VACCINATION**

The earliest report of transfection of cells, *in vivo*, with injected purified DNA, was made almost thirty years ago (Ito, 1960). The potential of this discovery, however, was largely disregarded until relatively recently. The first evidence for direct *in vivo* gene transfer was reported by Wolff in 1990 (Wolff et al. 1990). This group demonstrated expression of a reporter gene in murine skeletal muscle and biological activity of the

encoded enzymes for up to 60 days after inoculation. Meanwhile, the potential of this new technology to initiate immune responses was first described by Tang *et al* in 1992 (Tang et al. 1992) who observed that mice inoculated by gene gun delivery, with plasmids expressing the gene for human growth hormone, developed specific primary antibody responses. Moreover, these responses could be boosted by subsequent immunisations. Further reports described the generation of antibodies raised against influenza virus haemagglutinin (HA) glycoprotein and HIV gp120, in mice immunised by gene gun with the appropriate plasmids (Eisenbraun et al. 1993).

Meanwhile, Ulmer *et al* further developed the technique of delivering DNA immunogens intramuscularly (Ulmer et al. 1993). He reported that mice immunised with plasmids encoding the nucleoprotein (NP) gene of the A/PR/8/34 strain of influenza virus developed potent, specific CTL responses which were protective against a heterologous strain of influenza (Ulmer et al. 1993). These initial experiments marked the beginning of new and exciting developments in immunoprophylaxis. DNA vaccination was considered as a therapeutic tool, for the prevention and treatment of a wide range of conditions, including infectious disease, allergy, autoimmunity and cancer.

### **1.3.3 DNA VACCINE PLASMIDS**

Plasmid DNA constructs used for vaccination are similar to those used for the delivery of reporter or therapeutic genes. These constructs have five main features in common (Davis, 1997). Firstly, they possess a bacterial origin of replication that facilitates amplification of large quantities of plasmid DNA for purification. Secondly, a prokaryotic selectable marker gene, such as an antibiotic resistance gene, is present. Thirdly, they contain eukaryotic transcription regulatory elements; these are usually strong viral promoter/enhancer sequences which direct high levels of gene expression in a wide host cell range. Importantly, they also possess DNA sequences which encode the antigenic protein or peptide of interest. Finally, these plasmids contain a polyadenylation sequence to ensure that the transcribed mRNA is appropriately terminated.

Many vectors used for DNA vaccination also possess a signal sequence, to ensure that the expressed protein is secreted. However, this feature does not appear to be essential for the initiation of either cell mediated or humoral immunity (Michel et al. 1995), (Donnelly et al. 1996). Sequences which encode cytokines (Iwasaki et al. 1997), (Tsuji et al. 1997), (Kim et al. 1997), or B-7 costimulatory molecules (Iwasaki et al. 1997), which when adjacent to MHC molecules in the cell membrane lead to immune activation rather than the induction of tolerance, may also be included in DNA vaccines. The co-expression of these cytokines or costimulatory molecules may enhance and augment the immune response to the antigenic component of the vaccine. This will be described in more detail in section 1.4.3.2.

Another important feature of plasmid DNA is the presence of unmethylated CpG motifs, which have T-helper cell type 1 (Th1) immunostimulatory activity (Pisetsky, 1996), (Klinman et al. 1996), (Sato et al. 1996). These CpG motifs are 6-base unmethylated DNA sequences which have in common a cytosine preceding a guanosine, flanked by two 5' purines and two 3' pyrimidines (Klinman et al. 1996). In their unmethylated form these DNA motifs have been demonstrated to be potent stimulators of several types of immune cell. Monocytes and macrophages are stimulated to produce a range of cytokines, including IL-12 and TNF- $\alpha$  (Halpern et al. 1996) and, in turn, these cytokines induce the lytic activity of natural killer (NK) cells and stimulate their secretion of IFN- $\gamma$ . CpG motifs may also rapidly activate murine B cells to secrete IL-6 and IgM, as well as to proliferate (Krieg et al. 1995), (Yi et al. 1996). These motifs are present at much lower frequencies in vertebrate DNA and are almost always methylated. Therefore, the immunostimulatory activity of bacterial DNA is likely to enhance the host immune response against invading pathogens. In DNA vaccine plasmids, this immunostimulatory activity, may act to mobilise the immune response against the DNA encoded antigen. Therefore, DNA vaccines possess endogenous adjuvant activity that is antigen independent and potently induces Th1 type immune responses.

### 1.3.4 ADVANTAGES AND DISADVANTAGES OF DNA VACCINATION

DNA vaccination possesses a number of potential advantages over current immunisation methods. This new technology combines many of the most attractive aspects of modern vaccination strategies, with few of the disadvantages. Primary advantages are the purity, physiochemical stability and simplicity of production (Davis, 1997) of plasmid DNA and the ability to formulate single dose vaccines containing many different antigens (from the same or different pathogens) rapidly, inexpensively, and on a large scale (Ramsay et al. 1997). In addition to the immense versatility of DNA vaccines, their low production costs and heat stability make them ideal prophylactic agents for use in the field in developing countries, where expensive refrigeration stores are not routinely available (Davis, 1997).

An ideal vaccine would be able to initiate both cellular and humoral immune responses (Davis, 1997). Antigen based vaccines containing the whole pathogen, such as inactivated vaccines, or a component of it, such as subunit vaccines, are able to elicit antibody production, but generally not CTL activity. They are processed solely as exogenous antigen, and are therefore limited to presentation by MHC class II molecules, with only a few exceptions (Jondal et al. 1996). Expression in the context of the cell's MHC class I molecules, essential for the induction of MHC class I-restricted antigen-specific cytotoxic CD8+ T lymphocyte activity, does not occur. However, vaccines which are processed as endogenous antigen, such as live attenuated and live viral vector vaccines, while able to elicit both antibodies and CTL activity by the presentation of antigen in the context of MHC class I and II molecules, possess some potential disadvantages.

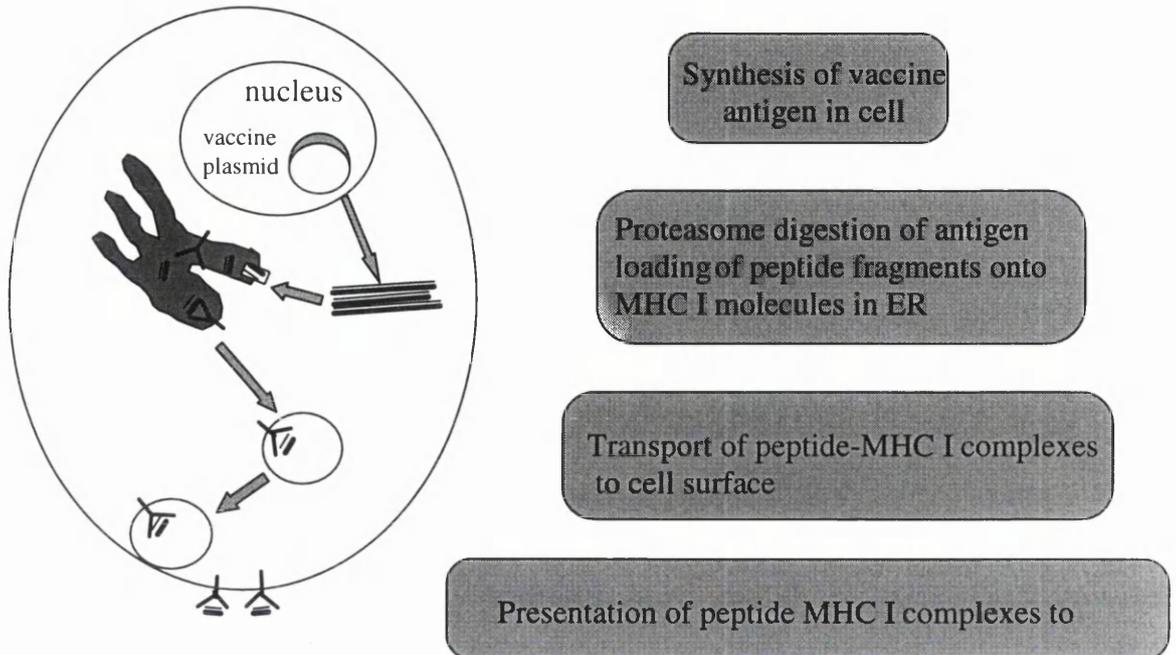
Live vaccine pathogens possess the potential to revert to a virulent form, of particular concern in immunosuppressed individuals, and live viral vector delivery systems may be associated with pre-existing vector immunity (Ramsay et al. 1997). Booster vaccinations may lead to the initiation of an inappropriate immune response to the delivery vector which may interfere with the immune response directed to the antigenic protein component of the vaccine. DNA vaccination, by contrast, utilises the host cells' transcriptional machinery to endogenously produce antigen, mimicking aspects of live

attenuated vaccination and allowing MHC class I and class II antigen presentation and the induction of CTL activity and antibody production. However, the attendant risk of reversion to virulence and potential pathogenic replication is completely abrogated (Lu, 1998).

Moreover, CTL activity elicited by DNA vaccination may provide cross-strain protection against viral pathogens, such as influenza A virus, by recognition of epitopes from conserved internal viral proteins, associated with MHC class I molecules (Ulmer et al. 1993). Both CD4+ helper T cells and CD8+ CTL are efficiently elicited by DNA vaccines (Pardoll and Beckerleg, 1995) and their ability to induce specific and long-lived cellular and humoral immunity (Ulmer et al. 1993), (Wang et al. 1993), (Ulmer et al. 1996) has been frequently reported. Figure 1.5. and figure 1.6. illustrate pathways for DNA vaccine antigen processing and presentation by MHC class I and II molecules.

# MHC I

## Synthesis of antigen in cell.



**Figure 1.5. Pathway for DNA vaccine antigen processing and presentation by MHC class I molecules.**

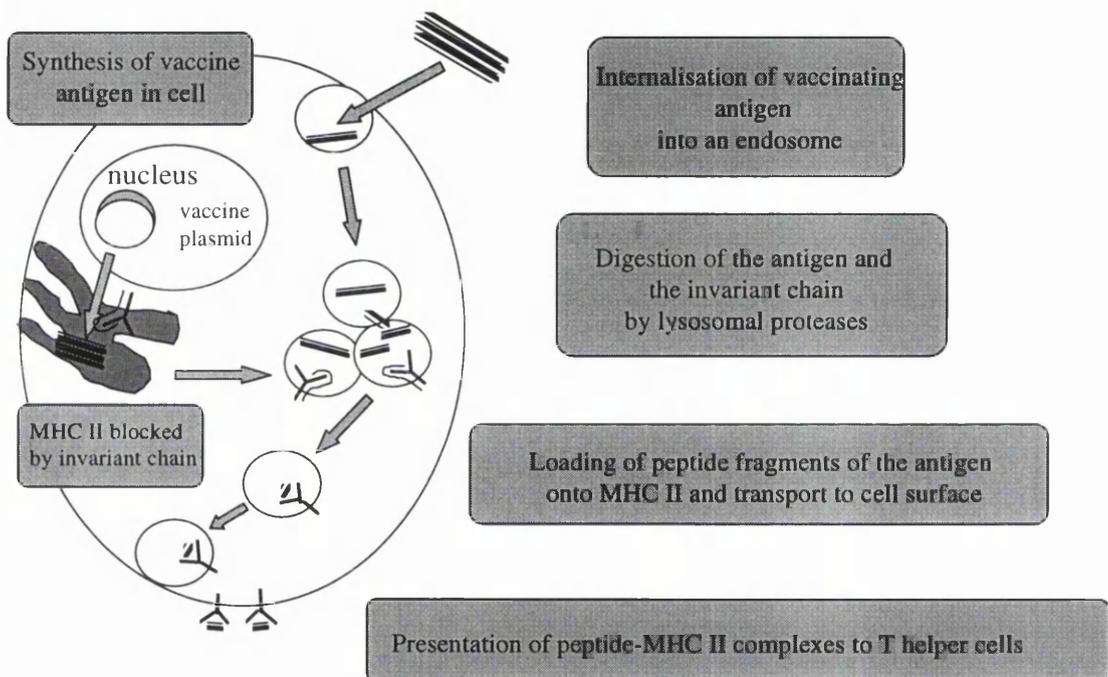
With relatively few exceptions, only vaccines which are processed as endogenous antigen, such as DNA vaccines, may be processed and presented by MHC class I molecules, as described above.

**ER**, endoplasmic reticulum; **CTL**, cytotoxic lymphocytes; **MHC**, major histocompatibility complex.

However, despite the huge potential advantages of DNA vaccination, some issues have been raised with regard to its safety. There is the theoretical possibility that integration of plasmid DNA into the host genome may occur, leading to insertional mutation, activation of oncogenes and, therefore, possible tumourigenesis (Ramsay et al. 1997). Similarly, there are concerns that DNA vaccination may lead to the development of

autoimmune disease due to the induction of anti-DNA antibodies, or the breaking of tolerance to self proteins due to prolonged expression of antigen (Ramsay et al. 1997). Of lesser importance is the concern that expressed antigens, for example, from non-viral intracellular pathogens, may not be correctly glycosylated leading to the generation in the host of antibodies of the incorrect specificity (Ramsay et al. 1997).

## Synthesis of antigen in cell or internalisation of antigen from outside of cell. **MHC II**



**Figure 1.6. Pathway for DNA vaccine antigen processing and presentation by MHC class II molecules.**

Vaccines which are processed as endogenous antigen, such as DNA vaccines, are able to elicit both antibodies and CTL activity, by the presentation of antigen in the context of both MHC class I and II molecules.

**T helper cells**, T helper lymphocytes; **MHC**, major histocompatibility complex.

As DNA vaccination is such a recently developed technology, emerging in the last six years, it is too early to unequivocally define it as a completely safe method of immunisation. However, to date, no experimental evidence for chromosomal integration of injected DNA has been produced (Nichols et al. 1995), (Wolff et al. 1992). Similarly, although bacterial DNA is immunogenic, no consequent pathology or exacerbation of autoimmune conditions has been observed following vaccination (Ramsay et al. 1997). Several human clinical trials are now in progress and in a recent study investigating a DNA-based vaccine for the treatment of HIV-1 infection (MacGregor et al. 1998), it was found that patients did not develop anti-DNA antibodies or raised muscle enzyme levels indicative of immune-mediated muscle damage. In fact, no laboratory test abnormalities or local or systemic adverse reactions were observed, at any time, during the course of the trial.

### **1.3.5 DNA VACCINE DELIVERY**

Direct delivery of plasmid DNA is preferred for the introduction of antigen-encoding DNA sequences, and this may be carried out by a number of different routes. Among the most popular delivery methods, are intramuscular (i.m.) and intradermal (i.d.) injections of DNA, and the use of a “gene gun” to bombard skin with DNA coated gold particles (Davis, 1997), (Robinson and Torres, 1997).

Intramuscular inoculation of pure plasmid DNA, encoding a wide range of antigens, has been used in a diverse range of species, from fish (Anderson et al. 1996), to cats (Hosie et al. 1998), to chimpanzees (Davis et al. 1996), with generally good results. Following i.m. inoculation, most antigen expression initially occurs in skeletal muscle (Wolff et al. 1990). This route, however, may be less effective in larger animals, due to lower muscle cell transfection efficiencies, arising as a result of cellular architectural differences between species (Davis, 1997). The amount of DNA needed to elicit an immune response, injected either intramuscularly or intradermally, appears to be fairly independent of the size of the immunised animal; similar doses of DNA, within a log of each other, have been used to immunise mice, calves and monkeys. Doses of between 1µg and 100µg of DNA have been used to inject mice, while between 10µg

and 1mg of DNA have been used to inject monkeys and calves (Robinson and Torres, 1997).

Intradermal inoculation of DNA vaccines can also induce potent cellular and humoral immune responses against the encoded protein (Raz et al. 1994), (van Drunen et al. 1998) although only a small number of cells are transfected (Raz et al. 1994). The efficacy of the immune response is likely to be related to the direct transfection of professional APCs in the skin, such as Langerhans' cells (Condon et al. 1996), as inoculation of plasmid DNA into the subcutaneous space, where such cells are absent, fails to generate effective immunity.

Another method employed for plasmid DNA delivery is the gene gun. This method involves the introduction of DNA coated gold particles into the epidermis. A particle accelerator or compressed helium is employed to fire the gold particles at high velocities into the epidermis (Haynes et al. 1996). This method of delivery is particularly efficient as very small amounts of DNA are able to elicit very potent immune responses (Fynan et al. 1993), (Pertmer et al. 1995). Gene gun inoculations in mice have used between 10ng and 10 $\mu$ g of plasmid DNA, between one hundred and one thousand times less than the amount of DNA required for an intramuscular or intradermal immunisation (Robinson and Torres, 1997). The reason for this enhanced efficiency is probably because the gene gun facilitates the direct and accurate penetration of many DNA coated gold particles into APCs such as Langerhans' cells (Fynan et al. 1993), thus allowing efficient antigen presentation and subsequent recognition in the draining lymph nodes.

Other, less invasive methods of plasmid DNA delivery are currently being developed. Administration of DNA to mucosal surfaces as DNA drops (Fynan et al. 1993), in liposomes (McCluskie et al. 1998), or in microspheres (Jones et al. 1997) has shown variable success. Schubbert demonstrated that it may be possible to deliver DNA vaccines orally, by administering microencapsulated DNA, targeted to the Peyers patches (Schubbert et al. 1994). Attenuated *Shigella* bacteria have been considered as a delivery vehicle for vaccine DNA to mucosal surfaces (Sizemore et al. 1997). These bacteria infect the colonic mucosa and due to an engineered mutation, burst open once

inside cells, releasing DNA expression plasmids. Intranasal DNA vaccination against measles virus with a highly attenuated *Shigella flexneri* vector has proved to be both safe and efficacious, eliciting cellular and humoral immunity (Fennelly et al. 1999). These mucosal methods of DNA delivery, via the respiratory and intestinal surfaces, hold great hope for the development of vaccines which selectively protect these main pathogen entry sites by inducing specific mucosal immunity.

### **1.3.6 IMMUNE RESPONSES RAISED BY DNA VACCINATION**

#### **1.3.6.1 Antibody responses raised by DNA vaccination**

As DNA vaccination effectively mimics natural infection, it is able to raise both strong humoral and cellular immune responses. Most successful DNA vaccination trials have demonstrated the generation of a significant antibody response against the encoded immunogen (Shiver et al. 1996), (Raz et al. 1994), (Xiang et al. 1994). Many factors have been reported to affect the efficacy and nature of the DNA-elicited antibody response, such as the route of DNA delivery (Pertmer et al. 1996), the DNA expression vector and the form of the DNA encoded antigen, whether the antigen is secreted, intracellular, or membrane associated. However, it appears that the most important factor influencing DNA-induced antibody responses is the expressed antigen itself. Some antigens, such as the influenza haemagglutinin antigen, are able to elicit potent and long-lived antibody responses in mice (Robinson et al. 1997) whereas others, such as HIV or SIV envelope antigen, raise only a transient, low-level antibody titre in mice (Lu et al. 1996). The difference in antibody responses may, in fact, reflect basic differences in the physical structure of antigens, and how they interact with the immune system (Robinson and Torres, 1997)

#### **1.3.6.2 T-helper cell responses raised by DNA vaccination**

T helper cells function by providing help, in the form of cytokines, to B cells and cytotoxic T cells. At least two different types of Th cells are thought to exist in humans and mice, Th1 and Th2, which support two different types of immune response. The

Th1 response, characterised by IFN- $\gamma$  synthesis and IL-2 production, controls cellular immunity, while the Th2 response, characterised by IL-4 production, is associated with humoral immune responses. Th1 cells stimulate the development of cytotoxic T cells, activate phagocytic cells and assist B cells to make IgG2a antibody, a complement binding IgG subclass, which acts to opsonise invading microbes (Feltquate, 1998). Th2 cells, meanwhile, activate non-phagocytic defences, such as mast cells, and assist B cells to produce IgE and IgG1 antibody, a non-complement binding subclass of IgG (Feltquate, 1998). The dominance of either a Th1 or Th2 response is governed, in part, by the nature of the antigen. There is, however, an argument to suggest that Th1 and Th2 are not distinct cell subtypes, but instead reflect different cytokine expression patterns.

In general, Th1 responses are more efficient in controlling bacterial and chronic viral infections, whereas Th2 responses are more effective in controlling parasitic or mucosal infections. It appears that certain types of pathology are associated with a particular T helper cell response. Aberrant Th1 responses may result in the development of autoimmune disease, while aberrant Th2 responses may support the development of allergic conditions. Thus, it appears that the predominance of either a Th1 or Th2 immune response may greatly influence the outcome of a particular disease process. Therefore, the ability to skew the immune response towards one or other of the T helper cell responses would be very valuable. The advent of DNA vaccination technology has facilitated this.

DNA vaccination with the same vaccine plasmid is able to raise responses biased towards either type 1 or type 2 T cell help, depending on the method of vaccine delivery employed; either saline inoculations of DNA, or gene gun delivery (Pertmer et al. 1996). To date, most reports have demonstrated that saline DNA inoculations by the intradermal or intramuscular routes stimulate predominantly Th1 immune responses, whereas gene gun inoculations stimulate Th2 responses (Feltquate et al. 1997). The type of response initiated by saline DNA inoculations may also be modified by changing the form of the DNA encoded antigen, from cell associated to secreted (Robinson and Torres, 1997) or by the coinoculation of cytokine or other immunostimulant plasmid DNA constructs (Chow et al. 1998).

Thus, DNA vaccination technology has given us the ability to engineer the immune response raised to vaccine antigens and to manipulate the errant immune responses responsible for the development of autoimmune and allergic conditions. Table 1.4. outlines the type of immune response elicited by different delivery methods and different forms of DNA encoded antigen. The mechanisms that support the different types of T cell help generated by DNA vaccination remain incompletely understood. Following DNA inoculation, it appears that different migration patterns of antigen presenting cells, the site of antigen presentation, and the nature of the antigen presenting cells, themselves, may influence whether a Th1 or Th2 response develops (Robinson and Torres, 1997).

Method of Delivery	Form of DNA encoded antigen		
	Intracellular	Plasma membrane	Secreted
Gene gun delivery	<b>Th2</b>	<b>Th2</b>	<b>Th2</b>
Intramuscular saline inoculation	<b>Th1</b>	<b>Th1</b>	<b>Mixed, Th1 and Th2</b>

**Table 1.4. Type of immune response elicited by different delivery methods and different forms of DNA encoded antigen.**

### **1.3.6.3 Cytotoxic T cell responses raised by DNA vaccination**

In contrast to many other types of vaccine, DNA vaccines are extremely efficient in eliciting cytotoxic T cell, CTL, activity. This is probably due to the fact that vaccine immunogens are presented by MHC class I molecules, a prerequisite for the activation of CD8+ cytotoxic T lymphocytes. There are many reports of potent and persistent cytotoxic T cell activity following DNA inoculation (Davis et al. 1997), (Raz et al. 1994), (Lu et al. 1996) which is possibly a result of vaccine expression vectors

expressing high levels of antigenic protein, and thus achieving high levels of MHC class I display.

### **1.3.7 DNA VACCINATION; MECHANISMS OF IMMUNISATION**

#### **1.3.7.1 Cellular transfection and antigen expression after DNA vaccination**

Depending on the route and method of DNA vaccination, a wide range of cells may be transfected. Between 1-3% of myocytes within a muscle bundle have been transfected and have shown prolonged antigen expression, following a single intramuscular DNA inoculation (Wolff et al. 1992). However, it is now known that a significant proportion of antigen expressing myocytes may be cleared from muscle tissue within weeks, by antigen-specific cytotoxic T cells (Davis et al. 1997) so these cells do not act as the main reservoir of antigen expression. Following gene gun and intradermal DNA vaccination, keratinocytes are the predominant cell type to be transfected (Raz et al. 1994). Antigen expression within these cells peaks at 24 hours post-transfection and is essentially absent after five to ten days due to natural epidermal sloughing. Several other types of epithelial cell have been transfected following saline DNA inoculations. These cells are only a tiny fraction of the total transfected cell population, after intramuscular or intradermal DNA vaccination. After intravenous saline DNA inoculation, the vascular endothelial cells are the predominant cell type to be transfected (Zhu et al. 1993).

Importantly, it appears that professional APCs such as tissue macrophages and dendritic cells are transfected during most methods of DNA vaccination. Macrophages resident in inoculated tissues as well as those in lymph nodes and spleen have been reported to contain plasmid DNA (Chattergoon et al. 1998). APCs in these specialised lymphoid tissues are thought to acquire plasmid DNA by either or both of the following ways. Plasmid DNA has been identified in draining lymph nodes only 20 minutes after DNA inoculation. This result suggests that DNA can leave the injection site in blood or lymph, travel to local lymph nodes and subsequently transfect macrophages and dendritic cells resident in the node (Kuklin et al. 1997).

Alternatively, APCs resident in the target site may be directly transfected and subsequently migrate to the draining node. In support of this hypothesis, labelled Langerhans' cells have been found in draining lymph nodes, 24 hours after gene gun inoculation of DNA into fluorescein coated skin (Condon et al. 1996).

### **1.3.7.2 Antigen presentation after DNA vaccination**

Optimal antigen presentation is mediated by APCs which are central to the production of most immune responses by taking up, processing and presenting antigen to T cells in the context of MHC class I and II molecules, with the costimulatory signals necessary for T cell activation. Antigen is presented in the context of the APC's MHC class I and/or class II molecules, depending on whether the antigen is synthesised endogenously, MHC class I and II presentation, or acquired exogenously, MHC class II presentation alone. MHC class I antigen presentation stimulates the cellular T cell response and activates CD8+ cytotoxic T lymphocytes, (CTLs). These CTLs, once activated, are able to directly lyse infected target cells. MHC class II molecules, meanwhile, present antigen to CD4+ T helper cells, which are divided into two subsets, Th1 and Th2, depending on the pattern of cytokines they produce. Th1 cells control cellular immunity and provide cytotoxic T cell help, while Th2 cells help B cells to proliferate and differentiate and are associated with humoral immune responses.

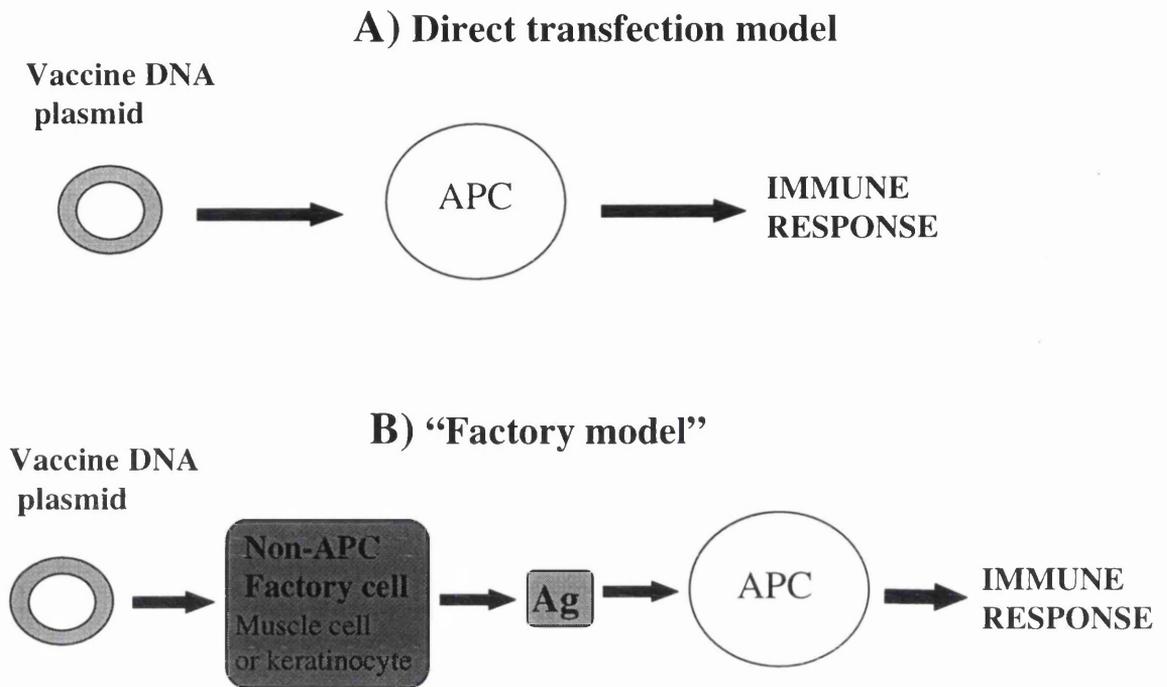
After DNA vaccination, DNA antigens expressed intracellularly are presented by both MHC classes, eliciting humoral and cellular immune responses. However, the question arises as to which cells actually serve as APCs following this method of inoculation. Experimental evidence suggests that non-professional APCs, such as muscle cells, do not act as antigen presenting cells following DNA vaccination (Corr et al. 1996). Studies involving bone marrow chimaeric mice have shown conclusively that bone marrow derived professional APCs function as antigen presenting cells following both gene gun and saline DNA inoculations (Iwasaki et al. 1997). Two theories have been proposed to explain how professional APCs acquire antigen for presentation following DNA vaccination (Pardoll and Beckerleg, 1995).

In the first model, directly transfected APCs are considered to be the cells responsible for eliciting the immune response. These cells can process and present antigen through MHC class I and II pathways. Antigen production by non-professional APCs, such as keratinocytes and muscle cells, would play only a minor role in the developing immunity. In support of this theory, it has been demonstrated that a DNA vaccine plasmid, containing an APC-specific promoter, was capable of eliciting an immune response (Xiang et al. 1997). Transfection and antigen expression by non-APCs was obviously not essential to the development of this response. Another group demonstrated that the transfer of transfected, antigen expressing APCs into mice was sufficient to raise a detectable immune response (Casares et al. 1997). Moreover, a study using timed muscle ablations, demonstrated that antigen production by muscle cells, non-APCs, was not essential for generating immunity (Torres et al. 1997). Removal of muscle tissue minutes after DNA inoculation resulted in the development of an immune response, equal in potency and longevity to that of non-ablated control mice, indicating that sufficient DNA may leave the injection site, in blood or lymph, to transfect APCs located elsewhere, such as in the spleen or lymph nodes.

In the second model, the majority of the transfected cells which are non-APCs such as muscle cells and keratinocytes, are thought to act as factories (Feltquate, 1998) producing antigen which is subsequently transferred to professional APCs. The APCs then process and present antigen to the immune system in the usual way. Directly transfected APCs would represent only a small proportion of the total population of APCs involved in the generation of immunity. Most evidence supporting this model illustrates that antigen production by non-APCs is necessary to maximise and enhance the developing immune response. In contrast to the results obtained in the muscle ablation studies, when skin was removed soon after gene gun inoculation, the immune response was completely abrogated (Torres et al. 1997). The longer skin was left in place following DNA inoculation, the greater the magnitude of the immune response raised. Thus, following gene gun inoculation, it appears that production of antigen by keratinocytes, the proposed “factory” cells, is essential for the generation of immunity.

Considering all the relevant experimental data, it appears that generation of the optimal immune response relies on a combination of both models. Although direct transfection

of professional APCs may be adequate to produce an immune response, APC distribution may be sparse at some injection sites and, therefore, it seems probable that the most potent, effective immunity would be elicited by a combination of both models (Feltquate, 1998). In fact a recent study using a transactivating plasmid system and bone marrow chimaeras has shown that both mechanisms appear to be involved (Corr et al. 1999), although the bulk of the immune response is dependent on the expression of antigen by non-lymphoid cells and the subsequent transfer to APCs. Figure 1.7. provides a schematic overview of the two models. Different delivery methods or routes of DNA vaccination may tip the balance in favour of one pathway, as compared with the other. Gene gun inoculation of skin appears to elicit immunity either by direct transfection of epidermal Langerhans' cells, of which there is a substantial number in the skin, or by direct transfection of keratinocytes, and subsequent transferal of expressed antigen to professional APCs, such as Langerhans' cells (Feltquate, 1998), (Robinson and Torres, 1997). Alternatively, both mechanisms may be employed.



**Figure 1.7. Schematic overview illustrating models of antigen acquisition by APCs, following DNA vaccination.**

Ag, antigen; APC, antigen presenting cell.

Two theories have been proposed, to explain how APCs acquire antigen for presentation, following DNA vaccination. In the first model, directly transfected APCs are considered to be the cells responsible for eliciting the immune response, while in the second model, the majority of transfected cells, non-APCs such as muscle cells and keratinocytes, are thought to act as antigen producing "factories". This antigen is subsequently transferred to professional APCs, which then process and present antigen to the immune system, in the usual fashion.

Intramuscular inoculation with plasmid DNA may raise an immune response in one or all of the following ways. Mentioned earlier in this section, following intramuscular inoculation, DNA and possibly some transfected blood cells, may rapidly leave the muscle in blood or lymph and transfect professional APCs located in lymphoid tissues, such as lymph nodes or spleen (Robinson and Torres, 1997). Alternatively, professional APCs resident in muscle tissue may be directly transfected. However, due

to the paucity of professional APCs in this tissue, it is unlikely that these cells are central to the generation of immune responses (Giese, 1998).

Muscle cells themselves may be the primary target cells for transfection following DNA vaccination. Although muscle cells are directly transfected and can express antigen (Giese, 1998), they lack the appropriate co-stimulation signals expressed by professional APCs necessary for T cell activation. Instead, it has been proposed that muscle cells express antigen and then deliver it, by means of secretion or apoptotic cell death, to professional APCs (Giese, 1998). These APCs are recruited to the inoculation site following local tissue damage by the injection needle. Several groups have now demonstrated that exogenous protein molecules are able to prime MHC class I restricted CTL responses (Martinez-Kinader et al. 1995). APCs may take up cell debris by phagocytosis, and this exogenous scavenger pathway may process these exogenous antigens for presentation by MHC class I molecules, to antigen specific CD8+ cytotoxic T lymphocytes (Giese, 1998). Thus, this cross-priming mechanism can circumvent the classical MHC class I processing pathway, via indirect antigen presentation.

### **1.3.7.3 Site of antigen presentation following DNA vaccination**

Classically, antigen presentation and immune activation occur in the lymph nodes and spleen, where a favourable organ architecture and rich cellular help enhance the process. Following gene gun inoculation, assays for DNA-expressing cells, using green fluorescent protein, and post-boost localisation of antibody secreting cells, have identified the inguinal lymph nodes, which drain the abdominal skin, as an important site for antigen presentation (Condon et al. 1996). In contrast, subsequent to intramuscular immunisations with plasmid DNA, studies involving post-boost localisation of antibody secreting cells, have identified the spleen, and not the draining lymph nodes, to be the primary site of antigen presentation (Robinson and Torres, 1997). Therefore, following gene gun inoculation of skin, directly transfected or antigen loaded epidermal Langerhans cells appear to migrate to the draining lymph nodes to present antigen. Following intramuscular DNA vaccination a significant proportion of DNA appears to leave the muscle, in blood or lymphatic fluid, and

travels to the spleen. Once there, the DNA may transfect resident APCs (Winegar et al. 1996) which then present antigen and initiate an immune response.

#### **1.3.7.4 Longevity of immunity raised by DNA vaccines**

The mechanism behind the longevity of immunity raised by DNA vaccines remains incompletely understood. Historically, after intramuscular inoculation, antigen expression has been demonstrated in transfected muscle cells for up to 19 months following DNA injection (Wolff et al. 1992). In contrast, after intradermal inoculation, transfected skin cells have been documented to support antigen expression for only five to ten days, coinciding with the normal sloughing of the epidermis (Eisenbraun et al. 1993). Interestingly, both methods of DNA inoculation have the ability to elicit long-lasting humoral immune responses (Raz et al. 1994) and (Davis et al. 1997).

It was demonstrated that a strong humoral response against hepatitis B virus surface antigen (HBsAg) persisted for up to 74 weeks, following a single intramuscular administration of DNA (Davis et al. 1997). Although the longevity of the response was thought to be due to sustained expression of antigen in muscle cells, this group documented the destruction of a portion of antigen expressing muscle fibres. This was probably due to their presentation of antigenic peptides on MHC class I molecules, and occurred between ten and twenty days after gene delivery (Davis et al. 1997). This result suggests that long-term humoral immunity does not require long-term DNA expression in muscle, and may be due instead to antigen expression and storage in the germinal centres on follicular dendritic cells (Davis, 1997).

Meanwhile, Raz reported that a single intradermal inoculation of free plasmid DNA, encoding the influenza nucleoprotein gene, induced anti-nucleoprotein-specific antibody (and CTLs) that persisted for at least 68-70 weeks after vaccination (Raz et al. 1994). This suggests that short-term expression of antigen in epidermal skin cells is sufficient to raise long-term humoral immunity. Alternatively, long-term antigen expression may occur in the dermis, or transfected resident and/or circulating immune cells such as Langerhans' cells or specialised circulating epidermotropic T lymphocytes, present in the target tissue at the time of gene delivery. This long-term

antigen expression may maintain the humoral immune response (Robinson et al. 1996). The maintenance of long-term CTL activity, meanwhile, appears to be independent of the maintenance of long-term antibody levels. A recent simian immunodeficiency virus DNA vaccine trial in macaques, demonstrated that while antibody responses fell with time, cytotoxic T-cell responses persisted. (Lu et al. 1996). Several trials have documented the ability of DNA inoculation to elicit long-lasting CTL activity (Raz et al. 1994), (Lu et al. 1996).

### 1.3.8 APPLICATIONS OF DNA VACCINATION

Since the inception of DNA vaccination technology, there have been a large number of successful experimental trials. Using this method of immunisation for prophylactic vaccination against infectious disease, immune responses to more than twenty viral antigens have been demonstrated, including those of influenza A viruses (Ulmer et al. 1993), (Fynan et al. 1993), hepatitis B virus (Davis et al. 1993) and rabies virus, (Xiang et al. 1994), as well as many bacterial and parasitic antigens (Donnelly et al. 1997b), (Ertl and Xiang, 1996). In most cases potent cellular and humoral immune responses have been initiated, and frequently protection against live pathogenic challenge.

It has also been reported that DNA vaccines can effectively immunise neonates (Wang et al. 1997) in situations where conventional vaccines have failed to elicit immunity, leading to immune activation rather than the induction of tolerance. This may be due to sustained antigen expression, more efficient antigen presentation and processing, or the presence of the CpG immunostimulatory motifs in the plasmid backbone. Several human phase I clinical trials are now in progress, or nearing completion, testing the safety and immunogenicity of DNA vaccination against HIV, influenza, hepatitis B and malaria. A recently published study, regarding the first human trial of a DNA-based vaccine for the treatment of HIV-1 infection, demonstrated the safety and potential immunogenicity of an DNA vaccine, encoding HIV-1 *env* and *rev* genes (MacGregor et al. 1998). These findings have encouraged further studies and, indeed, DNA

vaccination technology currently possesses a central role in the development of therapeutic strategies against retroviral disease, which is discussed in a later section.

DNA vaccines may also be useful in the treatment of individuals with chronic viral infections, such as hepatitis B. In many cases, failure to mount an adequate immune response in the early stages of viral infection results in incomplete clearance of the virus, and development of the chronic carrier state. DNA vaccination, with an encoded viral antigen, elicits potent, effective and specific CD8+ CTL activity, due to endogenous synthesis of antigen and subsequent MHC class I presentation. These antigen specific immune responses may then be able to break the state of immunological tolerance the immune system has developed towards the chronic viral infection. A study of a transgenic mouse model of the hepatitis B chronic carrier state demonstrated that DNA vaccination could break immunological tolerance, resulting in the complete clearance of circulating hepatitis B surface antigen and the long-term control of transgene expression in hepatocytes (Mancini et al. 1996).

DNA vaccination has also been considered as a useful tool in the immunotherapy for cancer. Idiotypic determinants of the immunoglobulin expressed on the surface of B-cell lymphomas are tumor-specific antigens which can be targeted by immunotherapy. A recent report demonstrated that immunization with DNA constructs encoding the idiotype (Id) of a murine B-cell lymphoma induced specific anti-Id antibody responses and protected mice against subsequent tumor challenge (Syrengelas et al. 1996). Use of DNA encoding an Id and GM-CSF cytokine (granulocyte-macrophage colony-stimulating factor) fusion protein improved vaccine efficacy. These results indicate that DNA vaccination may be an efficacious means of eliciting immune responses against a weak, previously unrecognised tumor antigen, enhanced by simultaneous cytokine DNA co-expression. Rakhmievich reported that gene gun delivery of plasmid DNA encoding murine interleukin 12, to the epidermis overlying implanted intradermal tumours, resulted in regression of both the established primary and the metastatic tumours (Rakhmievich et al. 1996).

DNA vaccination may also be useful in the treatment of allergic conditions. Raz demonstrated that DNA immunization with a plasmid encoding beta-galactosidase,

induced a Th1 response that dominated an ongoing protein-induced Th2 response and inhibited specific IgE antibody production (Raz et al. 1996). This suggests that immunization with plasmid DNA encoding allergens may provide a novel type of immunotherapy for allergic diseases. DNA vaccination technology has also been applied to the therapy of autoimmune disease. Prevention of autoimmune encephalomyelitis in mice by vaccination with DNA encoding a variable region gene of the T cell receptor, has been reported (Waisman et al. 1996). Analysis of T cells in vaccinated mice demonstrated a reduction in the Th1 cytokines interleukin-2 (IL-2) and interferon-gamma (IFN- $\gamma$ ). In parallel, there was an elevation in the production of IL-4, a Th2 cytokine associated with suppression of disease. The potential of DNA immunization to reverse the autoimmune response from Th1 to Th2, may make this approach attractive for treatment of Th1-mediated diseases like multiple sclerosis, juvenile diabetes and rheumatoid arthritis. DNA vaccination has also proved to be an effective research tool, for the generation of polyclonal (Sundaram et al. 1996) and monoclonal (Barry et al. 1994) antibodies. The simplicity of DNA vaccination, and the potent humoral immune responses it induces, make this technique ideal for antibody production, and circumvents the need to produce pure recombinant protein for immunisation, a costly and time consuming affair.

### **1.3.9 ENHANCEMENT OF DNA VACCINATION EFFICACY BY COINOCULATION OF DNA ENCODING CYTOKINE GENES**

Several studies *in vivo* in mice and cats have shown that the magnitude and nature of an immune response elicited by a DNA vaccine can be engineered by the coinoculation of DNA encoding cytokine genes, (Chow et al. 1998), (Chow et al. 1998), (Kim et al. 1997), (Tsuji et al. 1997), (Hosie et al. 1998). The next section introduces the cytokines, considers the immunoregulatory and immunomodulatory functions of these proteins and discusses their potential to act as adjuvants in DNA vaccination studies.

## **1.4 DNA PLASMIDS ENCODING CYTOKINE GENES; POTENTIAL ADJUVANTS IN DNA VACCINATION STUDIES**

### **1.4.1 THE CYTOKINES; AN OVERVIEW**

The cytokines are a large family of glycoproteins that may be produced by virtually every nucleated cell type in the body. They exert pleiotropic regulatory effects on many cell types involved in both host defence and normal and abnormal homeostatic mechanisms (Cohen and Cohen, 1996). Cytokines affect cells in a complex network of both positive and negative interactions by enhancing or suppressing cellular proliferation, differentiation, activation or motility (Cohen and Cohen, 1996). An understanding of the actions of a single cytokine is therefore not possible without consideration of these interactions. The rapidly expanding field of cytokine research has arisen from the former independent disciplines involving the study of lymphokines, interferons, haemopoietic growth factors and the more classical growth factors.

Originally, each cytokine was named according to the response it was first shown to produce in a biological assay (Campos et al. 1991). For example, the term interferon (IFN), was coined by Isaacs and Lindemann in 1957 to refer to a molecule which interfered with viral replication (Isaacs and Lindemann, 1957) and the term tumour necrosis factor (TNF) was given to a protein which was capable of inducing the necrosis of certain tumours (Carswell et al. 1975). Subsequently, cytokines were grouped according to the cell type in which they were produced. For example, the terms monokine and lymphokine identified cytokine proteins which were produced by monocytes and lymphocytes, respectively. However, it later became apparent that single cytokines had multiple biological actions (pleiotropy) and that in many cases a range of different cell types was capable of producing the same cytokine protein.

Cytokines are involved in orchestrating the immune response and may serve as chemical messengers between leukocytes. Therefore, the term interleukin was introduced in 1979 to reflect this basic property of cytokines and to help remove some

of the confusing nomenclature (Mizel and Farrar, 1979). Many well characterised cytokines are now referred to as interleukins, although others, such as colony stimulating factors (CSF) and interferons, have not been included in this system even though they are involved as mediators during immune responses. Therefore, the term "cytokine" is used to define a group of proteins which include lymphokines, monokines, interleukins, interferons, growth and colony stimulatory factors, and a variety of other proteins (Campos et al. 1991). Consequently, due to their diverse nature, it is difficult to formulate a precise definition as to what constitutes a cytokine. Vilcek and Le (1994) proposed a definition of cytokines as "regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of the cytokines include numerous effects on cells of the immune system and modulation of inflammatory responses" (Vilcek and Le, 1994). Table 1.5. describes some of the most important properties of cytokines.

- Most cytokines are secreted but some are also found associated with cell membranes.
- Cytokines are polypeptides, which may be glycosylated. The monomeric molecular mass of the mature cytokines ranges from 15 to 30kDa. Only one known cytokine, IL-12, is a heterodimer.
- The production of cytokines is not generally constitutive but is induced by altering the level of transcription or translation.
- Production of cytokines is generally transient and actions are mediated at close range, in either an autocrine or paracrine fashion.
- Cytokine actions are mediated via binding to high affinity cell-surface receptors.
- Actions are due largely to modulations in gene expression in target cells leading to alterations in cell proliferation, differentiation or function.
- Whilst their actions are often diverse, they generally include effects upon haemopoietic cells.

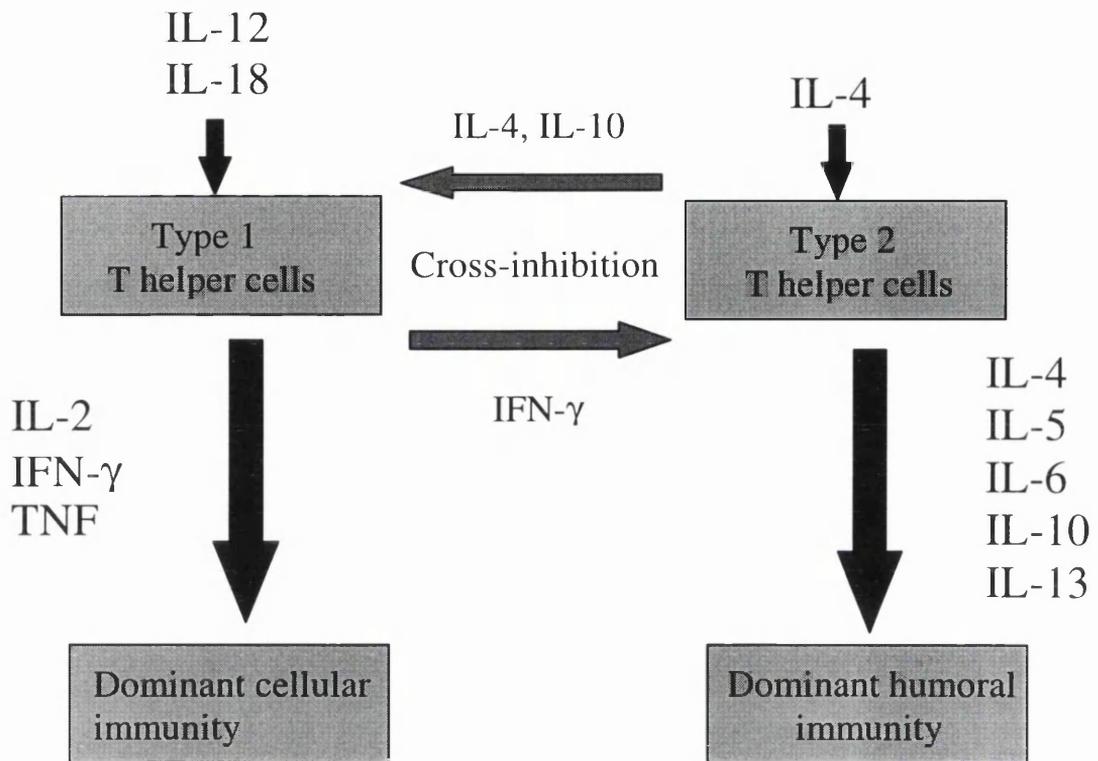
**Table 1.5. The characteristic features of cytokines**

#### **1.4.2 IMMUNOREGULATORY CYTOKINES; TH1 AND TH2 TYPES**

The cytokines which will be considered in depth here are the Th1 enhancing, immunoregulatory cytokines IL-12 and IL-18. Simplistically, T lymphocytes may be classified into functional groups: T helper cells, T suppressor cells, and cytotoxic T cells. Functionally distinct subsets of helper T cells, distinguishable by their different patterns of cytokine production, are found in both mice and humans (Abbas et al. 1997). These functionally polar subsets of T helper cells are the principal regulators of the immune system (Hnilica and Angarano, 1997). The T helper 1 (Th1), phenotype is characterised primarily by the production of IFN- $\gamma$  and tumour necrosis factor  $\beta$  (TNF $\beta$ ) and the Th2 phenotype by the production of IL-4, IL-5 and IL-13 (Lichtman and Abbas, 1997). A schematic representation of the cytokines involved in Th1 and Th2 type immune responses is provided in figure 1.8. As a direct result of the cytokines they produce, Th1 cells promote macrophage activation and stimulate

production of antibodies that bind the Fc $\gamma$  receptor and fix complement. Th1 responses are important for protective, cell-mediated immune responses against intracellular microbes, such as mycobacteria and viruses, but these responses may also lead to tissue injury, typified by the delayed-type hypersensitivity reaction (Lichtman and Abbas, 1997).

In contrast, the cytokines produced by Th2 cells promote IgE production and eosinophil rich inflammatory infiltrates, which together lead to the killing of parasites. Th2 type cytokines also act to downregulate Th1 dependent, cell-mediated immune responses. Th2 type immune responses, therefore, can protect against parasitic infections, and can limit the harmful effects of Th1 type responses, but may also promote the development of immediate hypersensitivity (allergic), reactions. Simplistically, then, Th1 type cytokines stimulate innate and cell-mediated immunity, while Th2 type cytokines stimulate humoral immunity (Hnilica and Angarano, 1997). When stimulated, each T helper subset inhibits the development of the alternative subset; therefore, diseases typically involve the predominance of either a Th1 or Th2 type immune response.



**Figure 1.8. The cytokines involved in Th1 and Th2 type immune responses**

**IFN- $\gamma$** , interferon gamma; **TNF**, tumour necrosis factor.

Different lines of evidence have shown that the early decision towards a Th1 or Th2 type immune response is mainly dependent on the balance between levels of IL-12 (which favours a Th1 response) and IL-4 (which favours a Th2 response (Trinchieri, 1993). IL-12 plays an essential role in the generation of Th1 cells (Trinchieri, 1994) and consequently the development of effective cell-mediated immune responses. Not only does IL-12 stimulate the development of naïve T lymphocytes into Th1 cells (type 1 helper T lymphocytes) (Manetti et al. 1993), this cytokine also facilitates antigen-induced activation of already differentiated Th1 cells, resulting in the proliferation and secretion of IFN- $\gamma$  (Murphy et al. 1994). Although IL-18 is not able to induce the development of Th1 cells itself, this cytokine is essential for the effective induction and activation of Th1 cells by IL-12 (Okamura et al. 1998). In fact, IL-12 and IL-18 synergise to dramatically enhance levels of IFN- $\gamma$  production (Micallef et al. 1996). Therefore, both IL-12 and IL-18 are essential for the development of a Th1 type

immune response. In chapter 3 the biological function of both these cytokines is discussed in greater depth.

### **1.4.3 CYTOKINES AS ADJUVANTS IN DNA VACCINATION**

#### **1.4.3.1 Introduction**

The ability to induce protective immunity against a pathogen by vaccination depends upon the expansion of a memory T lymphocyte population which, upon subsequent activation, will be capable of responding rapidly, leading to the elimination of the pathogen. Vaccine development has been revolutionised by the advent of techniques such as peptide chemistry and recombinant DNA technology. However, the new generation of vaccines possess a major disadvantage. Due to their small molecular structures, recombinant proteins, synthetic peptides and DNA vaccines are often less immunogenic than the killed or attenuated whole organisms contained in many traditional, successful vaccines. This has led, in turn, to interest in the development of novel, safe and effective adjuvants, to make the new vaccines more effective.

An adjuvant acts to enhance the efficacy of a particular vaccine by serving as a depot for the antigen and promoting the immune response raised to the vaccine immunogen. Many classes of adjuvants have been investigated, including ISCOMs (immunostimulatory complexes), liposomes and cytokines. Since Raz et al. reported that inoculation of cytokine genes into muscle resulted in the characteristic biological actions of these cytokines *in vivo* and could enhance the immune response raised to a protein antigen (Raz et al. 1993) it has been reported that in many cases the coinoculation of plasmids encoding cytokine genes could significantly enhance the immune response raised to DNA vaccine antigens (Cohen et al. 1998).

While, in most cases, cytokine adjuvanticity is not as powerful as that shown by the best experimental adjuvants, such as saponin or Freund's adjuvant, it rivals that of many of the adjuvants currently allowed for human use. Moreover, the use of particular cytokines as adjuvants has the major advantage that it may allow the engineering of *in*

*in vivo* immune responses, favouring either the development of cellular or humoral immunity, or both (Kim et al. 1998). The first studies using cytokine genes as adjuvants in DNA vaccination studies, involved using plasmids encoding the cytokine IL-2 (Smith, 1988). Chow demonstrated that inoculation of a vector encoding hepatitis B surface antigen (HbsAg) and IL-2 on the same plasmid induced significant increases in antibody responses, T cell proliferation and enhanced T cell production of IL-2 and IFN- $\gamma$ , compared to inoculation of the HbsAg expressing plasmid alone (Chow et al. 1997). These results suggested that coinoculation with plasmids expressing the IL-2 gene could augment the cellular and humoral immune responses elicited to a plasmid-encoded antigen and enhance Th1 type cytokine production.

Subsequently many different cytokine genes have been reported to have a significant effect on the immune response raised to a plasmid encoded antigen. Plasmids encoding three different types of cytokine have been studied: proinflammatory cytokines, such as IL-1 (Kim et al. 1998) and GM-CSF (Iwasaki et al. 1997), Th1 inducing cytokines, such as IL-12 (Kim et al. 1997) and IL-18 (Kim et al. 1998), and Th2 inducing cytokines, such as IL-4, IL-5 and IL-10 (Kim et al. 1998). In general, pro-inflammatory cytokines, such as IL-1, have enhanced the cellular and humoral immune responses raised to DNA vaccines (Kim et al. 1998), Th1 inducing cytokines, such as IL-12, have potently enhanced the cellular immune response (Kim et al. 1997) and Th2 inducing cytokines, such as IL-4, have augmented the humoral and downregulated the cellular immune response (Kim et al. 1998).

While these studies demonstrate the ability of cytokine genes to enhance the immune response raised to DNA vaccine antigens, these effects have mainly been examined in rodent models. Future research must investigate the effect of cytokines on DNA vaccination in target animals of different species to assess the long-term effects of cytokine gene expression, particularly from an efficacy and safety perspective. The use of cytokines as genetic adjuvants in humans is currently considered unsuitable due to concerns that persistent cytokine expression may lead to unwanted local and/or systemic immunological or haematopoietic side effects (Pasquini et al. 1997). The use of DNA plasmids containing suicide genes or inducible promoters may prove to be a safer approach for the use of cytokines as adjuvants in DNA vaccination studies in

humans (Pasquini et al. 1997). Thus, the information gained from studying the adjuvant effect of cytokines in experimental animal models, such as the feline model described in the course of this thesis, may prove extremely valuable when developing and planning future human DNA vaccination trials.

#### **1.4.3.2 Th1 cytokines as adjuvants in DNA vaccination**

As detailed above, both Th1 and Th2 inducing cytokines have proved to be effective adjuvants in DNA vaccination studies. This section will consider the use of Th1 inducing cytokines as immunological adjuvants in DNA vaccination studies.

The adjuvant effect of DNA plasmids expressing IL-12 is clear. In fact, it has been hypothesised that the adjuvanticity of commonly used and effective bacterial adjuvants, such as complete Freund's adjuvant, relates, in part, to the ability of bacterial molecules to stimulate IL-12 production (Scott and Trinchieri, 1997). The main biological actions of IL-12 are the potentiation of Th1 type cytokine production, particularly IFN- $\gamma$ , in T lymphocytes and NK cells, as a growth factor for activated T and NK cells, and in the generation of CTLs (cytotoxic T lymphocytes) and the activation of cytotoxicity in both CD8<sup>+</sup> and NK cells (Gherardi et al. 1999). Additionally, IL-12 has a central role in the generation of Th1 cells and the optimal differentiation of CTLs (Trinchieri, 1994).

Therefore, the use of plasmids delivering cytokines capable of triggering a Th1 response, such as IL-12 and IFN- $\gamma$ , in conjunction with plasmid encoded vaccine antigens, may help to induce a potent and stable cell-mediated immune response against the vaccine immunogens. Consequently, protection from challenge may also be enhanced. In fact, IL-12 expressing plasmids have been shown to increase the cellular immune response raised to plasmid encoded vaccine antigens in several different experimental models. This may be especially relevant in the development of a DNA vaccine against HIV-1. Several studies have emphasised the importance of CTL activity and cell-mediated immunity in combating HIV-1 infection and controlling the development of AIDS (Rowland-Jones et al. 1997).

A study involving the coinoculation of mice with a plasmid-encoding the HIV-1 envelope gene and a plasmid encoding the IL-12 gene clearly demonstrated that HIV-1-specific cell-mediated immunity was enhanced by inoculation of the IL-12 expressing plasmid (Tsuji et al. 1997). Similarly, Kim reported that the codelivery of IL-12 expression cassettes with DNA vaccine formulations for HIV-1 Ag, in mice, resulted in a shift in the specific immune responses induced. A decreased production of specific antibodies, increased T cell proliferation and a dramatic increase in virus-specific CTL responses were demonstrated, following combined HIV-1 antigen (*env* or *gag/pol*), and IL-12 inoculation (Kim et al. 1997). This work illustrates the power of DNA delivery *in vivo* for the production of new and effective vaccines and as an analytical tool for the molecular dissection of the mechanisms of immune function. Interestingly, Iwasaki has reported that coinoculation with an IL-12 encoding DNA plasmid could convert a weak plasmid DNA immunogen, a non-immunogenic mutant of influenza nucleoprotein (NP), into one capable of inducing a strong CTL response (Iwasaki et al. 1997).

Chow reported that mice immunised with a hepatitis B virus (HBV) DNA vaccine and the IL-12 or IFN- $\gamma$  gene exhibited a significant enhancement of Th1 cells, as well as a marked inhibition of Th2 cells (Chow et al. 1998). The CTL activity induced by HBV DNA vaccination was most significantly enhanced by codelivery of the IL-12 or IFN- $\gamma$  gene, whereas codelivery of the IL-4 gene, a Th2 inducing cytokine, suppressed the activity. Sin investigated the use of an IL-12 expressing plasmid as an adjuvant for a herpes simplex virus-2 (HSV-2) DNA vaccine, in a mouse challenge model (Sin et al. 1999). He reported that IL-12 coinjection with a plasmid encoding HSV-2 gD protein increased Th1 type cytokine secretion (IL-2 and IFN- $\gamma$ ), inducing significantly better protection from lethal HSV-2 challenge, compared with HSV-2 gD DNA vaccination alone, in both outbred and inbred mice. Thus, in this experimental model, IL-12, as a DNA vaccine adjuvant, potentiated an antigen specific Th1 type immune response, which resulted in reduced morbidity and mortality in the HSV-2 challenged mice.

In a similar murine DNA vaccination study, Sin investigated whether the Th1 or the Th2 type immune response, in general, was more important in protecting against HSV-

encoding Th1-type (IL-2, IL-12, IL-15, and IL-18) and Th2-type (IL-4 and IL-10) cytokines were studied in an effort to drive the immune response induced by vaccination towards the cellular or the humoral arm. (This is one of the very few published studies to utilise a plasmid encoding IL-18 as a DNA vaccination adjuvant. IL-18 is a recently discovered cytokine (Okamura et al. 1995) and its central role in the development of Th1 cells and the enhancement of cell mediated immunity has only recently been elucidated). Coinoculation with any of the Th1 type cytokine genes enhanced the survival rate and also reduced the frequency and severity of herpetic lesions following intravaginal HSV challenge, while coinjection with Th2 cytokine genes increased the rate of mortality and morbidity of the challenged mice. Of the Th1-type cytokine genes tested, IL-12 was a particularly potent adjuvant although IL-18 was also effective. in this HSV-2 murine challenge model. In contrast to these results, a recent paper reporting the efficacy of IL-18 as an adjuvant in an HIV-1 DNA vaccination trial noted that the immunomodulatory characteristics of IL-18 were similar to a Th2 type cytokine, rather than a Th1 type cytokine (Kim et al. 1998). The IL-18 expressing DNA plasmid induced dramatic increases in antibody production with only modest increases in CTL activity. The results of this study illustrate the important cautionary point that a particular cytokine may mediate different immunomodulatory effects in different systems and experimental models.

Plasmids expressing IFN- $\gamma$  have been utilised as adjuvants in several DNA vaccination trials. A DNA plasmid encoding feline IFN- $\gamma$  acted as an adjuvant when coinoculated with a FIV DNA vaccine consisting of a defective mutant provirus of FIV (FIVDeltaRT) (Hosie et al. 1998). In this trial, immunisation with FIV DNA and IFN- $\gamma$  gave the highest proportion of protected cats following challenge. As discussed above, Chow also reported the effective adjuvanticity of IFN- $\gamma$  in mice inoculated with a hepatitis B virus DNA vaccine which exhibited a significant enhancement of Th1 cells, a marked inhibition of Th2 cells and a significant enhancement of CTL activity compared to mice inoculated with the HBV DNA vaccine alone (Chow et al. 1998).

By contrast, dramatically different results were obtained when a plasmid encoding mouse IFN- $\gamma$  was coinoculated with a plasmid vector expressing the rabies virus glycoprotein (Xiang et al. 1997). Xiang found that the effect of IFN- $\gamma$  was dependent

glycoprotein (Xiang et al. 1997). Xiang found that the effect of IFN- $\gamma$  was dependent on the promoter driving expression of the viral antigen. The immune responses to antigen-expressing plasmids carrying a viral promoter such as the SV40 early promoter or the major histocompatibility (MHC) class I promoter were reduced in the presence of IFN- $\gamma$ , while the B and T helper cell responses to a plasmid expressing the antigen under the control of the MHC class II promoter were not affected. This result reiterates the important point that a particular cytokine may mediate different immunomodulatory effects in different systems and different experimental models.

In conclusion, several *in vivo* studies in mice and cats have shown that the amplitude and direction of an immune response elicited by a DNA vaccine can be engineered by the coinoculation of plasmid DNA constructs expressing cytokine genes (Chow et al. 1998), (Kim et al. 1997), (Tsuji et al. 1997), (Hosie et al. 1998). Codelivery of plasmids expressing IL-12, IL-18 and IFN- $\gamma$  with vaccine antigens has been shown to enhance the development of Th1 cell populations, the production of Th1 cytokines, and the generation of antigen-specific CTL responses, essential in the development of a functional cellular immune response (Chow et al. 1998), (Sin et al. 1999), (Kim et al. 1997). As discussed later, cell-mediated immunity may be extremely important in the generation of an effective immune response against FeLV (Charreyre and Pedersen, 1991), (Tompkins and Tompkins, 1985). Therefore, the coadministration of these Th1 type cytokine genes, IL-12, IL-18 and IFN- $\gamma$ , with FeLV vaccine antigens may steer the immune response towards the cellular arm, enhancing the efficacy of the FeLV DNA vaccine, which is described within this thesis.

## 1.5 SUMMARY

Feline leukaemia virus, identified by Jarrett in 1964, (Jarrett et al. 1964), is a significant pathogen of the domestic cat and represents a model of contagiously transmitted retroviral disease, in a natural outbred mammalian population (Neil et al. 1991). At present, none of the experimental or commercially available FeLV vaccines consistently provide 100% protection against the development of transient or persistent viraemia or latent bone marrow infection following exposure to virus (Sparkes, 1997).

Naked DNA vaccination, a recently described technology, presents a new and exciting approach in vaccine development. DNA vaccine constructs are created by the insertion of DNA encoding a desired antigen into an eukaryotic plasmid expression vector (Robinson and Torres, 1997). The purified plasmid DNA is inoculated directly into the host, and, as the host cells' transcriptional machinery is utilised, the protein is expressed directly in these cells with the appropriate post-transcriptional modifications and tertiary structure to induce potent humoral and cellular immune responses (Donnelly et al. 1997b). This approach to immunisation may allow the development of safe and efficacious prophylactic and therapeutic vaccines (Tang et al. 1992). Indeed, numerous animal models for DNA vaccines against viral, bacterial and parasitic diseases have now been described (Donnelly et al. 1997b) and, more recently, human clinical trials have been undertaken (MacGregor et al. 1998). Therefore, DNA vaccine technology may prove to be a useful approach in the development of a novel and efficacious FeLV DNA vaccine.

Cytokines are naturally occurring glycoproteins, which are involved in the orchestration of the immune response, regulation of haemopoiesis and in tissue repair and differentiation mechanisms. Cytokines possess a wide therapeutic potential and may act as potent vaccine adjuvants, modulating the amplitude and direction of an immune response elicited by a DNA vaccine (Kim et al. 1998). Interleukin-12, interleukin-18 and IFN- $\gamma$ , Th1 type cytokines, may enhance the development of Th1 cell populations and the development of cellular immune responses, when coinoculated with DNA vaccines (Chow et al. 1998), (Sin et al. 1999). As cell-mediated immunity

may be extremely important in the generation of an effective immune response against FeLV (Charreyre and Pedersen, 1991), the coinoculation of these cytokine adjuvants may enhance the efficacy of the novel FeLV DNA vaccine.

## **1.6 AIMS OF THE PROJECT**

Many trials examining the adjuvant effect of cytokine genes in DNA vaccination models are currently in progress. The aims of this project were to clone, sequence and express cDNAs encoding feline IL-12 and IL-18 cytokine genes with the ultimate goal of investigating their potential as adjuvants in feline leukaemia virus DNA vaccination studies. The primary aims of the FeLV DNA vaccination trial were to determine if the FeLV DNA vaccine alone could provide protection against viral challenge, and secondly, to establish if plasmids encoding feline Th1 type cytokines, IL-12, IL-18 and IFN- $\gamma$ , could act as adjuvants when coinoculated with the vaccine. This work formed part of a larger project within the Department of Veterinary Pathology which aimed to isolate a number of feline haemopoietic and immunomodulatory cytokines. The availability of a number of feline cytokines may facilitate the development of new methods for use in the prophylaxis and therapy of various feline diseases and assist in the understanding of the pathogenesis of diseases of the cat.

The objectives of the project were:

- To amplify, by the polymerase chain reaction, cDNA encoding feline IL-12 and IL-18, from mRNA derived from feline alveolar macrophages.
- To sub-clone the individual PCR products into a plasmid vector and sequence the recombinant DNA.
- To express the feline IL-18 cDNA as a glycosylated protein, using a mammalian expression vector system and to demonstrate this expression using Northern and western blotting techniques.
- To investigate the potential of these feline Th1 type cytokines, IL-12, IL-18 and IFN- $\gamma$ , to act as genetic adjuvants in FeLV DNA vaccination studies.

The following chapters will describe the experimental techniques used to achieve these objectives and the results that were obtained.

## **2. CHAPTER TWO; MATERIALS AND METHODS**

## **2.1 MATERIALS**

Materials in regular use, such as equipment, general reagents and solutions are detailed in this section.

### **2.1.1 CELL CULTURE MATERIALS**

#### **2.1.1.1 Cell lines**

QN10 cells were grown and maintained for use in the FeLV virus isolation technique, kindly performed by Mr Mathew Golder and Mr Mike MacDonald, in the Feline Virus Unit, University of Glasgow.

FEA cells were kindly supplied by M. Golder, as above.

293 cells were grown and maintained for use in transfection experiments by Dr Derek Bain (Department of Veterinary Pathology, University of Glasgow).

293T cells were kindly supplied by Christine M<sup>c</sup>Gillivray (Molecular Medicine Laboratory, Department of Veterinary Pathology, University of Glasgow).

#### **2.1.1.2 Plasticware**

Tissue culture flasks, 96 well plates etc. were supplied by Costar (Cambridge, MA). Cryotubes and 35mm petri dishes were supplied by Nunc (DK 400, Roskilde, Denmark). Falcon conical centrifuge tubes (15 and 50ml) were supplied by Becton Dickinson UK Ltd. (Oxford, UK).

All the plasticware used by the Feline Virus Unit staff in the virus isolation, bone marrow and cell culture techniques, was supplied by Gibco BRL Life Technologies.

#### **2.1.1.3 Solutions, media and supplements**

All solutions and media for cell culture were supplied by Gibco BRL Life Technologies, unless otherwise stated.

### 2.1.1.3.1 Media

All media were supplied as sterile solutions and stored at +4°C.

#### Alpha Minimal Essential Medium

Dulbecco's Modified Eagle's Medium (DMEM), containing HEPES buffer, 25mM, 4500mg/L D-glucose.

#### Leibovitz medium

### 2.1.1.3.2 Supplements

Foetal Calf Serum: (FCS), TCS Biologicals Ltd., (Buckingham, UK): virus screened, mycoplasma screened. FCS was heat inactivated at 56 °C for 30 minutes, then stored in 50ml aliquots at -20 °C until use.

L-glutamine: supplied 200mM (100X) stock solution. This was stored in five millilitre aliquots at -20 °C and routinely added to culture media prior to use.

Penicillin/streptomycin: supplied as a 100X stock solution of 10,000 units penicillin and 10,000 units streptomycin per millilitre. Stored in five millilitre aliquots at -20 °C.

Trypsin-EDTA: supplied as 10X liquid, stored at -20 °C. This was diluted 1:10 in sterile PBS prior to use and stored at +4 °C.

Preservative free heparin (Sigma)

Hydrocortisone succinate (Sigma).

Sodium pyruvate: supplied as a 100mM stock solution

Polybrene: supplied by Abbott laboratories, a registered trademark for hexadimethrine .

MEM Non Essential Amino Acids (NEAA): supplied as 100 x solution containing L-alanine (890 mg/L), L-asparagine (1320 mg/L), L-aspartic acid (1330 mg/L), L-glutamic acid (1470 mg/L), glycine (750 mg/L), L-proline (1150 mg/L) and L-serine (1050 mg/L). Stored at +4°C.

L-arginine: Supplied as lyophilised powder; reconstituted in 10 ml of culture medium to make solution of 20 mg/ml and stored at +4°C.

L-asparagine: Supplied as lyophilised powder; reconstituted in 10 ml of culture medium to make solution of 5 mg/ml and stored at +4°C.

Ciprofloxacin: Anti-mycoplasma antibiotic added to tissue culture medium as necessary to a final concentration of 10mg/ml (Miles, Bayer Diagnostics).

G418: Selection media for 293T cells added to tissue culture medium at a concentration of 400µg/ml.

### **2.1.2 RADIOCHEMICALS**

[ $\alpha$ -<sup>35</sup>S]-dATP (specific activity of > 37 TBq/mmol at reference date), for DNA sequencing was supplied by Amersham Life Science (Bucks, UK). [ $\alpha$ -<sup>35</sup>S]-dATP was stored in 2 µl aliquots, in screw-top eppendorf tubes, at -70°C, until use.

$\alpha$ (<sup>32</sup>P) dCTP specific activity 3000Ci/mmol, for labelling probes, was supplied by Amersham Life Science.

### **2.1.3 GENERAL CHEMICALS**

Chemicals used were of analytical or ultrapure quality and were supplied by Sigma Chemical Company (Dorset, England), Fisons Scientific Equipment (Loughborough, UK), Boehringer Mannheim, Pharmacia Biotech (Herts, UK), GibcoBRL or BDH Ltd. (Poole, England), unless stated otherwise.

### **2.1.4 COMPLETE KITS**

First-Strand cDNA synthesis kit and QuickPrep mRNA purification kit supplied by Pharmacia Biotech (Herts, UK).

Original TA Cloning Kit supplied by Invitrogen (NV Leek, The Netherlands).

Perkin Elmer GeneAmp PCR Core Reagents kit supplied in the UK by Applied Biosystems Ltd.

Perfectprep Plasmid DNA Preparation kit (Flowgen, UK).

QIAGEN Plasmid Purification Maxi kit supplied by Qiagen (UK).

QIAamp Blood Kit (Qiagen, UK).

QIAquick Gel Extraction kit (Qiagen, UK).

High Prime DNA labelling kit (Boehringer Mannheim): Premixed solution for random primed DNA labelling using ( $a^{32}P$ ) dCTP and random oligonucleotides as primers (Boehringer Mannheim).

ThermoSequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Life Science).

Sequenase Version 2.0 DNA Sequencing Kit (USB, Cleveland, Ohio) distributed in the UK by Amersham Life Science.

ProFection Mammalian Transfection System (Promega, UK).

### 2.1.5 BACTERIAL STRAINS

*E.coli* INV $\alpha$ F': One Shot Competent cells, (Invitrogen). F' *endA1 recA1 hsdR17*( $r_k^-$ ,  $m_k^+$ ) *supE44 thi-1 gyrA96 relA1*  $\phi 80lacZ\Delta M15 \Delta(lacZYA-argF)U169 deoR^+ \lambda^-$ .

*E.coli* DH5 $\alpha$ : MAX Efficiency DH5 $\alpha$  Competent Cells, Life Technologies, (GibcoBRL). F'  $\phi 80lacZ\Delta M15 \Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17$ ( $r_k^-$ ,  $m_k^+$ ) *supE44*  $\lambda^- thi-1 gyrA96 relA1$ .

Epicurian Coli XL2-Blue MRF' ultracompetent cells (Stratagene):  $\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac$  [F' *proAB lacI<sup>q</sup>Z* $\Delta M15 Tn10$  (Tet<sup>r</sup>) Amy Cam<sup>r</sup>] <sup>a</sup>

All of the above strains have the *lacZ* $\Delta M15$  marker, enabling blue-white screening by  $\alpha$ -complementation of  $\beta$ -galactosidase encoded by vector DNA (e.g. pCR<sup>TM</sup> II). The genotypes *endA1* and *hsdR17* give improved quality of miniprep DNA; *recA1* denotes recombination negative, recommended for stable replication of high copy number plasmids

### 2.1.6 DNA

Plasmid, molecular weight marker and oligonucleotide DNAs were stored at -20°C.

### 2.1.6.1 Plasmid Vectors

pCR2.1 (Invitrogen): *lacZ*<sup>+</sup>, *amp*<sup>r</sup>, *kan*<sup>r</sup>, T7 promoter and priming site, M13 forward and reverse primer annealing sites. Plasmid designed for direct cloning of PCR products with 3' deoxyadenosine residues (A - overhangs), generated by the non-template dependent activity of *Taq* polymerase. The vector is supplied as linearised DNA with single 3' deoxythymidine (T) residues allowing for efficient ligation of target sequence to vector.

pCRII: As above but also has SP6 promoter and priming sites (Invitrogen).

pUC18/19 (Invitrogen): pBR322 based cloning vector. *lacZ*<sup>+</sup>, *amp*<sup>r</sup>, multiple cloning site and M13 forward and reverse primer annealing sites. Plasmid supplied with TA cloning kit for use as positive control for verifying the transformation efficiency of competent bacteria; concentration of 0.1 µg/ml.

pCI-neo (Promega): The pCI-neo Mammalian Expression Vector carries the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote constitutive expression of cloned DNA inserts in mammalian cells (Promega, 1998). The pCI-neo vector contains the neomycin phosphotransferase gene, a selectable marker for mammalian cells, and can be used for transient expression or for stable expression by selecting transfected cells with the antibiotic G-418. The pCI-neo plasmid contains the T7 and T3 promoter and priming sites, a chimeric intron and the SV40 late polyadenylation signal.

### 2.1.6.2 Molecular Size Standards

φX174 RF DNA/Hae III fragments (size range 72-1,353 bp) and λ DNA/HindIII fragments (size range 125-23,130 bp) were supplied by Gibco BRL.

### 2.1.6.3 Oligonucleotide Primers

Oligonucleotides: Unlabelled and IRD-41 fluorescent labelled oligonucleotides, for use in PCR amplification reactions and cycle sequencing, were synthesised by MWG Biotech. Primers were reverse phase purified and supplied as lyophilised DNA.

Primers were reconstituted in dH<sub>2</sub>O, quantified by spectrophotometry and unlabelled oligonucleotides were resuspended to 1mg/ml, while IRD-41 labelled oligonucleotides were resuspended to 1pmol/μl. Aliquoted primers were stored at -20°C and IRD-41 labelled oligos were also protected from light.

β actin primers (Clontech): for use in PCR amplification reactions were supplied by Cambridge Bioscience. Primers were supplied at 20 μM concentration.

### **2.1.7 ENZYMES**

All enzymes were stored at -20°C, being removed immediately before use.

Restriction enzymes and their associated reaction buffers were supplied by Gibco BRL.

T4 DNA Ligase was provided by Gibco BRL or Invitrogen (as part of the TA Cloning Kit).

Taq DNA polymerase was provided by Perkin Elmer, as part of the GeneAmp PCR Core Reagents kit.

Pfu DNA polymerase was provided by Stratagene Ltd, Cambridge, UK.

Murine Moloney Virus Reverse Transcriptase Enzyme was supplied by Pharmacia Biotech (as part of the First-Strand cDNA synthesis kit).

RNAse A was supplied by Sigma.

### **2.1.8 PROTEIN SDS-PAGE STANDARDS**

Kaleidoscope Prestained SDS-PAGE standards (7.5 - 216 KDa) were supplied by Biorad (Hercules, CA). 10 μl of standards were loaded onto each gel intended for blotting.

## **2.1.9 EQUIPMENT**

### **2.1.9.1 Major Equipment**

Benchtop centrifuges: Omnifuge 2.0 RS and Megafuge 1.0 (Heraeus Sepatech - Germany).

Microcentrifuge: Biofuge 13 (Heraeus Sepatech).

Incubators for tissue culture: supplied by Heraeus Sepatech and Leec Ltd. (Nottingham, UK).

Water baths: supplied by Grant Instruments (Cambridge) Ltd.(England).

Spectrophotometer: Model DU640, Beckman.

Vacuum dessicator: Hetovac, Heto Laboratory Equipment, Denmark.

Manual Sequencing Apparatus: Flowgen.

Automatic Sequencing Apparatus: Li-Cor model 4000 DNA sequencer (MWG-Biotech).

Automated Processor: Kodak X-omat processor, model ME-3, Eastman Kodak Co., New York, USA.

Gel dryer: Model 583 gel dryer, Biorad, Hercules, CA.

Pipette man (P20, P200, P1000): supplied by Gilson Medical Electronics (Villiers-le-Bel, France).

Automatic Sarpette: supplied by Sarstedt.

Ultraviolet Transilluminator: supplied by UV Products Inc. (San Gabriel, CA).

Autoradiography (Film) cassettes: with intensifying screens (Cronex), supplied by Dupont.

MWG 2000i gel documentation system (MWG-Biotech).

### **2.1.9.2 Consumables**

Bottle top filters (0.22 $\mu$ m pore size) were supplied by Sigma; for sterilisation of tissue culture media.

Screw top 1.5 ml eppendorf tubes, 0.5 ml and 1.5 ml flip top tubes were supplied by Treff AG (Degersheim, Switzerland).

Pipette tips were supplied by Sarstedt.

Syringes (two, five, 10, 20 and 50 ml) were supplied by Becton Dickinson.

Flat ended gel loading tips were supplied by Sorenson Bioscience Ltd.

Filter tip pipette tips (30  $\mu$ l and 200  $\mu$ l) were supplied by Rainin Instrument Co. (Woburn, MA); for use in setting up PCR reactions.

Acrodisc syringe filters (0.22 and 0.4  $\mu$ m) were supplied by Gelman Sciences (Ann Arbor, MI); used for filter sterilising of small volumes of solutions.

Petri dishes, bijoux and universals were supplied by Greiner (Stonehouse, Glos., UK).

Disposable, sterile scalpels were supplied by Swann-Morton (Sheffield, England).

## **2.1.10 EXPERIMENTAL ANIMALS**

Specific pathogen free (SPF) cats were obtained from a commercial breeding unit, housed at Glasgow University and fed a commercial diet. All procedures were carried out in accordance with Home Office regulations.

## **2.1.11 BUFFERS, SOLUTIONS AND GROWTH MEDIA**

### **2.1.11.1 Water**

Tissue culture grade distilled water was supplied by Gibco BRL. Ultrapure water (for procedures involving recombinant DNA, PCR etc.) was provided by a Millipore Q50 water purification system (Millipore (UK) Ltd., Watford, UK). A Millipore RO10 system was used to supply water for preparation of general solutions and media.

### **2.1.11.2 Antibiotics**

*Ampicillin* (Penbritin<sup>TM</sup> - Beecham Research (Herts, England)): Solution of 100 mg/ml prepared by addition of 5 ml of dH<sub>2</sub>O to vial of ampicillin; filter sterilised and stored in aliquots at -20°C until use.

### 2.1.11.3 Buffers and solutions

Ammonium persulphate: 10% (w/v) stock solution in ddH<sub>2</sub>O, freshly made.

10 x TBE Buffer: Tris base 216 g, boric acid 110 g, EDTA 16.9 g. pH 8.2/8.3, made up to 2 L.

50 x TEA Buffer Solution: Tris base 484.5g, NaOAc 272.15 g, NaCl 116.8 g, Na<sub>2</sub>EDTA 74.45 g. pH adjusted to 8.15 with glacial acetic acid and made up to 2 L volume.

Lysis Buffer: 25 mM Tris HCl pH 8.0, 10 mM EDTA, 50 mM Glucose.

1% SDS/ 0.2M NaOH: Made up immediately prior to use by combining equal volumes of 2% SDS and 0.4M NaOH.

KoAc solution: 60 ml 5M Potassium acetate, 11.5 ml acetic acid, 28.5 ml dH<sub>2</sub>O.

1M Tris HCl: 121g Tris base, 800ml dH<sub>2</sub>O. Adjusted to desired pH with concentrated HCl and made up to 1L.

TE Buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

1 x PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.3).

10 x DNA Gel Loading Buffer: 20% w/v Ficoll 400, 0.1 M Na<sub>2</sub>EDTA, pH 8, 1.0% w/v sodium dodecyl sulphate, 0.25% bromophenol blue, 0.25% xylene cyanol. Stored at room temperature and used at a 1:10 dilution.

RNA gel Loading Buffer: 50% formamide, 2.2M formaldehyde, 1X MOPS, in ddH<sub>2</sub>O. Made fresh.

Ethidium bromide: made to a working solution of 3mg/ml with dH<sub>2</sub>O in a fume cupboard. Stored away from light.

10 x SDS-PAGE Electrode (Running) Buffer: tris base 60 g, glycine 288 g, SDS 20 g. Made up to 2 L by addition of dH<sub>2</sub>O.

5x Protein Sample Loading Buffer: SDS Reducing Buffer: 62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol. dH<sub>2</sub>O 3.0 ml, 0.5M Tris-HCl, pH 6.8 1.0 ml, glycerol, 1.6 ml, 10% SDS 1.6 ml, β-mercaptoethanol 0.4 ml, 0.5% (w/v) bromophenol blue (in dH<sub>2</sub>O) 0.4 ml; stored at 4°C. Sample diluted at least 1:4 with buffer and heated at 100°C for 5 minutes prior to loading gel.

10 x Tris Buffered Saline (TBS): Tris base 24.2 g, NaCl 80.0 g, HCl 38.0 ml. pH 7.6. dH<sub>2</sub>O to 1L.

10 x Semi Dry Transfer Buffer: Tris base (48mM) 58 g, glycine (39mM) 29 g, SDS (0.01%) 1 g. dH<sub>2</sub>O to 1L. Working stock was prepared prior to use by the addition of 100 ml 10 x stock to 200 ml methanol and 700 ml dH<sub>2</sub>O.

NT buffer: 10mM Tris, 1.21g/litre, 0.15M NaCl, 8.7g/litre, pH to 8.0 with HCl.

ACK lysis buffer: 8.29 g NH<sub>4</sub>Cl (0.15 M), 1 g KHC<sub>3</sub> (1.0 mM), 37.2 mg Na<sub>2</sub>EDTA (0.1 mM). Dissolved in 800 ml dH<sub>2</sub>O, pH adjusted to 7.2 - 7.4 with 1 M HCl, then dH<sub>2</sub>O added to one litre.

X-gal solution (5-bromo-4-chloro-3-indolyl-b-galactoside): prepared as 40 mg/ml stock in dimethylformamide; stored at -20°C in the dark.

X-Gal assay solution: 5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl<sub>2</sub>, 1mg/ml X-Gal, made up in 0.1M PBS pH 7.4. (X-Gal was dissolved at 40mg/ml in DMSO fresh, when required).

Polyacrylamide solution: Acrylamide/Bis acrylamide stock solution. 30% (w/v) acrylamide, 1.579% (w/v) bis acrylamide, ratio 19:1. Severn Biotech Ltd., stored in the dark at 4 °C.

Polyacrylamide (4%) solution with urea for automated sequencing: 4.8ml Sequagel XR ultra pure concentrate (National Diagnostics), 25.2g urea, 7.2ml 10X TBE in a total volume of 60ml with ddH<sub>2</sub>O. The solution was filtered through a 0.4 µm syringe filter. 400µl of 10% ammonium persulphate and 40µl of TEMED was added for each sequencing gel using the 66cm Li-Cor gel apparatus.

Sequencing gel solution (6%; for manual sequencing): prepared with 21 g ultrapure urea, five millilitres 10 x TBE buffer, six millilitres Long Ranger Gel solution (AT Biochem, Malvern, PA) and dH<sub>2</sub>O to 50 ml. This was filtered through a 0.4 µm syringe filter, and stored at +4°C for no longer than one week.

Pre-hybridisation buffer for Northern blots: 50% deionised formamide, 4 X Denhardt's solution, 4 X SSC, 1.6 X Gene Screen, 0.1% SDS, 8% Dextran Sulphate and 30mg/ml heat denatured, sheared salmon sperm DNA.

RNA ladders: 0.24-9.5kb RNA ladder, stored at -70°C. 3-5mg/lane (GibcoBRL).

RNase A: Prepared as a 10mg/ml stock in 10mM Tris-HCl pH7.5 and 15mM NaCl. Boiled for 15 minutes and cooled slowly. Stored at 20°C.

SSC (20X): 3M NaCl, 0.3M Sodium Citrate in dH<sub>2</sub>O and adjusted to pH7.0. Stored at room temperature.

Denhardt's solution (50X): 1% bovine Serum Albumin (BSA), 1% Ficoll, 1% polyvinyl pyrrolidone in ddH<sub>2</sub>O. Aliquoted and stored at -20°C.

Gene screen (20X): 0.5M NaH<sub>2</sub>PO<sub>4</sub>, 0.5M Na<sub>2</sub>HPO<sub>4</sub>, pH6.5. Stored at room temperature.

Hybond N/N<sup>+</sup>: Nylon membrane for the transfer of nucleic acids (Amersham Life Science).

MOPS buffer (10X): 200mM MOPS pH7.0, 50mM potassium acetate, 10mM EDTA. Stored at 4°C in the dark.

Nick column: Sephadex G-50, Pharmacia Biotech, stored at room temperature.

The ProFection® Mammalian Transfection System, Calcium Phosphate System, (Promega). Store at -20°C

2X HBS (HEPES-Buffered Saline): for use with the above kit. 50mM HEPES, pH 7.1, 280mM NaCl, 1.5mM Na<sub>2</sub>HPO<sub>4</sub>. The final pH should be 7.1.

#### Materials and solutions for anti-gp70 antibody ELISA

Monoclonal antibody: 3-17 (EVL BV, The Netherlands).

FeLV: FeLV-A/F422, purified by sucrose density gradient centrifugation. Resuspended in TBS and stored in 100µl volumes at -80°C, until use.

Coating buffer: 1.59g Na<sub>2</sub> CO<sub>3</sub>, 2.93g NaHCO<sub>3</sub>, to 1 litre of water and pH 9.0.

Tris buffered saline (TBS): 0.1M NaCl, 0.01M Tris HCl, pH 7.4.

TBT Wash buffer: 1 x TBS and 0.1% Tween

Blocking buffer: Non-fat milk proteins (Marvel) 2% w/v in TBS.

Virus disruption buffer: 1% w/v Empigen in TBS.

Goat serum: Scottish Antibody Production Unit, Law Hospital, Carlisle.

Conjugate: Goat anti-cat IgG-alkaline phosphatase (Sera-Lab), diluted in wash buffer.

Substrate: Phosphate substrate (KPL).

Stop solution: 0.4M NaOH.

Plates: Immulon 2.

#### **2.1.11.4 Bacteriological Media**

Media was sterilised by autoclaving at 121°C for 15 minutes, unless stated otherwise.

LB Medium: 20 g tryptone, 20 g NaCl, 10 g Yeast Extract, to 2 L with dH<sub>2</sub>O, pH adjusted to 7.0 with NaOH.

SOC Medium: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose.

## **2.2 METHODS**

Methods used throughout the thesis are described in this chapter, whilst techniques specific to one area are described in later chapters. Many of the methods described are based on standard techniques, which are detailed in several laboratory manuals (Maniatis et al. 1982), (Ausubel et al. 1994).

### **2.2.1 GROWTH AND MANIPULATION OF MAMMALIAN CELLS**

#### **2.2.1.1 Basic Techniques**

All procedures involving manipulation of mammalian cells were carried out using standard aseptic procedures. Where possible all procedures were performed in a laminar flow hood.

##### *2.2.1.1.1 Cryopreservation of cells*

In order to preserve stocks of cell lines for long term use, cells were stored over liquid nitrogen. Cells to be frozen were grown to mid-log phase (as described below) and removed into a sterile 50 ml centrifuge tube (using trypsin-EDTA where necessary). The cells were centrifuged at 400 x g for five minutes, the supernatant discarded and the cells resuspended in freezing medium (appropriate culture medium supplemented with FCS to 20% and 10% DMSO) to a concentration of approximately  $2 \times 10^6$  cells/ml. The cell suspension was transferred in one millilitre aliquots, to labelled cryovials and brought to  $-70^{\circ}\text{C}$  in a controlled rate cell freezer (Kryo 10 - Planer Products Ltd., Sunbury on Thames, UK). The vials were then transferred to a liquid nitrogen freezer. Cell stocks were revived by rapid thawing in a  $37^{\circ}\text{C}$  water bath and subsequently cultured using standard techniques (described below).

#### *2.2.1.1.2 Cell counting*

Cells were counted using a haemocytometer (Weber Scientific International), as follows. Cells were diluted in PBS to give an approximate concentration of  $5 \times 10^5$  to  $1 \times 10^6$  cells/ml. The cell suspension was introduced to the haemocytometer chamber and cells counted under an inverted microscope with 4 x or 10 x objective; cells lying on the top and right hand perimeter of each large (one millimetre) square were included, those on the bottom or left hand were excluded. Cell concentration (cells/ml) was calculated by multiplying the mean number of cells per large square by  $10^4$  and correcting for the dilution factor. Where an estimate of live cell numbers was required 0.4% trypan blue (Gibco BRL) was added to the cell suspension (1:1) and allowed to incubate for five minutes at room temperature prior to counting; dead cells take up the stain and therefore appear blue.

#### **2.2.1.2 FEA cell line**

The FEA cell line is a fibroblast cell line derived from whole feline embryos (Jarrett et al. 1973). The cells grow as an adherent monolayer in culture. Cells were cultured in 20- 30 ml Dulbecco's MEM containing 10% foetal calf serum (FCS), 10 mM HEPES, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin in 75 cm<sup>2</sup> tissue culture flasks at 37°C, 5% CO<sub>2</sub>. Cultures were split, typically 1:3 to 1:4, every three to four days, when sub-confluent. The medium was decanted from the cell monolayer, the cells washed with trypsin-EDTA and then incubated at 37°C with approximately one millilitre of fresh trypsin-EDTA for three to five minutes. The detached cells were then washed in fresh medium and pelleted by centrifugation at 400 x g for five minutes, prior to resuspending in fresh medium and seeding new tissue culture flasks.

#### **2.2.1.3 QN10 cell line**

The QN10 cell line is derived from a clone of AH927 feline fibroblasts into which had been introduced the provirus of the Moloney murine sarcoma virus (Jarrett and Ganieri, 1996). The cells grow as an adherent monolayer in culture. Stock cultures of cells were cultured in 20 - 30 ml growth media, consisting of Dulbecco's MEM containing 25mM HEPES buffer, 10% Foetal calf serum, 1% 100mM sodium,

pyruvate, 1% 200mM L-Glutamine, and 400 IU/ml penicillin/streptomycin, in 75 cm<sup>2</sup> tissue cultures flasks at 37°C, 5% CO<sub>2</sub>. Cultures were split, typically 1:5 every three to four days, when sub-confluent and were trypsinised and seeded into new flasks as described above.

#### **2.2.1.4 293 cell line**

The 293 cell line is a permanent line of primary human embryonal kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA (Graham et al. 1977). The cells are highly permissive for adenovirus DNA, and contain and express the transforming genes of Ad 5. Stock cultures of cells were cultured in 20 - 30 ml growth media, consisting of Dulbecco's MEM, 10% heat-inactivated foetal calf serum, 1% 100mM sodium pyruvate, 1% 200mM L-Glutamine, 400IU/ml penicillin/streptomycin and 1X MEM Non Essential Amino Acids (NEAA) in 75 cm<sup>2</sup> tissue cultures flasks at 37°C, with 5% CO<sub>2</sub>. The cells grow as an adherent monolayer in culture. Cultures were split, typically 1:3 when sub-confluent and were trypsinised and seeded into new flasks as described above.

#### **2.2.1.5 293T cell line**

The 293T cell line, a highly transformed human renal epithelial cell line, is derived from the 293 cell line and expresses the SV40 large T antigen (DuBridgde et al. 1987). The cells grow as an adherent monolayer in culture. Stock cultures of cells were cultured in 20 - 30 ml growth media, consisting of Dulbecco's MEM, 10% Foetal calf serum, 1% 100mM sodium pyruvate, 1% 200mM L-Glutamine, 400IU/ml penicillin/streptomycin, 1X MEM Non Essential Amino Acids (NEAA) and 400µg/ml G418 in 75 cm<sup>2</sup> tissue cultures flasks at 37°C, with 5% CO<sub>2</sub>. Cultures were split, typically 1:10 every three to four days when sub-confluent and were trypsinised and seeded into new flasks as described above.

### **2.2.1.6 Feline alveolar macrophage culture**

Specific pathogen free (SPF) domestic cats were bled out under deep anaesthesia, then euthanased via intracardiac injection of pentobarbitone. The lungs with the trachea and larynx intact were then removed. In a laminar flow tissue culture hood the larynx and proximal trachea were removed under sterile conditions. A sterile funnel was inserted into the trachea and medium, Dulbecco's MEM, (with sodium pyruvate), 10% FCS, 20mM Hepes buffer, 2mM glutamine and 100IU/ml penicillin/streptomycin, was poured in until the lungs were inflated to approximately normal inspiratory volume. The medium was then poured off into a sterile beaker and the cells spun down in falcon tubes at 1700rpm for 5-10 minutes. The cell pellet was washed in 20ml of medium and resuspended in approximately 150X the volume of medium, before being transferred to tissue culture flasks (approximately 20ml per 75cm<sup>2</sup>). The flasks were then gased with 5% CO<sub>2</sub> and incubated at 37°C. The medium was replaced after four hours to remove any contaminating red blood cells, as the macrophages were adherent after this time. The macrophage cultures were then stimulated the following morning with 10µg/ml LPS and after four hours the cells were harvested for mRNA isolation.

## **2.2.2 RECOMBINANT DNA TECHNIQUES**

### **2.2.2.1 Storage and growth of bacteria**

Plasmids were maintained in *E.coli* strains DH5α or INVαF'. To enable storage of these *E.coli* host strains and of transformants obtained during this work, glycerol stocks were prepared. The desired bacterial culture was streaked onto a 1.5% agar plate (1.5% agar in LB medium); in cases where the bacterial stock contained a plasmid conferring ampicillin resistance (all vector strains used in this project) the medium was supplemented with 50 - 100 µg/ml ampicillin. The plate was incubated overnight at 37°C and the following day single colonies were picked using a pipette tip, into 10 ml LB medium (supplemented as appropriate with 50 - 100 µg/ml ampicillin) in a sterile universal. The cultures were incubated at 37°C overnight in an orbital incubator. Confirmation that the overnight culture was derived from bacteria containing the desired recombinant plasmid was achieved by DNA extraction and

restriction digest (section 2.2.2.4). Glycerol stocks were prepared by the addition of 200 µl of 80% glycerol to one millilitre of culture; stocks were stored at -70°C. Bacterial stocks were revived for subsequent work by using a sterile platinum wire to scratch the surface of the stock, following which it was streaked onto an agar plate as outlined above.

### **2.2.2.2 Extraction and purification of plasmid DNA**

Plasmid DNA was isolated using a modification of the alkali lysis technique described by Birnboim and Doly (Birnboim and Doly, 1979).

#### *2.2.2.2.1 Large Scale Plasmid Preparations.*

Large quantities of high quality plasmid DNA, for sequencing purposes or transfections, were purified from 500ml of an exponentially growing overnight culture of recombinant bacteria using the QIAGEN Plasmid Purification Maxi kit. A 10 ml overnight culture of the desired transformant was grown and used to seed a 500 ml culture which was then grown overnight at 37°C, with shaking. The remainder of the protocol was performed according to the manufacturer's instructions. DNA was stored at -20° C.

#### *2.2.2.2.2 Small-Scale Preparations.*

One and a half millilitres of an overnight culture of the desired transformant was removed to an eppendorf and spun at 13,000 rpm in a microcentrifuge for two minutes. The supernatant was discarded and the tubes inverted for two minutes to ensure that the pellet was media free. The pellet was resuspended in 150 µl of lysis buffer containing approximately 0.5 mg of lysozyme. Lysis was effected by addition of 300 µl of 1% SDS/0.2 M NaOH to the resuspended cells. Following gentle mixing the tubes were left on ice for five minutes. Protein, bacterial chromosomal DNA and cellular debris were precipitated by addition of 225 µl of 3M KoAc and following gentle mixing the tubes were left on ice for five minutes. After centrifugation at

13,000rpm for five minutes the supernatant was removed into a clean eppendorf. The plasmid DNA was recovered by addition of 630  $\mu$ l of 100% ethanol followed by centrifugation at 13,000 rpm for 10 minutes. The pellet was washed with one millilitre of 70% ethanol and again centrifuged (13,000 rpm, two minutes). The ethanol was removed and the pellet dried in a vacuum desiccator for one minute. The DNA was then resuspended in 20 - 40  $\mu$ l dH<sub>2</sub>O.

#### *2.2.2.2.3 Preparation of DNA for cycle sequencing*

Small scale purification of sequencing grade plasmid DNA was purified from 1.5 - 3ml of an overnight culture using the PERFECTprep Plasmid DNA purification Kit (Flowgen), according to the manufacturer's instructions. 500ng of DNA was used per cycle sequencing reaction. DNA was stored at -20°C.

### **2.2.2.3 Determination of nucleic acid concentration**

#### *2.2.2.3.1 Determination by spectrophotometry*

The nucleic acid sample was diluted 1:100 by addition of 4  $\mu$ l of nucleic acid to 396  $\mu$ l of dH<sub>2</sub>O. The optical density was measured at 260 nm and 280 nm, in comparison to a blank of dH<sub>2</sub>O. An OD reading of 1.0 at 260 nm corresponds to an approximate nucleic acid concentration of 50  $\mu$ g/ml for double stranded DNA, 40  $\mu$ g/ml for RNA, or 33  $\mu$ g/ml for single stranded oligonucleotides. The ratio of the OD readings at 260 nm and 280 nm (OD<sub>260</sub>/OD<sub>280</sub>) was used to estimate the purity of the nucleic acid. Pure preparations of DNA and RNA have an OD<sub>260</sub>/OD<sub>280</sub> of 1.8 and 2.0, respectively; a lower value suggests possible protein or phenol contamination.

#### *2.2.2.3.2 Estimation of double stranded DNA concentration via gel electrophoresis*

In cases where there was insufficient sample to permit quantification via spectrophotometry or where it was desired to verify the purity of DNA fragments of a certain size, the concentration of dsDNA was determined by running the sample on a

polyacrylamide or agarose gel (see section 2.2.2.5) and comparing the intensity of the fluorescence of the unknown DNA to that of a known quantity of the appropriate size marker ( $\phi$ X174 RF DNA/Hae III fragments or  $\lambda$  DNA/HindIII fragments), following staining with ethidium bromide and visualisation by UV transillumination.

#### **2.2.2.4 Restriction endonuclease digestion**

Typically, 1 - 2  $\mu$ g of DNA was digested in a 20  $\mu$ l reaction mix containing the appropriate buffer, 5 mM spermidine and 5 - 10 units of the desired restriction enzyme. The reactions were incubated at 37°C for a minimum of one hour. Where the isolation of restriction fragments was required, larger quantities of DNA, generally 5 - 10  $\mu$ g, were digested, with the reaction volume and components being increased proportionally. When digesting plasmid DNA prepared by the small scale procedure, likely to contain significant RNA contamination, this was followed by the addition of 10  $\mu$ g of RNase A with a further 15 minute incubation at 37°C.

#### **2.2.2.5 Electrophoresis of DNA**

##### *2.2.2.5.1 Agarose gel electrophoresis*

DNA fragments of 1.0-10 kb were separated and identified by agarose gel electrophoresis using a submarine agarose gel kit (Mini the Gel Cicle - Hoefer Scientific Instruments, San Francisco, CA). Typically, 0.5 - 0.75 g agarose was added to 50 ml of TAE buffer, melted in a microwave and mixed to produce a 1 - 1.5% gel. Once the gel mix had cooled to 55°C, the gel was poured into a 100 x 65 mm gel support in its casting tray and an appropriate gel comb (eight or twelve well) inserted. The gel was allowed to solidify before transferring to an electrophoresis tank; the gel was immersed in TEA buffer and the comb carefully removed. DNA samples were prepared by the addition of an appropriate volume of 10 x gel loading buffer. Molecular size standard DNA was prepared similarly and the samples loaded into the wells using a micropipette. Gels were run at 40 - 50 volts for 60 - 120 minutes, then removed from the gel apparatus and stained in buffer solution containing 0.5  $\mu$ g/ml

ethidium bromide for 30 minutes. Following destaining for 30 minutes in dH<sub>2</sub>O, gels were visualised on a UV transilluminator and photographed using black and white Polaroid film (Type 667 - Polaroid UK Ltd., St Albans, Herts, UK).

#### *2.2.2.5.2 Polyacrylamide gel electrophoresis*

In order to separate, visualise and determine the size of DNA fragments under approximately 1.2 kb (including PCR products and products of restriction digests), non-denaturing polyacrylamide gel electrophoresis was employed. Glass plates of 16 cm x 16 cm size were assembled with a 0.75 mm spacer in a casting stand (Atto). Five to six percent gels were prepared with five to six millilitres of 30%:0.8% acrylamide/bisacrylamide solution (Scotlab, Strathclyde, Scotland), three millilitres of 10 x TBE buffer and dH<sub>2</sub>O added to 30 ml total volume. Following the addition of 25 µl of TEMED and 250 µl 10% APS, the gel solution was poured between the assembled gel plates and a comb (12 or 20 well) inserted. After polymerisation, the gel plates were removed from the casting apparatus, the spacer removed and the plates transferred to the gel electrophoresis apparatus. The apparatus was filled with 1 x TBE buffer, the gel comb removed and the wells flushed with buffer. Samples were prepared as described in 2.2.2.5.1 and loaded onto the gel using 0.4 mm flat ended gel loading tips; φX174 RF DNA/Hae III fragments were used as a molecular size standard. Gels were electrophoresed at 220 V for 45 - 90 minutes then removed and stained in buffer solution containing 0.5 µg/ml ethidium bromide for 15 minutes. Following destaining for 30 minutes in dH<sub>2</sub>O, gels were visualised on a UV transilluminator and photographed using black and white Polaroid film.

#### **2.2.2.6 Purification of restriction enzyme fragments**

Where purification of DNA fragments was required for construction of recombinant plasmids, DNA was purified from agarose gels using the QIAquick Gel Extraction kit (Qiagen). Following electrophoresis, DNA fragments of interest were excised from agarose gels using a clean scalpel, and the remainder of the procedure was performed according to the manufacturers instructions.

### **2.2.2.7 Ligation of vector and target DNA**

Fragments of DNA generated by restriction digestion were ligated into approximately 50ng of vector using T4 DNA ligase (GibcoBRL) according to the manufacturers instructions. Vector and insert DNA were mixed at a molar ratio of 1:1 to 1:5 (typically using 50 - 100 ng vector DNA), with an appropriate volume of ligation buffer and 4 units DNA ligase, in a volume of 10 - 20  $\mu$ l. Vector DNA was linearised using an appropriate restriction enzyme. To prevent re-circularisation of the vector DNA, both 5`-phosphate groups were hydrolysed with 0.5 units of calf intestinal alkaline phosphatase (CIAP, Promega) at 37<sup>0</sup>C for 30 min. If one or both ends of a DNA fragment generated by a restriction enzyme digest needed to be converted into blunt ends for cloning, the 5` or 3` protruding ends were 'filled-in' or removed using T4 DNA polymerase (GibcoBRL) which has a 5`-3` polymerase activity as well as a 5`-3` exonuclease activity. Ligation of DNA fragments generated by PCR were carried out according to the manufacturer's instructions with the Original TA Cloning Kit (Invitrogen). Reactions were allowed to proceed overnight at 14°C and stored thereafter at -20°C if not used immediately. A control ligation, omitting insert DNA was generally set up in parallel to the above, in order to check for 'background' when performing subsequent bacterial transformations.

### **2.2.2.8 Transformation of bacteria with plasmid DNA**

In addition to the transformation with recombinant plasmid, the bacteria were also transformed with a control plasmid (as a positive control) and a ligation reaction from which the insert DNA had been omitted (as a negative control).

*Transformation of DH5 $\alpha$  cells:* Cells were thawed on wet ice, gently mixed and 20  $\mu$ l of cells aliquoted to a chilled microcentrifuge tube for each transformation required. Unused cells were refrozen in 20  $\mu$ l aliquots in a dry ice/ethanol bath for five minutes before returning them to the -70 °C freezer. One microlitre of ligation reaction or control plasmid (pUC18) was added to the cells (1 - 10 ng DNA), moving the pipette through the cells while dispensing in order to facilitate mixing. Cells were left on ice for 30 minutes then heat shocked in a 42°C water bath for 40 seconds. After heat

shocking the cells were placed on ice for 2 minutes, 80 µl of SOC medium was added and the tubes were incubated at 37°C for 1 hour with shaking at 225 rpm. Cells were plated onto LB plates (containing 50 µg/ml ampicillin; 25 µl of X-Gal stock solution was spread on the plate one hour prior to use if blue-white colour selection was used) and incubated at 37°C overnight.

*Transformation of INVαF' cells:* The transformation procedure for these cells was essentially as described for DH5α cells with minor modifications: one vial of cells (50 µl) was used for each transformation; 2 µl of 0.5 M 2-ME was added to the cells prior to incubation on ice; cells were heat shocked for 30 seconds; 450 µl of SOC medium was added to the cells; typically 100 µl of cells was spread on each LB agar plate.

*Transformation of Epicurian Coli XL2-Blue MRF' ultracompetent cells:* The transformation procedure for these cells was essentially as described for DH5α cells with minor modifications. 100µl of cells per transformation were aliquoted into prechilled 15 ml Falcon polypropylene tubes. 1.7 µl of 0.5 M 2-ME was added to the cells prior to a ten minute incubation on ice. After adding the DNA to the cells the tubes were incubated for a further 30 minutes on ice. The cells were heat shocked for 30 seconds and 900 µl of SOC medium was added to the cells; typically 100 µl of cells was spread on each LB agar plate.

### **2.2.2.9 Screening of transformants for desired recombinant plasmids**

All plasmid strains used in this project conferred ampicillin resistance upon host bacteria, allowing selection and maintenance of transformed bacteria with ampicillin supplemented media.

#### 2.2.2.9.1 *α*-complementation

The pCRII and pCR2.1 plasmids contain genes encoding the *lacZα* fragment of β-galactosidase and the *lac* promoter and therefore are capable of complementation with the  $\phi$  fragment encoded by the *E.coli* host strains DH5 $\alpha$  and INV $\alpha$ F', giving active β-galactosidase. The incorporation of X-gal into LB agar plates allows the selection of transformants based on blue-white screening. Disruption of *lacZα* expression occurs with the cloning of fragments into the multiple cloning site of this vector, hence recombinants with plasmid containing insert DNA appear white whilst non-recombinants, expressing a functional β-galactosidase, appear blue.

#### 2.2.2.9.2 *Restriction analysis of small-scale plasmid preparations*

Plasmid DNA, isolated as described in 2.2.2.2.2., was subjected to restriction digest with the appropriate enzyme(s), and the resulting products of digestion were run on a polyacrylamide or agarose gel. Bacteria with plasmids containing inserts of the desired size were stored as glycerol stocks as detailed in section 2.2.2.1.

### 2.2.3 PREPARATION OF NUCLEIC ACIDS

#### 2.2.3.1 Preparation of mRNA

For procedures involving RNA preparation and subsequent manipulation, care was taken to avoid degradation by ribonucleases. All plasticware used was either new or was treated by soaking overnight in DEPC treated water, followed by autoclaving twice at 121°C for 15 minutes. All solutions were prepared using DEPC treated water. Gloves were worn and changed frequently.

The preparation of high quality mRNA was facilitated by the use of the QuickPrep mRNA Purification kit (Pharmacia). Tissue is disrupted in guanidinium isothiocyanate to ensure rapid inactivation of endogenous RNase activity and dissociation of cell

components from the mRNA (based on the method of Chirgwin *et al* (Chirgwin et al. 1979)). After adjustment of the buffer concentration and pelleting of cellular debris and insoluble proteins by centrifugation, polyadenylated mRNA is extracted by binding to oligo(dT) cellulose columns (Aviv and Leder, 1972). Finally, following washes to remove DNA, protein and non-poly A+ RNA, the mRNA is eluted from the column.

The manufacturer's instructions were followed, briefly, as follows. Cultured cells ( $< 5 \times 10^7$  cells) were pelleted by centrifugation at  $400 \times g$  for five minutes. The cells were resuspended in 1.5 ml extraction buffer (aqueous solution containing guanidinium thiocyanate and N-lauroyl sarcosine) and homogenised by passing through a 21 G needle attached to a syringe. To the sample was added three millilitres of elution buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA); after brief homogenisation the sample was transferred to a sterile polypropylene centrifuge tube and centrifuged at 10,000 rpm in a Beckman JA-20 rotor for 10 minutes. An oligo(dT)-cellulose spun column was prepared by centrifugation (all centrifuge steps involving the spin column were at  $350 \times g$  for two minutes) with both top and bottom closures removed; the supernatant was then applied to the column and mixed gently for 15 minutes. The column was centrifuged with both closures on and the supernatant discarded. The column was then washed (followed each time by centrifugation) three times with high-salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5 M NaCl) and twice with low-salt buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl). The mRNA was then eluted with three sequential washes of 0.25 ml elution buffer warmed to  $65^\circ\text{C}$ . The mRNA was precipitated overnight at  $-70^\circ\text{C}$  following the addition of 10  $\mu\text{l}$  glycogen solution (5 -10 mg/ml glycogen in DEPC treated water), 75  $\mu\text{l}$  2.5 M KoAc solution and 1.5 ml of 95% ethanol. The mRNA was then pelleted by centrifugation at 13 K for 15 minutes, the ethanol removed, and the mRNA dried for one minute in a vacuum desiccator. The mRNA was then resuspended in 20 - 40  $\mu\text{l}$  DEPC treated water and its concentration determined by spectrophotometry (section 2.2.2.3.1).

### **2.2.3.2 First-strand cDNA synthesis**

In order to maximise the likelihood of obtaining full-length cDNA copies of mRNA, a commercial cDNA synthesis kit was employed (First-strand cDNA synthesis kit -

Pharmacia Biotech). The kit contained all components required for first strand cDNA synthesis, including a pre-assembled reaction mix containing Moloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase (Roth et al. 1985), RNAGuard (an RNase inhibitor), RNase/DNase free BSA, and dNTPs in an aqueous buffer. An oligo-dT primer (Not I-d(T)<sub>18</sub> primer) supplied with the kit was used to prime cDNA synthesis; the sequence is as follows:

5'-d[ AAC TGG AAG AAT TCG CGG CCG CAG GAA T<sub>18</sub> ]-3'

Typically, 200 ng mRNA was placed in a microcentrifuge tube and brought to 20 µl with RNase free water. The RNA was denatured at 65°C for 10 minutes then chilled on ice. To the RNA was added 11 µl bulk first strand reaction mix, 1 µl of 200 mM DTT and 1 µl (0.2 µg) Not I-d(T)<sub>18</sub> primer. The reaction mix was incubated for one hour at 37°C, then kept on ice (or stored at -70°C) prior to amplification by the polymerase chain reaction.

## **2.2.4 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION**

The polymerase chain reaction (PCR) is a powerful technique for amplification of specific DNA sequences from a complex mixture of DNA. The procedure was developed by Mullis and co-workers in the mid 1980s (Mullis et al. 1986), (Mullis and Faloona, 1987) enabling large amounts of a single copy gene to be generated from genomic (Saiki et al. 1985), (Saiki et al. 1986) or viral DNA (Kwok et al. 1987). The initial method used the Klenow fragment of DNA polymerase I, which had to be replenished during each cycle as it is readily denatured by the amplification conditions used. The substitution of thermostable *Taq* polymerase, isolated from *Thermus aquaticus*, circumvented this problem and allowed the automation of thermal cycling (Saiki et al. 1988).

PCR enables the amplification of unknown DNA sequence by the simultaneous extension of a pair of primers, flanking the unknown sequence, each complimentary to opposite strands of the DNA. The uses of PCR are many and it has superseded more conventional molecular biological methods in many areas, including sequencing (Innis

et al. 1988), cloning (Scharf, 1990) and detection and analysis of RNA (Veres et al. 1987). An extensive overview of PCR, its applications and detailed protocols are given in Innis *et al.* (Innis et al. 1990). An overview of the procedure is given below, with more detail in the appropriate chapters.

#### **2.2.4.1 Primer design**

Primer design was aided by some basic guidelines as suggested by Innis and Gelfand (Innis and Gelfand, 1990). Primers were generally 18 - 28 nucleotides in length, with a G + C composition of 50 - 60% where possible. For a given primer pair, the annealing temperatures ( $T_m$ ), were balanced and complementary regions between and within primers were avoided; design of primers in this respect was aided by the Oligo primer analysis software program (Version 4.1 - Medprobe AS, Oslo, Norway).

#### **2.2.4.2 Preparation of PCR reactions**

As PCR is such a sensitive procedure it is essential to take stringent precautions to avoid PCR contamination from tube to tube or carry over of PCR products (Saiki et al. 1988). PCR reactions were set up in a designated area, at a site distant from the main laboratory area, where the PCR products were handled. A set of micropipettes were kept for the sole purpose of setting up PCR reactions. Filter tip pipette tips were used to decrease the risk of carry over of reaction components from one tube to the next. A bulk reaction mix was used in order to minimise the number of pipetting steps. Reaction components (including primers) were aliquoted prior to use and aliquots stored at  $-20^{\circ}\text{C}$ .

#### **2.2.4.3 Reaction conditions**

The use of high quality reagents is essential to the success of PCR; to facilitate this the Perkin Elmer Gene Amp Kit, containing all necessary reagents, was used. The manufacturer's instructions were followed. Typically a reaction mix was set up in 50  $\mu\text{l}$  volume in 0.5 ml tubes containing 1.25 mM each dNTP, 1 x PCR buffer (10 mM

Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.01% gelatin), 2.5 units *Taq* DNA polymerase, 0.2 - 2.0 μM each primer and an appropriate volume of DNA or cDNA template. The reaction was then overlaid with mineral oil. Thermal cycling was carried out in a DNA thermal cycler (Perkin Elmer), with a typical cycle consisting of an initial denaturation of 94°C for five minutes, followed by 30 - 35 cycles of: denaturation at 94°C, for one minute; annealing at 45 - 60°C for one minute; extension at 72°C for one minute; with a final extension step of 72°C for five minutes. Reaction products were visualised by polyacrylamide gel electrophoresis as detailed in 2.2.2.5.2, generally using five-ten microlitres of reaction product per well.

## **2.2.5 DNA SEQUENCE ANALYSIS**

### **2.2.5.1 Manual sequencing by the chain termination method**

#### *2.2.5.1.1 Sequencing reactions*

Manual sequencing employed the Sequenase version 2.0 DNA sequencing kit, which is based on the chain termination method originally described by Sanger et al. (Sanger et al. 1977). This method of sequencing was performed in an attempt to resolve the compressions in DNA sequences encountered when using automated sequencing methods. The latter method was employed for all other sequencing procedures undertaken in the project. The kit uses Sequenase 2.0 DNA polymerase, a modified version of the original Sequenase enzyme, described by Tabor and Richardson (Tabor and Richardson, 1987). The enzyme lacks 3' - 5' exonuclease activity, present in the wild type enzyme, and shows higher speed and processivity. The manufacturer's instructions were followed; the procedure is described briefly below, divided into four stages.

*Denaturation of dsDNA template:* Double-stranded plasmid DNA, prepared as described in 2.2.2.2.2, (approximately six to ten micrograms contained in 36 μl dH<sub>2</sub>O) was denatured by the addition of four microlitres of 2 M NaOH followed by incubation

at 37°C for 15 minutes. Eight microlitres of 5 M ammonium acetate was added to neutralise the mixture, and the DNA precipitated with 2 - 2.5 volumes ethanol at -70°C for 30 minutes. The DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes, the supernatant discarded and the pellet washed in 500 µl 70% ethanol. After a brief centrifugation at 13,000 rpm, the pellet was dried in a vacuum desiccator, then dissolved in 14 µl dH<sub>2</sub>O, providing sufficient DNA for two sequencing reactions.

*Annealing step:* Plasmid DNA, contained in seven microlitres of dH<sub>2</sub>O, was mixed with two microlitres of reaction buffer (200 mM Tris-HCl (pH 7.5), 100 mM MgCl<sub>2</sub>, 250 mM NaCl) and one microlitre (0.5 - 2.0 pmol) of primer in a microcentrifuge tube. The mixture was brought to 65°C for two minutes, then removed to a beaker of water at 65°C and allowed to cool slowly to 35°C, at which point the reaction was considered complete and the tube placed on ice.

*Labelling step:* The primer annealed to the DNA template was extended using limiting concentrations of a mix of dNTPs, including radioactively labelled dATP. This step results in the generation of a mix of labelled DNA chains, varying from several to hundreds of nucleotides in length. To the above template-primer mix was added one microlitre 0.1 M DTT, 0.5 µl of [ $\alpha$ -<sup>35</sup>S]-dATP and two microlitres of labelling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP) diluted five-fold with dH<sub>2</sub>O. Finally Sequenase enzyme was diluted 1:8 with ice cold enzyme dilution buffer (10 mM Tris-HCl (pH 7.5), 5 mM DTT, 0.5 mg/ml BSA), two microlitres was added to the labelling mix, and the reaction was incubated for five minutes at room temperature.

*Chain-termination step:* DNA synthesis was continued using a mix of dNTPs and a dideoxynucleoside triphosphate; this results in the termination of DNA synthesis with a known ddNTP. Two and a half microlitres of each termination mix (80 µM each dNTP, 50 mM NaCl plus 8 µM appropriate ddNTP) was aliquoted into labelled screw-top microcentrifuge tubes and pre-warmed to 37°C for one minute. Three and a half microlitres of the above labelling reaction was then added to each tube, mixed by gentle pipetting, and incubated at 37°C for 15 minutes. The reactions were terminated by the addition of four microlitres of stop solution (95% formamide, 20 mM EDTA,

0.05% bromophenol blue, 0.05% xylene cyanol FF). Reactions were stored at -20°C for up to one week prior to gel electrophoresis.

#### *2.2.5.1.2 Gel electrophoresis*

The completed sequencing reactions were run on six percent denaturing polyacrylamide gels, incorporating Long Ranger polyacrylamide gel solution (AT Biochem, Malvern, USA). Long Ranger gel mix contains modified acrylamide monomers and a modified crosslinker that results in a gel that produces longer readable sequence, is stronger and more elastic than conventional gels, and does not require fixing or removal of urea prior to drying. Glass sequencing plates (50 x 22 cm) were cleaned with 1% SDS, rinsed thoroughly then cleaned with ethanol. Repelcote (BDH) was applied to the surface of one plate to ensure that the gel would not stick to the glass plate. The sequencing plates were assembled with 0.2 mm spacers and plastic adhesive tape. To 50 ml of sequencing gel mix was added 25 µl of TEMED and 250 µl of 10% APS. The gel was poured using a 50 ml syringe, and a 24 well sharktooth comb was inserted in an inverted position and clamped in place using 'bulldog' clips. The gel was then allowed to polymerise in a near-horizontal position for at least one hour at room temperature. The tape at the bottom of the gel plates was removed, the plates mounted into the sequencing apparatus and the upper and lower gel tanks filled with 1 x TBE. The comb was removed, the surface of the gel rinsed with buffer and the comb reinserted in the correct orientation. The gel was pre-electrophoresed for 15 minutes at 35 Watts. The DNA samples were denatured by heating to 75°C for two to five minutes immediately prior to loading two to three microlitres of sample per well. Aliquots of each reaction were run at 35 Watts for approximately two hours and four hours; this generally allowed 350 - 500 bp to be read from each sequencing reaction.

Following electrophoresis, the plates were removed from the sequencing apparatus, allowed to cool briefly then separated. The gel was transferred to Whatman 3 MM filter paper (Whatman International Ltd., Maidstone, England), covered with Saran wrap (Dow Chemical Co.) and dried, gel side uppermost, under vacuum in a gel drier for 30 - 60 minutes at 80°C. The plastic wrap was then removed and the top of the gel trimmed to fit an autoradiography cassette. A sheet of autoradiography film (Biomax

HR single sided emulsion film or X-omat AR double sided emulsion film (both 35 x 43 cm) - IBI Limited, A Kodak Company, Cambridge, England) was placed in contact with the gel and exposed overnight, at room temperature, prior to developing in an automated processor. The sequence was read manually over a light box, and the data stored on a UNIX computer system. DNA sequence was analysed using the GCG package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin), using notably 'SeqEd', 'Bestfit', 'Lineup' and 'Pileup' programs and database searches were carried out by FastA searches in GCG.

### **2.2.5.2 Automated sequencing**

The Licor model 4000 automated sequencer was employed for almost all sequencing procedures undertaken in this project. The sequencer utilises an infrared detection system, whereby DNA fragments are detected following labelling with IRD41 labelled primers, as they run through the denaturing polyacrylamide gel. Cycle sequencing reactions were carried out using IRD41-labelled primers (MWG-Biotech) and the ThermoSequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Life Science). Cycle sequencing is based on the chain termination method of Sanger (Sanger et al. 1977), but uses a thermostable DNA polymerase to give multiple rounds of high temperature DNA synthesis. This variation on the original method of chain termination sequencing was first described by Innis *et al.* (Innis et al. 1988). The method allows direct sequencing of dsDNA without alkali denaturation, requires less template and is generally more efficient at sequencing templates that are G/C rich or have high secondary structure.

Briefly, 500ng of plasmid DNA, 1pmol of IRD-41 labelled primer (MWG-Biotech) and 2 µl of reaction mix which contained 45mM each of dGTP, dATP, dTTP and dCTP, reaction buffer and thermostable DNA polymerase, were mixed in a 0.5ml reaction tube. The reaction was made up to 8µl with ddH<sub>2</sub>O, overlaid with 20µl of mineral oil, denatured for 5 min at 95°C then passed through 25 cycles of 95°C for 30 seconds, 48-60°C for 30 seconds and 72°C for 30 seconds. The mineral oil was then removed by running each sample down a parafilm gradient and collecting the aqueous phase at the bottom of the slope. 4µl of formamide loading buffer supplied with the kit

was then added to each reaction tube. The gel mix was prepared before use with 4.8ml Sequagel XR ultra pure concentrate (National Diagnostics), 25.2g urea, 7.2ml 10X TBE in a total volume of 60ml with ddH<sub>2</sub>O. To the gel mix was added 400µl 10% APS. Three millilitres of the gel mix was removed to a bijou and 32 µl 10% APS and four microlitres of TEMED added; a pasteur pipette was then used to inject the mix between the bottom of the glass plates to form a plug. After the plug had polymerised, 36µl TEMED was added to the remaining gel solution, mixed and the gel poured.

A comb was inserted and clamped in place using two 'bulldog' clips; the gel was then inclined at approximately 30° from horizontal, and allowed to polymerise for a minimum of 90 minutes. The gel was then transferred to the electrophoresis apparatus, both buffer tanks filled with 1 x TBE, the comb removed and the wells flushed. The gel was pre-electrophoresed for 30 - 45 minutes, during which time the scanning microscope was focused and the gain controls adjusted. Sequencing reactions were denatured at 95°C for three minutes prior to loading; generally 0.8 - 1.5 µl was loaded per well. Data recording was performed on a Li-Cor model 4000 DNA sequencer (MWG-Biotech). Typically 800-1000bp of sequence was generated from one run. DNA sequence was analysed as described above, using the GCG package, and database searches were carried out by FastA searches in GCG.

## **2.2.6 ANALYSIS OF RECOMBINANT PROTEINS**

### **2.2.6.1 SDS - polyacrylamide gel electrophoresis of proteins**

The separation and analysis of proteins was facilitated by one dimensional denaturing discontinuous gel electrophoresis, as originally described by Laemmli (Laemmli, 1970). Proteins are denatured by boiling in the presence of SDS and β-mercaptoethanol. The sample is then loaded onto a discontinuous gel consisting of a stacking buffer which concentrates the loaded protein sample into a narrow band and a separating gel which separates proteins on the basis of molecular size, with smaller proteins migrating faster towards the anode.

Minigels (8.0 x 7.3 cm) were formed and run using the Mini-PROTEAN II electrophoresis system (Biorad, Herts, UK) as recommended by the manufacturer. Glass plates were assembled with 0.75 mm spacers in a casting stand. The separating gel was poured to a depth of approximately 5 cm; consisting of 4 ml 30%:0.8% w/v acrylamide/bisacrylamide (giving a 12% gel), 3.35 ml dH<sub>2</sub>O, 2.5 ml 1.5 M Tris-HCl (pH 8.8), 50 µl 20% SDS, 100 µl 10% APS and 10 µl TEMED. This was overlaid with tris-saturated butanol and allowed to polymerise. The butanol was then poured off, the surface of the separating gel rinsed with dH<sub>2</sub>O and the stacking gel poured. The stacking gel consisted of 650 µl 30%:0.8% w/v acrylamide/bisacrylamide, 3.0 ml dH<sub>2</sub>O, 1.25 ml 0.5M Tris-HCl (pH 6.8), 25 µl 20% SDS, 25 µl 10% APS and 7 µl TEMED. A 10 well comb was inserted and the gel allowed to polymerise. The gel was then transferred to the electrophoresis tank, both buffer tanks filled with running buffer, the comb removed and the wells flushed. Protein samples (typically 5 - 30 µg of protein in 5 - 25 µl) were prepared by addition of an appropriate volume of 5 x protein sample loading buffer, followed by heating to 100°C for five minutes. Samples were then loaded onto the gel using 0.2 mm flat ended gel loading tips. A protein molecular weight standard (5 -10 µl) was loaded in one or both outer wells to allow estimation of the size of sample proteins. Gels were electrophoresed at 140V for 60 - 80 minutes until the bromophenol blue dye reached the bottom of the separating gel. The gel was then removed from the glass plates, the stacking gel discarded, and the protein bands detected by immunodetection (section 2.2.6.2).

### **2.2.6.2 Detection of proteins by immunoblotting**

The detection of proteins by immunoblotting (western blotting) is a rapid and sensitive technique that exploits the inherent specificity of antigen recognition by antibodies (Towbin et al. 1979). Proteins were transferred to PVDF membrane by electroblotting, following electrophoretic separation and detected using ECL reagents (Amersham Life Science). This detection system is based on the emission of light following the oxidation of luminol by horse radish peroxidase (HRP labelled antibodies), in the presence of chemical enhancers such as phenols (enhanced chemiluminescence), (Durrant, 1990) and is particularly sensitive (Gillespie and Hudspeth, 1991). The light

emitted can be detected by a short exposure to blue-light sensitive film (Hyperfilm ECL, Amersham).

Following SDS-PAGE, as described in section 2.2.6.1, the gel was removed from the glass plates and rinsed in TBS. Hybond-ECL membrane was prewetted in 100% methanol for 15 seconds, with distilled water for 2 minutes and then allowed to equilibrate with transfer buffer for 10 minutes prior to blotting. Proteins were transferred to the membrane using a semi-dry electroblotting system (Transblot SD - Biorad Laboratories, Hercules, CA) The gel and membrane were sandwiched in close apposition between two sheets of extra thick filter paper (Biorad), pre-soaked in transfer buffer, and transferred at 10 V for 45 minutes.

The membrane was then rinsed in TBS and non-specific binding sites blocked by immersing the membrane for 30 minutes in 10% low fat dried milk (Marvel - Premier Beverages, Stafford, UK) and TBS-T (0.1% Tween in tris buffered saline) solution at room temperature on an orbital shaker. The membrane was rinsed briefly with TBS-T, washed once for 15 minutes then twice for five minutes, with shaking at room temperature. The membrane was then incubated with the primary antibody, at a pre-determined dilution in 5% low fat dried milk, TBS-T, for two hours at room temperature, with shaking. The membrane was then washed as detailed above prior to incubating with the secondary antibody (HRP labelled), appropriately diluted in 5% low fat dried milk, TBS-T, for one hour, at room temperature, with shaking.

The membrane was washed (as above) before detection by the ECL method, which was carried out in a darkroom. An equal volume of detection reagent A was mixed with reagent B (typically 0.5 ml each). Excess buffer was drained from the membrane and the detection solution was pipetted onto the surface of the membrane carrying the protein. After incubation for one minute at room temperature, excess reagent was drained from the membrane which was then wrapped in plastic film. The membrane was placed, protein side up, in a film cassette and a sheet of autoradiography film (Hyperfilm-ECL) placed on top, in the dark. The cassette was closed and the film exposed for 15 - 30 seconds, before developing in an automated processor. A second

sheet of film was then exposed, generally for 2 - 20 minutes, the time being estimated from the appearance of the first autoradiograph.

## **2.2.7 TRANSIENT TRANSFECTION OF MAMMALIAN CELLS**

### **2.2.7.1 Calcium phosphate mediated transfection**

Calcium phosphate mediated transfection involves mixing DNA directly with  $\text{CaCl}_2$  and a phosphate buffer to form a fine precipitate which is dispersed over the cultured cells (Graham and Eb, 1973). This technique for DNA transfer was extended by Wigler *et al.* when they demonstrated that the thymidine kinase gene could be transfected and stably expressed in mouse cells (Wigler *et al.* 1977). The ProFection® Mammalian Transfection System, designed for high efficiency transfer of DNA into mammalian cells, was used for the transient transfection of genes into 293T cells.

The 293T cells were split 1 in 5 the day before the transfection experiment, so the cells were 30-40% confluent on the day of transfection. G-418 was omitted from the medium, at this stage. Three hours prior to transfection medium was removed from the cells and replaced with fresh culture medium. All system components were thawed and then warmed to room temperature, mixing each component thoroughly by vortexing. DNA to be transfected was resuspended in TE buffer at a final concentration of 0.5-1.0 $\mu\text{g}/\mu\text{l}$  and DNA purity was checked by determining the ratio of absorbance at 260nm ( $A_{260}$ ) to 280nm ( $A_{280}$ ), using a spectrophotometer. For each transfection, the DNA and 2X HBS solutions were prepared in two separate sterile tubes. 6 $\mu\text{g}$  of DNA and the appropriate volume of sterile, deionised water to a volume of 300 $\mu\text{l}$ , were added to the first tube and mixed well, before adding 37 $\mu\text{l}$  of the 2M  $\text{CaCl}_2$  and mixing again. Meanwhile, 300 $\mu\text{l}$  of 2X HBS solution was added to the second tube. Working in a sterile tissue culture hood, the tube containing the 2X HBS solution was vortexed gently while the DNA solution was slowly added dropwise to the 2X HBS. This transfection solution was vortexed again, immediately prior to the addition of the solution, dropwise, to the plates of cells. The plates were swirled to distribute the precipitate evenly over the cells and then were returned to a 37°C  $\text{CO}_2$  incubator. 18

hours after transfection the culture media was changed and the cells were finally harvested 48 hours after transfection.

The cell supernatants were removed from the flasks, placed into 15ml Falcon tubes and spun at 1000 rpm for 5 minutes to remove cell debris. The resulting supernatants were pipetted into fresh tubes and stored at -20°C. The cell monolayers were washed briefly with 5ml of 1X PBS and 250µl of 1X protein sample reducing buffer was added to the cell lysates which were to be subjected to western blot analysis. The 1X protein sample reducing buffer was swirled over all the cells and then scraped off with a cell scraper into 1.5 ml screw top eppendorfs. The lysates were then boiled for ten minutes, spun for 5 minutes at 13000rpm in a microcentrifuge, and finally the cell lysate supernatants were aliquoted into fresh eppendorfs and stored at -20°C for future western blot analysis. Cell lysates to be subject to Northern blot analysis were washed briefly in 1X PBS as above, then 1.5ml extraction buffer from the Quickprep mRNA purification kit was added. The cells were scraped off using a cell scraper and the resultant suspension was pipetted into sterile 15ml Falcon tubes. These were homogenised with a 21' gauge needle, before 3ml of elution buffer from the kit was added. Finally, homogenisation with the needle and syringe was performed again, before proceeding with the manufacturer's protocol, as detailed in section 2.2.3.1 .

#### **2.2.7.2 $\beta$ -galactosidase assay to assess transfection efficiency**

To monitor transfection efficiency, a control transfection was performed alongside all the test transfections, using a plasmid expressing  $\beta$ -galactosidase. Cells expressing this enzyme could then be visualised *in situ* by staining with X-Gal substrate. Cells were transfected, as described above, with the pSV- $\beta$ -galactosidase Control Vector (Promega) and the  $\beta$ -galactosidase assay was performed 48 hours after transfection, when the test transfection cells were harvested. All tissue culture medium was aspirated from the cells and they were rinsed quickly with warm 0.1M PBS pH 7.4. The cells were then fixed for 15 minutes with 4% w/v paraformaldehyde 0.1M PBS 0.12M sucrose pH 7.4. The fix was subsequently removed and the cells subjected to three five minute washes with warm 0.1M PBS pH 7.4. The cells were then permeabilised for five minutes with 0.1% v/v Triton/PBS. After aspiration of the

Triton solution, the cells were washed three times, as above, before the X-Gal assay solution was applied to just cover the cells. The flask of cells was then incubated at 37°C overnight, checking the reaction process after three hours. Finally, the assay solution was aspirated and the cells washed as described previously. The cells were then examined under a microscope with the 4 x or 10 x objective and the number of blue cells (transfected cells) in the dried monolayer counted and compared to the number of unstained cells (non-transfected cells). Transfection efficiency for a particular set of transfections was thus determined.

## **2.2.8 RNA HYBRIDISATION ANALYSIS**

### **2.2.8.1 Northern blot transfer of RNA**

1-5µg of polyA<sup>+</sup> mRNA was lyophilised (VR-1 Hetovac, Heto) then resuspended in 20µl of RNA loading buffer and denatured for 15 min at 65°C. Subsequently, 3-5µl of RNA running dye was added and the samples electrophoresed for 3 hours at 100V in 200ml of a 1% agarose gel containing 1 X MOPS and 2.2M formaldehyde in 1X MOPS buffer that was continually recirculated. 5µg of RNA ladders (GibcoBRL), which were treated in the same way as the RNA samples, were used as molecular weight markers. Following electrophoresis, the marker lanes were removed using a clean scalpel and stained in 3µg/ml ethidium bromide for 30 min, then destained overnight and photographed. The rest of the gel was washed twice in ddH<sub>2</sub>O for 20 min to remove formaldehyde, then equilibrated in 10 X SSC and transferred overnight onto Hybond-N in 10 X SSC. The membrane was removed and rinsed briefly in 2 X SSC and the RNA crosslinked to the membrane (Spectrolinker XL-1500 UV Crosslinker, Spectronics Corporation). All northern blots were probed with a rat GAPDH probe to control for RNA loading and integrity. The GAPDH probe is a 750bp *Eco*RI fragment purified from the plasmid pGAPDH.

### **2.2.8.2 Preparation of radiolabelled DNA probes**

PCR generated or restriction fragment DNA probes were gel-purified, using the QIAquick Gel Extraction kit (Qiagen), and radioactively labelled using a 'random prime' DNA labelling kit (High Prime, Boehringer Mannheim), and  $\alpha(^{32}\text{P})$  dCTP, specific activity 3000Ci/mmol (Amersham). Generally, 20-50ng of heat denatured DNA was radiolabelled using 50 $\mu\text{Ci}$  (1.85Mbq) of  $\alpha(^{32}\text{P})$  dCTP and 4 $\mu\text{l}$  of High Prime in a final volume of 20 $\mu\text{l}$ , following the manufacturer's instructions. Unincorporated nucleotides were removed by gel-filtration through Sephadex-G50 beads (Nick Columns, Pharmacia) and labelled fragments were eluted in 400 $\mu\text{l}$  of TE buffer and the activity of 2 $\mu\text{l}$  was determined. Typically, incorporations of  $10^8$  -  $10^9$  cpm/ $\mu\text{g}$  were achieved.

### **2.2.8.3 Hybridisation of labelled probes to membrane bound nucleic acids**

Standard high stringency conditions for the hybridisation of specific radiolabelled probes on nucleic acids immobilised on nylon membranes were as follows. Membranes which had been pre-wetted in 2 X SSC and rolled into Hybaid hybridisation bottles were pre-hybridised in 10-20ml of the northern blot pre-hybridisation buffer (section 2.1.11.3.) at 42°C for at least 2 hours in a Hybaid oven with continual rotation. Freshly boiled DNA probe was added to the pre-hybridisation solution:  $10^6$  cpm/ml was used for Northern. Filters were hybridised overnight as above. After rinsing briefly with 2 X SSC, the membrane was washed for 20 min with three changes of 0.1X SSC, 0.5% SDS at 60°C, unless otherwise stated. Membranes were then sealed in polythene and exposed to X-ray film (Kodak). Following hybridisation, filters were stripped of probe by continuous shaking in boiling dH<sub>2</sub>O containing 0.1%SDS until the solution had cooled to room temperature.

**3. CHAPTER THREE; CLONING, SEQUENCING AND  
EXPRESSION OF FELINE INTERLEUKIN 12 AND  
INTERLEUKIN 18**

## 3.1 INTRODUCTION

### 3.1.1 FELINE CYTOKINES

The application of recombinant human cytokine proteins in the treatment of feline disease has been of limited success, mainly due to the poor degree of conservation between the human and feline proteins, resulting in poor therapeutic efficacy and the generation of neutralising antibodies in treated cats (Dunham, 1999). To facilitate the production of feline specific cytokines, as therapeutic agents and to aid studies into the role of cytokines in feline disease, the isolation and cloning of feline cytokines has been undertaken by many different research groups, (reviewed by Dunham 1999). Determination of the unique feline nucleotide sequence allows techniques such as quantitative RT-PCR, Northern assays or *in situ* hybridisation to be performed. The cytokine protein may also be expressed in an appropriate expression system and antibodies generated, to evaluate both the *in vitro* and *in vivo* effects of the cytokine. Ultimately many cytokines are likely to form a new array of therapeutic agents to treat feline disease. As discussed in the introduction, the isolation of the feline Th1 type cytokines IL-12 and IL-18, cytokines integral to the development of cell-mediated immunity, was undertaken to investigate their potential to act as genetic adjuvants in FeLV DNA vaccination studies.

## **3.1.2 INTERLEUKIN 12**

### **3.1.2.1 Introduction**

Interleukin 12 (IL-12), a recently discovered heterodimeric cytokine, is a pivotal regulator of T and NK cell function (Gubler et al. 1991), stimulating proliferation, cytolytic activity and cytokine induction. IL-12 was identified by two independent research groups at the Wistar Institute (Kobayashi et al. 1989) and Hoffmann-La Roche (Stern et al. 1990), who named it natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF), respectively. Subsequent cloning of the cDNAs encoding these proteins revealed that NKSF and CLMF were the same molecule (Gubler et al. 1991), now generally known as IL-12. Although IL-12 shares many of its activities with other interleukins, such as IL-2, IL-12 has, more recently, been found to play a critical and unique role in promoting Th1 type cytokine responses (Manetti et al. 1993), leading to the enhancement of the cell-mediated immune response. Based on these immuno-enhancing activities, the therapeutic potential of IL-12 in the treatment of infectious disease (Khan et al. 1994) and cancer (Brunda et al. 1993) has been evaluated in animal models, and protective effects have been observed.

While experimental work to isolate the feline IL-12 gene was ongoing, two independent research groups cloned and sequenced the feline IL-12 cDNA (Schijns et al. 1997), (Fehr et al. 1997). However, efforts to elucidate the full length sequences of both subunits of the cytokine, p35 and p40, continued, as differences between the published p40 nucleic acid sequences and the p40 nucleic acid sequence obtained during the course of this project were discovered. As data regarding the molecular biology and biological activity of feline IL-12 were extremely limited at the time of writing, the current data regarding human and murine IL-12 are reviewed below.

### **3.1.2.2 Structure and molecular biology**

IL-12 is a disulphide-linked heterodimeric cytokine comprised of 40kDa (p40) and 35kDa (p35) chains (Kobayashi et al. 1989). The mature human p40 subunit is 306 amino acids long and contains ten cysteine residues and four potential N-linked

glycosylation sites, while the mature p35 chain is 197 amino acids long with seven cysteine residues and three potential N-linked glycosylation sites (Gubler et al. 1991). Murine IL-12 possesses a similar structure to human IL-12, with the p40 and p35 chains sharing 70% and 60% of the amino acid sequence identity, respectively (Schoenhaut et al. 1992). Meanwhile, Fehr *et al.* demonstrated that feline interleukin-12 (IL-12) possessed similar structure and mediated similar biological activities to the human and murine molecules (Fehr et al. 1997). Schijns *et al.* determined that the feline IL-12 p35 cDNA contained an open reading frame encoding a 222 amino acid protein, while the p40 cDNA contained an open reading frame encoding a 329 amino acid protein (Schijns et al. 1997). Comparison of the predicted amino acid sequence revealed that the feline IL-12 p35 protein shared 90.5%, 81.5%, 85.1% and 55.4% identity with the dog, bovine, human and murine IL-12 p35 chain, respectively, while the predicted feline IL-12 p40 protein shared 92.1%, 84.8%, 84.2% and 68.1% identity with the dog, bovine, human and murine IL-12 p40 chain, respectively (Schijns et al. 1997).

The primary amino acid sequence of the IL-12 p35 chain indicates an  $\alpha$ -helix-rich structure, similar to most cytokines (Trinchieri, 1994). Interestingly, the gene for feline IL-12 p35 was cloned in 1994, and it was found that this molecule shared many conserved leucine-zipper motifs with human and murine p35 (Bush et al. 1994). The genes for human p40 (chromosome 5, 5q31-q33) and p35 (chromosome 3, 3p12-3q13.20) are found on separate chromosomes (Sieburth et al. 1992). There is no sequence homology between the p35 and p40 subunits, but the p35 subunit is distantly related to the cytokines IL-6, granulocyte colony stimulating factor (G-CSF) and chicken myelomonocytic growth factor (Hendrzak and Brunda, 1995). Meanwhile, p40 possesses sequence homology with the extracellular domain of the IL-6 receptor and the ciliary neurotropic factor receptor. It has therefore been suggested that IL-12 is structurally analogous to a secreted disulphide linked complex of a cytokine with a cytokine receptor (Gately and Brunda, 1995).

Coexpression of both subunits of IL-12 is required to generate the bioactive heterodimer (Wolf et al. 1991). Cells transfected with only one subunit gene did not secrete active IL-12 (Gubler et al. 1991) and structural analysis of IL-12 using site-

specific chemical modification revealed that intact disulphide bonds were essential for bioactivity (Podlaski et al. 1992). However, mixing recombinant monomeric p40 with monomeric p35 did result in IL-12 biological activity, but at concentrations from 2-5 orders of magnitude higher than those at which the covalently linked heterodimer IL-12 was active. (Trinchieri, 1994). It has been observed that mouse IL-12 p40-containing culture supernatants could specifically inhibit the biological activity of the mouse IL-12 heterodimer in several *in vitro* assays (Mattner et al. 1993). Similarly, a disulphide linked homodimer of human IL-12 p40 has been shown to act as a specific antagonist of IL-12 (Ling et al. 1995). Lymphoid cells that have been induced to secrete IL-12 heterodimer are known to produce p40 in substantial excess of the heterodimer (Podlaski et al. 1992), so perhaps p40 homodimers may act as physiological regulators of IL-12 activity *in vivo*.

### **3.1.2.3 Interleukin 12 receptor and signalling by interleukin 12**

IL-12 receptors (IL-12Rs) have been detected on activated human T cells and on resting or activated Natural Killer (NK) cells, while no IL-12Rs have been found on resting T cells, resting or activated B cells or monocytes (Desai et al. 1992). The IL-12R can be upregulated by mitogens or alloantigen stimulation of T cells and on NK cells activated by IL-2 or IL-12 (Naume et al. 1993). Ligand binding studies have identified three classes of IL-12R with apparent affinities of 5 to 20pM (high affinity), 50 to 200pM (medium affinity) and 2 to 6nM (low affinity) (Chua et al. 1994). The structure of the IL-12 receptor is not yet completely understood. The cDNAs for two subunits of the IL-12R have been cloned from human and mouse T cells and have been designated IL-12R $\beta$ 1 (Chua et al. 1994), and IL-12 $\beta$ 2 (Presky et al. 1996) Both of these subunits belong to the gp130 subgroup of the cytokine receptor superfamily. They are type I transmembrane glycoproteins, with molecular sizes of about 100kDa (IL-12R $\beta$ 1) and 130kDa (IL-12 $\beta$ 2). On the cell surface, each of the two recombinant IL-12R subunits occur as dimers/oligomers and the formation of these higher order structures was found to be ligand independent (Gately et al. 1998). When expressed in recombinant form on COS cells, each human subunit binds labelled IL-12 with only low affinity. Coexpression of both the IL-12R $\beta$ 1 and IL-12 $\beta$ 2 subunits is required for the generation of human high affinity IL-12 binding sites (Presky et al. 1996). A

proposed model for IL-12 signalling indicates that IL-12R $\beta$ 1 interacts with Janus-family kinase 2 (JAK2) and IL-12 $\beta$ 2 interacts with TYK2 and with signal transducers and activators of transcription 3 (STAT3) and STAT4. IL-12 activates JAK2 and TYK2 and induces tyrosine and serine phosphorylation of STAT3 and STAT4 (Lamont and Adorini, 1996).

#### **3.1.2.4 Production of IL-12**

The requirement for expression of two different genes to produce the biologically active IL-12 heterodimer renders the genetic control of the production of this cytokine particularly complex. EBV transformed cell lines, stimulated PBMCs and neutrophils produce the free p40 chain in a 10-50 fold excess over the biologically active p70 heterodimer (D'Andrea et al. 1992). Furthermore, although the stimulation of PBMCs with bacterial products upregulated the production of p35 and p40 mRNA transcripts, the induction of the p40 gene was much more marked. The abundance of p40 transcriptions was up to 200 fold higher than that of the p35 transcripts, explaining the excess production of the p40 protein (Cassatella et al. 1995). As discussed in section 3.1.2.2., perhaps the excess p40 protein forms homodimers, which may act as physiological regulators of IL-12 activity *in vivo*.

Bioactive IL-12 is produced mainly by dendritic cells (DCs), macrophages and neutrophils (D'Andrea et al. 1992). Keratinocytes and Langerhans cells are also capable of low levels of IL-12 production, but normal B cells produce very little, if any, of this cytokine. Production of IL-12 by DCs and macrophages can be induced by interaction with activated T cells, which provide costimulatory signals via molecules such as the CD40 ligand (Shu et al. 1995). These signals appear to be essential, as their inhibition can abrogate IL-12 production. In phagocytic cells, IL-12 production is induced by many stimuli, but particularly by bacteria, bacterial products and intracellular parasites (Vieira et al. 1994). Cytokines such as IFN- $\gamma$  and GM-CSF may also upregulate the ability of phagocytic cells to produce IL-12 (Trinchieri and Gerosa, 1996).

### 3.1.2.5 Biological activities of interleukin 12

The major target cells for IL-12 are NK and T cells, on which IL-12 mediates three major effects: firstly, the induction of cytokine production, secondly, a proliferative effect on activated T and NK cells, and finally, the enhancement of cytotoxic functions (Kobayashi et al. 1989). Among the cytokines induced by IL-12, IFN- $\gamma$  clearly predominates, due to the potency of IL-12 in inducing IFN- $\gamma$  gene transcription in resting and activated T and NK cells, and in its ability to synergise with other IFN- $\gamma$  inducers, such as IL-2, antigen stimulation, mitogens and the recently described cytokine, IL-18 (Yoshimoto et al. 1998). Indeed, the ability to induce the production of large amounts of IFN- $\gamma$  is one of the most important properties of IL-12 and this cytokine mediates many of its biological activities through IFN- $\gamma$ . However, other cytokines, such as TNF- $\alpha$ , GM-CSF and IL-2 are also induced by IL-12. In terms of its proliferative effects, IL-12 activity can be easily detected on preactivated T or NK cell blasts, possibly due to the upregulation of the IL-12 receptor on these cells. IL-12 has also been shown to act as a cofactor mediating proliferation of T cells in conjunction with other mitogens (Kobayashi et al. 1989), and is often required for the optimal proliferation of T cell clones. The enhancing effect of IL-12 on cell-mediated cytotoxicity can be demonstrated by a short-term enhancement of NK cell-mediated cytotoxicity and by its ability to enhance the generation of lymphokine-activated killer cells and cytotoxic T lymphocytes (Kobayashi et al. 1989), (Stern et al. 1990). Finally, IL-12 is also known to have a direct enhancing effect on colony formation by early pluripotent and committed haemopoietic progenitor cells induced by other colony-stimulatory factors (Jacobsen et al. 1993).

The most distinctive and perhaps the most important of IL-12's activities is its ability to regulate the balance between Th1 and Th2 cells, described in section 1.4.2. Studies in both human (Manetti et al. 1993) and murine systems (Heinzel et al. 1993) have shown that IL-12 promotes Th1 responses, resulting in the production of IL-2 and IFN- $\gamma$ , the promotion of cell mediated immunity, macrophage activation and the production of opsonising immunoglobulin IgG2a. However, IL-12 inhibits the generation of Th2 cells, which predominantly produce IL-4, IL-5, IL-10, and promote humoral immunity and the production of IgG1, IgE and IgA isotypes. In addition to promoting the

commitment of naïve Th cells to the Th1 pathway, IL-12 has also been shown to serve as an important costimulus for the activation of fully differentiated Th1 cells (Murphy et al. 1994). It is likely that the Th1/Th2 dichotomy is mainly regulated by the balance, early during an immune response, between levels of IL-12, favouring Th1 responses, and IL-4, favouring Th2 responses.

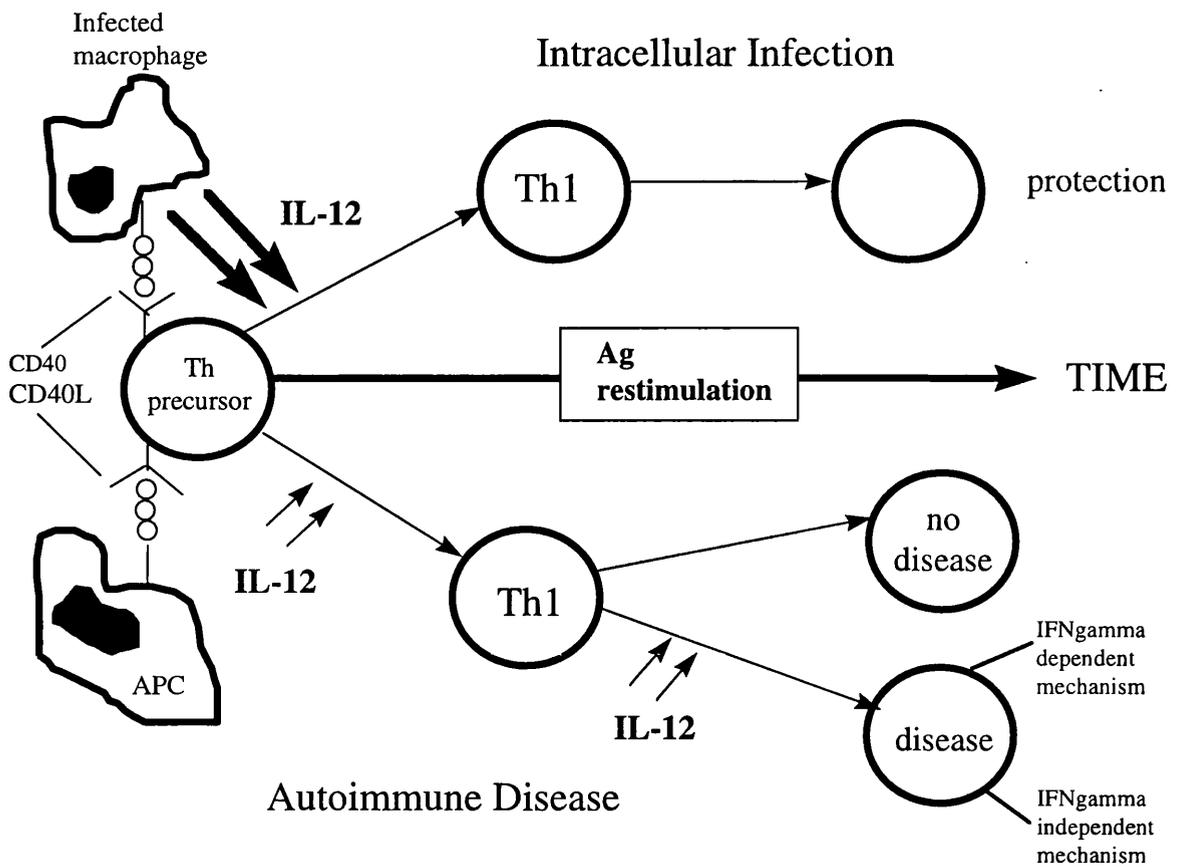
### **3.1.2.6 Therapeutic potential of interleukin 12**

Numerous studies have demonstrated that endogenous IL-12 and IFN- $\gamma$  are required to induce a protective Th1 type immune response to many intracellular pathogens in murine models, such as *Leishmania major* (Sypek et al. 1993), *Toxoplasma gondii* (Gazzinelli et al. 1993), *Mycobacterium tuberculosis* (Cooper et al. 1995), *Histoplasma capsulatum* (Zhou et al. 1997) and *Candida albicans* (Romani et al. 1994). Similarly, exogenous IL-12 has been shown to be an effective therapeutic, as well as vaccine, adjuvant in many of these murine models of disease (McDyer et al. 1998). IL-12 has been most extensively studied in the infectious disease model of murine leishmaniasis (Heinzel et al. 1993), (Sypek et al. 1993), the progression of which is associated with the development of IL-4 producing Th2 cells, and the resolution associated with the presence of IFN- $\gamma$  producing Th1 cells. Experimental studies suggest that IL-12 effectively cured murine leishmaniasis by promoting a protective Th1-type cytokine response, leading to IFN- $\gamma$ -induced macrophage activation, while inhibiting the development of a deleterious Th2-type response. In this disease model, IL-12 has also been demonstrated to be an effective vaccine adjuvant, when administered with soluble leishmania antigen; protection again correlated with the development of Leishmania-specific Th1 cells (Afonso et al. 1994). IL-12 is also effective against some infectious diseases via a T cell independent mechanism, involving the production of IFN $\gamma$  by NK cells, as is the case in infection with the intracellular parasite *Toxoplasma gondii* (Gazzinelli et al. 1993).

IL-12 has proven to be effective in therapy against viral infections, such as type 2 herpes simplex virus (HSV-2), murine cytomegalovirus (Gately and Mulqueen, 1996) and a model of retrovirus-induced murine acquired immunodeficiency syndrome (MAIDS) (Gazzinelli et al. 1994). IL-12 has also been widely tested as a vaccine

adjuvant, enhancing the immune responses raised in mice against pathogens as diverse as hepatitis B virus, HSV-2 and HIV, as reviewed in section 1.4.3.2, and has been considered as an immunotherapeutic agent in the treatment and/or prevention of atopic and allergic disorders (McDyer et al. 1998).

Due to its multiple immunoregulatory functions, including activation of NK cells, CTLs and Th1 cells, IL-12 has also been utilised as an antitumour agent. In fact, the antimetastatic and antitumour activities of IL-12 have been demonstrated in a number of murine models. Both the inhibition of established experimental pulmonary or hepatic metastases and a reduction in spontaneous metastases have been achieved by treating with murine IL-12 (Brunda et al. 1996). Systemic treatment of mice bearing subcutaneous tumours with IL-12 results in tumour growth inhibition, prolonged survival and, in some models, tumour regression. Experiments have demonstrated that both T cells and IFN- $\gamma$  induction are required for the optimal antitumour effects of IL-12. Interestingly, a recent paper describing the cloning and expression of canine IL-12 reported that this cytokine possessed strong cytotoxic activity against an established canine mammary tumour cell line (Okano et al. 1997). The encouraging results in animal models have prompted the use of IL-12 as an anti-tumour agent in human oncology and Phase I and II clinical trials in patients suffering from renal cell carcinomas and breast carcinomas are ongoing (Lamont and Adorini, 1996). However, although IL-12 is an effective therapeutic agent in many different disease models, it has been implicated in the induction of pathology associated with Th1 cell mediated autoimmune diseases, such as experimental allergic encephalomyelitis (EAE) and insulin-dependent diabetes mellitus (IDDM) (Trembleau et al. 1995). Figure 3.1. illustrates the role of IL-12 in the development of Th1 effector cells in infectious and autoimmune disease.



**Figure 3.1.** A schematic presentation of the role of IL-12 in the development of Th1 effector cells in infectious and autoimmune disease.

**Key:** Ag, antigen; IFN gamma, interferon gamma; APC, antigen presenting cell; Th, T helper lymphocyte. The thinner arrows represent the pathway of Th1 cell differentiation.

### **3.1.3 INTERLEUKIN 18**

#### **3.1.3.1 Introduction**

Interleukin 18 (IL-18), originally designated as interferon gamma inducing factor (IGIF), has been recently cloned by Okamura and colleagues. (Okamura et al. 1995). This novel pro-inflammatory cytokine is produced predominantly by activated macrophages, such as Kupffer cells, and acts to augment IFN- $\gamma$  production in spleen cells (Okamura et al. 1995), enhance their natural killer cell activity (NK), enhance the expression and function of Fas ligand on T cells and NK cells (Dao et al. 1996), and activate nuclear factor- $\kappa$ B, (NF- $\kappa$ B) in murine T helper type 1 cells (Matsumoto et al. 1997). Most activities are due to a receptor complex that recruits the IL-1 receptor-activating kinase (IRAK), leading to translocation of NF- $\kappa$ B. This property and others described later, such as the fact that one of the IL-18 receptor chains is the IL-1 receptor-related protein, (IL-1Rrp) (Torigoe et al. 1997) support the hypothesis that IL-18 is related to the IL-1 family (Dinarello et al. 1998). In conclusion, the ability of IL-18 to induce tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$  and both CXC and CC chemokines (Puren et al. 1998), together with the properties already described, suggest that this cytokine is an important contributor in the generation of local and systemic inflammatory responses, participating in both innate and acquired immunity. Again, as data regarding the molecular biology and biological activity of feline IL-18 were unavailable at the time of writing, the current data regarding human and murine IL-18 are reviewed below.

#### **3.1.3.2 Structure and molecular biology of IL-18**

IL-18, a 24kDa single peptide chain, is enzymatically cleaved from the inactive precursor form, to a mature active protein, with a molecular weight of 18kDa (Bazan et al. 1996). Cloned murine and human IL-18 cDNA encode the novel proteins consisting of 192 and 193 amino acids, respectively (Okamura et al. 1995), (Ushio et al. 1996) and possess an unusual leader sequence 35 amino acids in length. The human

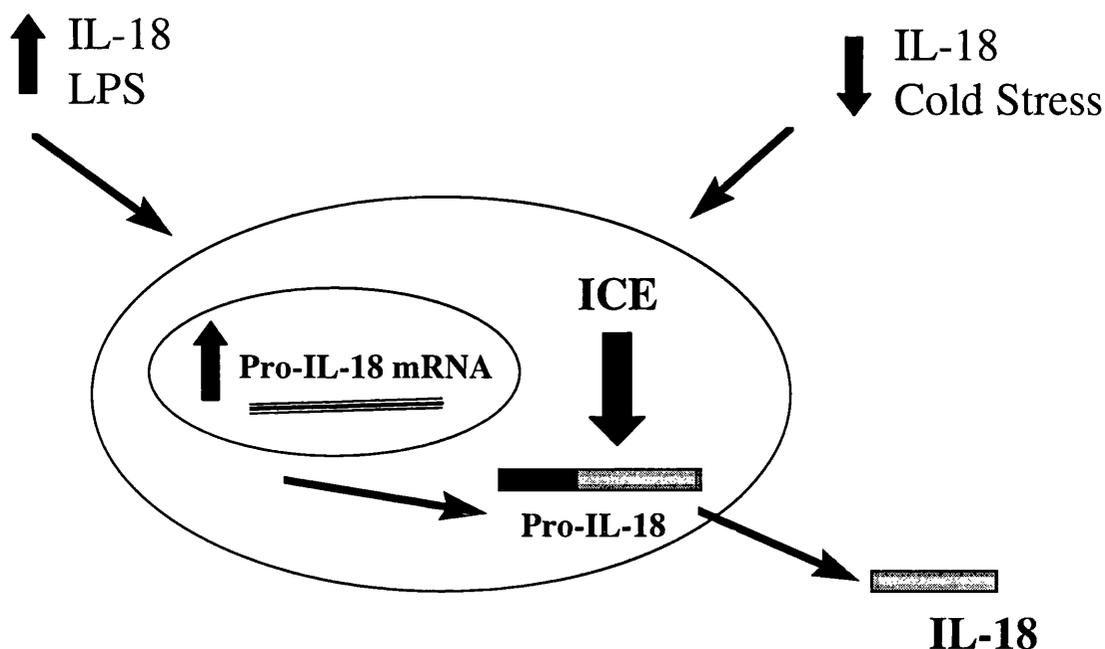
IL-18 gene has recently been mapped to 11q22.2-q22.3, closely linked to the DRD2 locus and distinct from the currently identified human IDDM loci, and the mouse and human genes map to syntenic chromosomal locations (Nolan et al. 1998). IL-18 is related to the IL-1 family in terms of structure (Dinarello, 1999). IL-18 and IL-1 $\beta$  share significant primary amino acid sequences, are similarly folded as all-beta pleated sheet molecules, and Bazan demonstrated that IL-18 shared a 12 $\beta$  sheet structure in common with that of the IL-1 family (Bazan et al. 1996). However from sequence alignments of IL-1 $\alpha$ , IL-1 $\beta$ , IL-1Ra and IL-18 it became apparent that IL-18 did not share substantial identity with the regions of IL-1 $\beta$  which interacted with the IL-1 receptor (IL-1R) (Dinarello, 1994). Moreover, a 1000 fold excess of IL-1 $\beta$  was unable to effect the biological activity of IL-18, indicating that IL-18 acted through a receptor distinct from the IL-1 receptor (Udagawa et al. 1997).

At the amino acid level, murine and rat IL-18 share 64% identity with the human molecule, porcine and human IL-18 share 76% identity (Gillespie and Horwood, 1998), and the canine IL-18 sequence was found to be 74% and 62% homologous to the human and mouse amino acid sequences, respectively (Argyle et al. 1999), (Okano et al. 1999). Each of these homologues of IL-18 share features of the IL-1 signature-like sequence, although IL-18 differs from IL-1 at three key amino acids within this signature motif (Gillespie and Horwood, 1998). Due to these differences, IL-18 appears to exhibit its own signature motif which is modified but distinct from that of IL-1 (Gillespie and Horwood, 1998). In conclusion, although IL-18 does possess a significant relationship with the IL-1 family, the fact that the former cytokine exhibits different biological activities to IL-1 and utilises a different cellular receptor signifies that IL-18 should be considered as an independent cytokine and not simply as a member of the IL-1 family (Gillespie and Horwood, 1998).

### **3.1.3.3 Expression and activation of IL-18.**

Although only limited tissue distributions for IL-18 have been performed, IL-18 mRNA appears to be widely, but not universally expressed. mRNA has been detected in the pancreas, kidney, skeletal muscle, liver, lung, bone and skin, indicating constitutive expression in these tissues (Gillespie and Horwood, 1998). The cell types

expressing IL-18 in many of these tissue compartments have yet to be identified, although activated macrophages appear to be the predominant source. The human IL-18 precursor protein, termed pro-IL-18, is 193 amino acids long and lacks a conventional signal sequence. The N-terminus of the mature protein occurs after Asp<sup>36</sup> (Asp<sup>35</sup> in the murine sequence) and it has been demonstrated that interleukin 1 $\beta$  converting enzyme (ICE, or caspase-1), an aspartic acid-specific protease, cleaves the IL-18 molecule C-terminal to Asp<sup>36</sup> (human) or Asp<sup>35</sup> (mouse) (Gu et al. 1997), to facilitate the removal of the pro sequence and liberate biologically active, mature IL-18. In fact the presence of bioactive ICE is an absolute requirement in the generation of active IL-18; when wild type pro-IL-18 was coexpressed in cells with an inactive form of ICE, no IL-18 bioactivity was detected in the culture supernatants (Gu et al. 1997). The activation of IL-18 by ICE raises further parallels to IL-1 $\beta$ , which is also activated by ICE.



**Figure 3.2. Regulation and processing of IL-18**

LPS and cold stress are known to regulate IL-18mRNA levels in different cells. Increased gene expression leads to synthesis of inactive pro-IL-18 which is cleaved by ICE in the cytoplasm and is secreted from cells as mature IL-18.

**Pro-IL-18**, full length inactive IL-18 precursor; **ICE**, Interleukin-1 $\beta$  converting enzyme; **LPS**, lipopolysaccharide.

### 3.1.3.4 IL-18 receptor

The IL-18 receptor has recently been identified (Torigoe et al. 1997), although it was originally characterised as an IL-1-receptor-related protein (IL-Rrp) (Parnet et al. 1996). Torigoe *et al.* reported that IL-1Rrp is the functional component of the IL-18 receptor. However, the  $K_d$  for IL-18 binding to IL-1Rrp transfected into COS cells was 45nM, an unusually low affinity for a cytokine with a biological activity in the picomolar range. Nakanishi has recently reported that there are both high (0.4nM) and low (40nM) affinity binding sites for IL-18 in murine primary T cells (Nakanishi et al. 1997). Therefore, the IL-1Rrp component could account for the low affinity IL-18

binding sites, and an unidentified second receptor component could account for the high affinity binding sites (Dinarello et al. 1998). It is likely that the IL-18 receptor complex is comprised of a high affinity ligand binding IL-18R $\alpha$  chain, with the second chain being the IL-1Rrp component. Recently, an IL-18 binding protein has been purified from urine, and this is thought to be the soluble (extracellular) form of the IL-18R $\alpha$ -chain (Novick et al. 1999).

### **3.1.3.5 Biological activities of IL-18**

IL-18 is presently considered as a costimulatory factor that induces IFN- $\gamma$  production from T cells, B cells and NK cells (Okamura et al. 1998). This cytokine was originally described as a factor that enhanced IFN- $\gamma$  production from anti-CD3-stimulated Th1 cells, particularly in the presence of IL-12 (Okamura et al. 1995). Naive T cells respond meagrely to IL-18 or IL-12 alone, on anti-CD3 coated plates, although IL-18 appears to induce IFN- $\gamma$  more potently than does IL-12 (Okamura et al. 1995). However, the combination of both these cytokines induced these T cells to proliferate and to produce IFN- $\gamma$  in a synergistic manner (Okamura et al. 1995) and, subsequently, it was discovered that the presence of the anti-CD3 stimulation was not required in this process. Recently, investigations into the mechanism of this synergism have revealed that IL-12 upregulates the expression of the IL-18 receptor on cells producing IFN- $\gamma$  (Ahn et al. 1997), rendering these T cells more responsive to IL-18. Interestingly, under certain conditions IL-18 is able to trigger the production of IFN- $\gamma$  independently of IL-12 and augment IFN- $\gamma$  production and proliferation of Th1 clones, even in the presence of saturating amounts of IL-12, demonstrating that IL-18 and IL-12 are functionally distinct with respect to receptor binding and signal transduction pathways (Kohno et al. 1997). Furthermore, IL-18 also enhances the production of GM-CSF and IL-2 by T cells (Ushio et al. 1996).

IL-18, like IL-12, acts as a costimulatory factor on Th1 cells, but not on Th2 cells (Kohno et al. 1997). Kohno and colleagues have revealed that IL-18 itself cannot induce Th1 differentiation, but instead serves as a strong costimulatory factor (Kohno et al. 1997). Robinson *et al* corroborate this fact and claim that IL-18 is unable to

independently drive Th1 cell development, and merely potentiates IL-12 induced Th1 development (Robinson et al. 1997). This group and others have also suggested that T cell subsets differ in their expression of the receptors for IL-1 family molecules: IL-18R being expressed on Th1 cells (Xu et al. 1998) and IL-1R (type 1R) on Th2 cells. This may help to explain the differential responsiveness of Th1 and Th2 cells to IL-18 and IL-1, both of which activate the common transcription factor NF- $\kappa$ B.

IL-18 also mediates important effects on B cells. In combination with IL-12, IL-18 induces anti-CD40-activated highly purified murine B cells to produce IFN- $\gamma$ , which inhibits IL-4-dependent IgE and IgG1 production and enhances IgG2a production without inhibiting the B-cell proliferative response (Yoshimoto et al. 1997). Turning to NK cells, this cell type appears to be the primary target for IL-12 and IL-18 when infection or other stresses occur. IL-18 increases the cytotoxic activity of spleen cells against YAC cells (Okamura et al. 1998). This effect was not inhibitable by treatment with antibodies against IL-2, a potent NK cell stimulatory factor, indicating that IL-18 directly, or at least not via IL-2, upregulates the cytotoxicity of lymphocytes. As IL-18 had no such effect on the spleen cells derived from NK cell-depleted mice, IL-18 facilitates the cytotoxicity of NK cells. Furthermore, IL-18 acts on NK cells independently of IL-12 and activates the perforin/granzyme system (Okamura et al. 1998). Additionally, IL-18 enhances the action of a second killing apparatus of NK cells, Fas ligand (FasL). IL-18 acts directly and independently of IFN- $\gamma$  on NK cells to induce the expression of FasL and to augment their cytotoxicity against Fas-transfected cells (Tsutsui et al. 1996). Finally, as discussed above, in an *in vitro* system, it has been demonstrated that IL-12 and IL-18 induce anti-CD40-activated B cells to produce IFN- $\gamma$ , which inhibits IL-4-dependent IgE and IgG1 production and enhances IgG2a production. This result indicates that B cells can act as regulatory cells in the immune response and, as IgE responses are an important factor in the pathogenesis of allergic conditions, IL-12/IL-18 treatment may present a unique approach in the therapy of these debilitating conditions (Yoshimoto et al. 1997).

### 3.1.3.6 Therapeutic potential of IL-18

As IL-18 is a multifactorial cytokine, having a major effect on both innate and acquired immunity, its use in therapy has been considered by several researchers (Qureshi et al. 1999), (Kremer et al. 1999), (Tanaka-Kataoka et al. 1999), (Osaki et al. 1999), (Tan et al. 1998). To date, IL-18 has been evaluated as an effective anti-tumour agent (Osaki et al. 1998), (Tan et al. 1998), (Micallef et al. 1997), (Fukumoto et al. 1997), (Coughlin et al. 1998), (Osaki et al. 1999) an anti-microbial agent (Qureshi et al. 1999), (Kremer et al. 1999), (Tanaka-Kataoka et al. 1999), and as a vaccine adjuvant (Kim et al. 1998), (Sin et al. 1999). Strategies to prevent the activity of IL-18 in inflammatory disorders, such as rheumatoid arthritis, have also been devised (Dinarello et al. 1998). Many of these therapeutic strategies have investigated the effects of IL-18 in combination with IL-12. Direct injection of an adenoviral vector expressing the mature form of murine IL-18 into an established MCA205 murine fibrosarcoma completely eradicated tumours in all animals and induced protective systemic immunity (Osaki et al. 1999). Interestingly, coadministration of systemic IL-12 provided synergistic anti-tumour effects in this model. Another study investigated the mechanism of the anti-tumour effects of murine IL-18 on the growth of several mouse tumour cell lines *in vivo* (Osaki et al. 1998). Mice receiving IL-18 before or after challenge with mouse melanoma or sarcoma cell lines were found to have significantly suppressed tumour growth. IL-18 appeared to mediate these effects through the action of NK and CD4<sup>+</sup> T cells and independently of IL-12 and IFN- $\gamma$ . Recent experiments with canine IL-18 have demonstrated that the growth of canine tumours transplanted into SCID mice may be inhibited by the inoculation of recombinant canine IL-18 (Okano et al. 1999).

Recently, a study to investigate the *in vivo* antiviral effects of IL-18 in a mouse model of vaccinia virus infection has been conducted (Tanaka-Kataoka et al. 1999). Intraperitoneal inoculation of IL-18 at 0, 2 and 4 days after infection with vaccinia virus, was found to significantly suppress pock formation on the tails of BALB/c mice, perhaps through the action of NK and CTL cells. Another study has examined the effect of injecting the cDNA of full length pro-IL-18, (cloned into a plasmid containing the CMV promoter), into the skin of mice (Kremer et al. 1999). When mice were

subsequently infected with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), they produced lower levels of anti-BCG antibody than control animals, but higher amounts of antigen-specific IFN- $\gamma$  after *in vitro* restimulation. Therefore, it appeared that IL-18 increased the Th1 type response, indicating that this cytokine may be useful in the development of new immunoprotective strategies against infection by intracellular parasites, such as mycobacteria. IL-18 has also proved to be an effective therapeutic agent against *Cryptococcus neoformans*, (in combination with IL-12), (Zhang et al. 1997), (Qureshi et al. 1999), and *Yersinia enterocolitica* infection (Bohn et al. 1998). The use of IL-18 as an adjuvant in DNA vaccination studies has been discussed in section 1.4.3.2.

### 3.1.4 CLONING OF NOVEL CYTOKINES

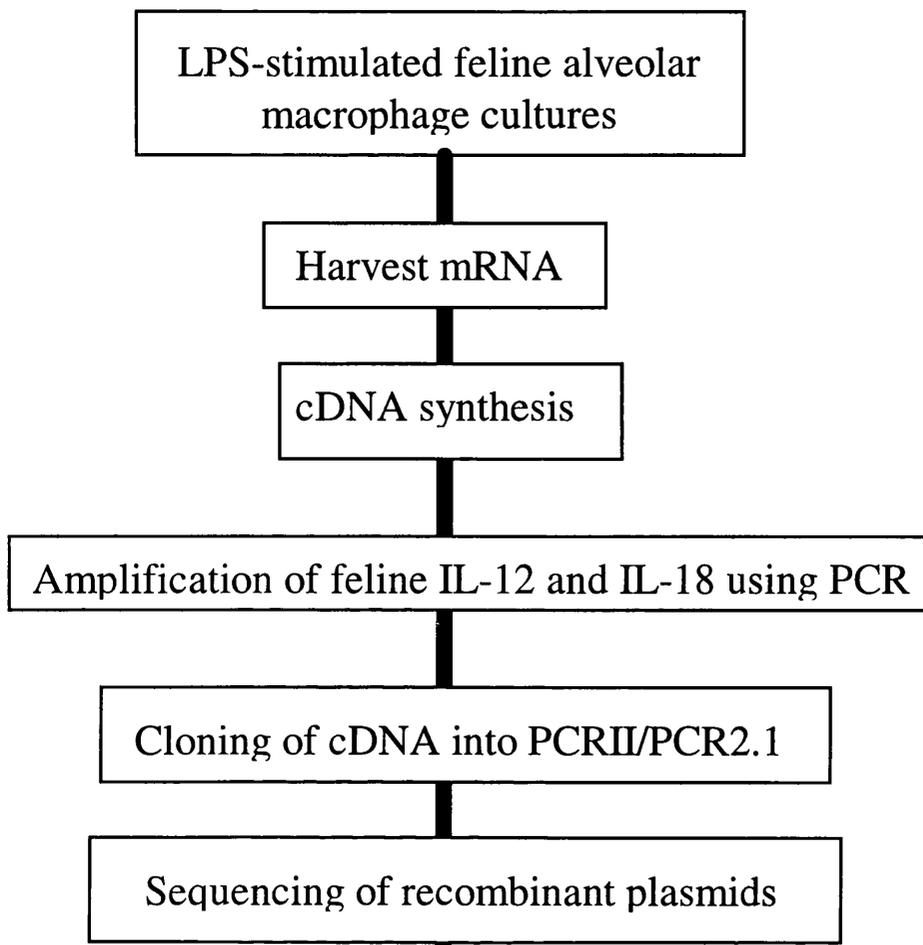
The earliest of human cytokines to be described and studied in detail were the interferons; in particular interferon- $\alpha$ . Studies were initially limited by the availability of sufficient quantities of pure preparations of interferon- $\alpha$ , which had to be purified from the supernatants of human leukocyte cultures. However, for most cytokines such large scale production was impossible, until the advent of recombinant DNA technology. The ability to clone and manipulate genes of interest has enabled the production of many cytokines in sufficient quantities to allow the characterisation of their biological activities and subsequent use in clinical therapy. Traditionally, the isolation of novel genetic sequences using recombinant DNA technology relied upon the generation of genomic or cDNA libraries, followed by screening using one of a number of techniques. The isolation of a given genetic sequence is particularly difficult when the gene exists only rarely, as a single copy gene in a complex genome, or as a rare mRNA species in an mRNA population. Where an mRNA of interest is strongly inducible it may represent a much higher proportion of the total mRNA population and isolation may be possible using techniques such as subtractive hybridisation. While, rare mRNAs can be obtained by screening cDNA libraries, ideally the library should utilise mRNA that contains the mRNA sequence of interest at a high level, to simplify the screening procedure and to maximise the chance of obtaining a full length clone.

Advancements in protein sequencing techniques have allowed the determination of *N*-terminal amino acid sequence from increasingly smaller quantities of protein. The availability of such amino acid sequence data permits the design of degenerate oligonucleotide probes which may then be used to screen a cDNA library. With the advent of the polymerase chain reaction (Mullis et al. 1986), peptide sequence data could be used to design degenerate primers for PCR amplification of specific oligonucleotides, with complete homology to the desired gene. These oligonucleotides may then be utilised as hybridisation probes to screen cDNA libraries. If a cDNA of interest has already been cloned and sequenced in a limited number of species, it is possible to use a probe derived from known sequence data to screen a cDNA library of another species under reduced stringency conditions. This technique has been widely used, for example in the isolation of canine IL-18 (Okano et al. 1999). Furthermore, use of the polymerase chain reaction may enable the direct amplification of cDNA clones from mRNA using the reverse transcriptase - polymerase chain reaction (RT-PCR). This technique avoids the frequently difficult and laborious process of cDNA library generation and screening and significantly reduces the quantity of mRNA that is needed to successfully clone a gene of interest. This approach has recently been used in this department to isolate a number of cytokine cDNAs (Curran et al. 1994), (Argyle et al. 1995), (Dunham et al. 1995).

### **3.1.5 THE CLONING OF FELINE IL-12 AND IL-18**

The technique of RT-PCR was chosen to clone feline IL-12 and IL-18, for several reasons. Firstly, both IL-12 and IL-18 show high sequence homology between species and, consequently, the use of PCR primers, designed from conserved regions flanking the protein coding sequence, was considered to have a good chance of success. Secondly, the RT-PCR technique offered a simpler and potentially faster approach than techniques involving the generation and screening of cDNA libraries. Thirdly, as one of the ultimate aims of this project was to express the feline cytokines as proteins, using a mammalian expression vector system, it was necessary to obtain only the protein coding sequence (i.e. DNA sequence data from introns or non-coding 5' and 3'

flanking mRNA was not required). An important consideration when attempting the isolation of any gene is the choice of a suitable cell line or tissue. Feline alveolar macrophages stimulated with LPS were used as a substrate for the isolation of feline IL-12 and IL-18 by RT-PCR. This choice was based on the knowledge that activated macrophages are a potential source of both these cytokines (Okamura et al. 1998). This chapter describes the cloning and sequencing of both IL-12 and IL-18 using an RT-PCR based technique. An overview of the experimental procedure employed is illustrated in figure 3.3., overleaf.



**Figure 3.3. Overview of the experimental procedure used to clone cDNAs for feline IL-12 and IL-18**

**PCR**, polymerase chain reaction; **cDNA**, complementary DNA; **mRNA**, messenger RNA; **LPS**, lipopolysaccharide.

### **3.1.6 GENE EXPRESSION SYSTEMS**

Heterologous protein expression systems are frequently used to produce proteins for biological uses. They have the advantages of ease of manipulation and frequently enable the production of protein in higher levels than would be achieved by direct isolation of the native protein. Recombinant proteins have been successfully expressed in a wide variety of different systems which include bacterial, yeast, fungal, insect, plant and mammalian cells. In order to evaluate the potential of IL-12 and IL-18 DNA

constructs to act as *in vivo* genetic adjuvants in FeLV DNA vaccination studies, these cytokines were cloned into the mammalian expression vector pCI-neo, and IL-18 protein and mRNA expression was evaluated in an *in vitro* mammalian expression system. Ideally, *in vitro* expression of the IL-12 gene would also have been studied at this time. However, the limited time scale of this project precluded this evaluation.

The expression of proteins from higher eukaryotes in an *in vitro* mammalian system has certain inherent advantages. Proteins are usually expressed in the correct cellular compartment and appropriately modified. Authentic modifications reduce the likelihood of the expressed protein proving immunogenic or having altered biological activity or pharmacokinetics. However, mammalian expression systems tend to be more technically demanding and expensive to use. Transient expression systems are commonly used to produce small amounts of proteins to evaluate the functional activity of a novel cDNA, as is the case with feline IL-18. 293T cells are often used for such short term expression. The 293T cell line, a highly transformed human renal epithelial cell line (Marazzi et al. 1998), has been transformed with SV40 virus carrying a defective origin of replication. The cells do not, therefore, produce whole virus, but do produce large quantities of the viral protein, SV40 large tumour (T) antigen. This protein directs the amplification of vectors containing the SV40 origin of replication to high levels (10,000 - 100,000 copies per cell) 48 - 72 hours following transfection. Plasmids containing a cDNA or genomic DNA insert can therefore direct the synthesis of large amounts of protein in a short time, under the control of an appropriate promoter. The mammalian expression vector pCI-neo, into which feline IL-18 was cloned, contains the SV40 origin of replication, and thus optimises the amount of IL-18 protein expressed from transfected cells. However, a major disadvantage of this system is that transfected cells generally die after a number of days, because the protein production machinery of the transformed cell is effectively 'hijacked'. Therefore, the large scale production of proteins in mammalian systems requires the establishment of stably transfected cell lines and gene amplification.

### 3.1.7 EXPRESSION OF FELINE IL-18

The reasons for choosing a mammalian system to express feline IL-18 are discussed in section 3.1.6. This chapter describes the expression of three forms of feline IL-18; pro-IL-18 + pCI-neo (inactive, full-length precursor), mature IL-18 + pCI-neo (active form) and PsecI IL-18, (mature IL-18 cloned into a vector derived from pCI-neo, containing a synthetic immunoglobulin secretory component to facilitate the secretion of the active cytokine). PCR and restriction enzyme digests were employed in the synthesis of these constructs. To assess *in vitro* expression of IL-18 before *in vivo* administration to cats, 293T cells were transfected with the three feline IL-18 DNA constructs and, subsequently, cell lysates and supernatants were harvested and mRNA isolated from the transfected cells. Expression of the feline IL-18 mRNA and protein was then confirmed using Northern and western blotting techniques, respectively.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 ISOLATION OF mRNA FROM FELINE ALVEOLAR MACROPHAGES**

Messenger RNA (mRNA) was isolated from LPS stimulated feline alveolar macrophages using the Quickprep mRNA isolation kit (Pharmacia) as described in section 2.2.3.1. Feline alveolar macrophages were cultured as described in section 2.2.1.6. Cells were harvested from a 162 cm<sup>2</sup> tissue culture flask by applying 1.5 ml extraction buffer directly to the cell monolayer, after removal of culture medium. Upon completion of mRNA extraction the yield and purity of the RNA was measured by spectrophotometry. The harvested mRNA was then used as a template for cDNA synthesis.

### **3.2.2 SYNTHESIS OF cDNA**

Complementary DNA (cDNA) was synthesised from feline alveolar macrophage mRNA using a First-strand cDNA synthesis kit (Pharmacia) as described in section 2.2.3.2. The completed first strand reaction was heated to 90°C for five minutes then chilled on ice immediately prior to use as a PCR template; this was performed to denature the RNA-cDNA duplex.

### **3.2.3 PCR AMPLIFICATION OF FELINE IL-12 p35 AND p40 SUBUNITS**

The amplification of both subunits of feline IL-12 will be described in this section, but each subunit will be considered separately.

### 3.2.3.1 Design of primers

In order to amplify the p35 and p40 subunits of feline IL-12 it was necessary to design oligonucleotide primers which flanked the protein coding sequence of both subunits. For the p40 subunit, primers were chosen which flanked the 5' and 3' ends of the protein coding sequence of the canine IL-12 p40 cDNA (Buttner et al. 1998), (GenBank accession number U49100, Buttner), as the canine and feline sequences were thought to be highly conserved.

5' primer: 5'-GCAAGATGCATCCTCAG-3'

3' primer: 5'-CATCCTGGGGGTGGAACC-3'

These primers were expected to amplify a p40 IL-12 cDNA of approximately 1010 nucleotides in size, containing the complete protein coding sequence.

The design of primers for the amplification of the p35 subunit was more complicated, as the use of primers based on the known sequences of canine, human, murine and even feline p35 cDNAs, which flanked the entire protein coding sequence, did not amplify the full length p35 cDNA. (The feline p35 IL-12 sequence, accession number Y07761, became available in the later stages of this project (Schijns et al. 1997)). Therefore, the full length gene sequence of the p35 subunit of feline IL-12 was amplified by PCR in two overlapping fragments, using a nested PCR strategy. Primers were designed using the canine and human (earlier stages of the project) and feline (later stages of the project) p35 subunit sequences. cDNAs were aligned using the 'Lineup' and 'Pileup' programs (UWGCG software) and primers were chosen from conserved areas of the protein coding sequence.

To amplify the 3' end of the p35 gene nested PCR was performed using the primers

5' primer: 5'-CAGTGCCGGCTCAGCATGTG-3'

3' primer: 5'-GACCTCMGCWTTYTAGGAAC-3'

A 5µl aliquot of this completed PCR reaction was then used in second round amplification using the primers

5' primer: 5'-CAAGAATGAGAGTTGCCT-3'

3' primer: 5'-GCTTTTTAGGAAGCATTTCAGATAGC-3'

These primers were expected to amplify a 3' fragment of p35 IL-12 cDNA of approximately 390 nucleotides in size, containing the partial p35 subunit protein coding sequence.

To amplify the 5' end of the p35 gene nested PCR was performed using the primers

5' primer: 5'-CAGTGCCGGCTCAGCATGTG-3'

3' primer: 5'-GAAGTATGCAGAGCTTGA-3',

A 5µl aliquot of this completed PCR reaction was then used in second round amplification using the primers

5' primer: 5'-GCATGTGCCCGCCGCGTG-3'

3' primer: 5'-CCAGTTCTTTCAGGGAG-3'

These primers were expected to amplify a 5' fragment of p35 IL-12 cDNA of approximately 550 nucleotides in size, containing the partial p35 subunit protein coding sequence.

### **3.2.3.2 Positive and negative controls**

In order to assess the efficiency of the PCR process a number of controls were set up each time PCR amplification of the feline IL-12 p35, IL-12 p40 or IL-18 cDNA was attempted.

#### *3.2.3.2.1 Positive controls*

##### β-actin primers:

β-actin is a gene that is constitutively expressed at high levels in many cell and tissue types, making it a suitable choice for a positive control. The gene is highly conserved between species, making it possible to amplify feline β-actin using primers designed for the amplification of human β-actin (Clontech Laboratories Inc., Palo Alto, CA).

The following primers were provided at a concentration of 20 µM.

5' primer: 5'-ATCTGGCACCCACCTTCTACAATGAGCTGCG-3'

3' primer: 5'-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3'

These primers amplify a PCR fragment of 838 bp in size when used to amplify human cDNA.

### Lambda DNA primers:

A further positive control is included with the GeneAmp PCR reagent kit; whole bacteriophage lambda is used as a template for PCR amplification using the primer pair:

Primer 1: 5'-GATGAGTTCGTGTCCGTACAACCTGG-3'

Primer 2: 5'-GGTTATCGAAATCAGCCACAGCGCC-3'

These primers amplify a 500 bp fragment (nucleotides 7131 to 7630) of the lambda target DNA.

These “internal controls” ensured that should any PCR failures occur, then the cause of failure could be localised. For example, if both positive controls failed to amplify efficiently this would suggest a problem involving the bulk reaction mix (e.g. *Taq* polymerase, dNTPs or PCR buffer) or the PCR cycler, while failure of the  $\beta$ -actin positive control alone (with alveolar macrophage cDNA template) would suggest either that the starting mRNA was of poor quality or had been inefficiently reverse transcribed to cDNA (e.g. due to inefficient denaturation of the mRNA template or failure of the reverse transcriptase enzyme). Finally, if both positive controls worked efficiently but there was no or poor amplification of cDNA using the IL-12 p35 or IL-12 p40 primers this would suggest that the reaction conditions were inappropriate (e.g. annealing temperature too high/low or PCR primer concentration too low), that IL-12 p35 or p40 was expressed at low levels in the starting mRNA or that the PCR primers did not anneal efficiently to the cDNAs of interest, due to mismatches.

#### 3.2.3.2.2 *Negative controls*

Each time a series of PCR reactions was set up a negative control was also included. A reaction mix was set up containing all PCR components (primers, dNTPs, PCR buffer and *Taq* polymerase) except template. This control was performed to check that there was no contamination of the PCR reactions with extraneous template that might serve as a template for PCR amplification.

### 3.2.3.3 Reaction conditions

Reactions were performed in a 50 µl volume in 0.5 ml tubes. A master reaction mix was prepared by combining five microlitres 10 x PCR buffer, 4µl of 25mM MgCl<sub>2</sub> solution and five microlitres of deoxynucleotide (dNTP) mix (1.25 mM each dNTP). The use of such a master mix minimises losses and inaccuracies associated with pipetting and ensures consistency from tube to tube. 14µl of this mix was then pipetted into each reaction tube, which contained 20pmoles of each primer and 5µl DNA template, and the volume was made up to 45 µl by the addition of dH<sub>2</sub>O. The reaction mix was then overlaid with mineral oil and the tubes were transferred to the thermal cycler. After a 5 minute denaturation incubation at 94°C, the tubes were removed and 2.5U (0.5µl) of *Taq* polymerase in 4.5 µl dH<sub>2</sub>O was added to each tube. The tubes were then returned to the thermal cycler and cycled as described below.

To isolate the full length gene sequence of the p40 subunit of feline IL-12, the thermal cycler was programmed to give 30 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1 minute) and extension (72°C for 2 minutes), followed by a 7 minute extension step at 72°C and finally by a 4°C 'soak'. PCR reaction products were visualised by polyacrylamide gel electrophoresis as detailed in 2.2.2.5.2, using five microlitres of reaction product per well. A series of optimisation experiments was carried out investigating the effect of annealing temperature on the amplification of feline IL-12 p40.

The full length gene sequence of the p35 subunit of feline IL-12 was amplified by PCR in two overlapping fragments. To amplify the 3' end of p35 the thermal cycler was programmed to give 30 cycles of denaturation (94°C for 1 minute), annealing (55°C for 1.5 minutes) and extension (72°C for 1 minute), followed by a 7 minute extension step at 72°C. A 5µl aliquot of this completed PCR reaction was then used in second round amplification, using cycling conditions as for first round amplification, except 35 cycles were performed and annealing was conducted at 60°C. PCR reaction products were visualised by polyacrylamide gel electrophoresis as detailed in 2.2.2.5.2, using five microlitres of reaction product per well. To amplify the 5' end of p35 the thermal cycler was programmed to give 30 cycles of denaturation (94°C for 1 minute),

annealing (60°C for 1.5 minutes) and extension (72°C for 1 minute), followed by a 7 minute extension step at 72°C. A 5µl aliquot of this completed PCR reaction was then used in second round amplification, using cycling conditions as for first round amplification. Five microlitres of completed reaction products were then visualised by polyacrylamide gel electrophoresis, as described previously.

### **3.2.4 PCR AMPLIFICATION OF FELINE IL-18**

#### **3.2.4.1 Primer design**

In order to amplify the full length feline IL-18 cDNA it was necessary to design oligonucleotide primers which flanked the protein coding sequence. 5' and 3' primers were designed which were based on the human, murine and especially the canine IL-18 cDNA sequences, (recently cloned in the department of Veterinary Pathology, University of Glasgow, by David Argyle and Christine M<sup>c</sup>Gillivray), (Argyle et al. 1999), as the canine and feline sequences were thought to be highly conserved. Initially, the complete coding sequence was obtained in two overlapping fragments.

To amplify the 5' end of the IL-18 gene PCR was performed using the primers

5' primer: 5'-GCAGGAATAAAGATGGCTGC-3'

3' primer: 5'-GCGTTTTGAACAGTGAACAT -3'

These primers were expected to amplify a 5' fragment of feline IL-18 cDNA of approximately 580bp nucleotides in size.

To amplify the 3' end of the IL-18 gene PCR was performed using the primers

5' primer: 5'-GACAATACGCTTTACTTTAT -3'

3' primer: 5'-GGCATGAAATTTTAATAGCTA -3'

These primers were expected to amplify a 3' fragment of feline IL-18 cDNA of approximately 540bp nucleotides in size.

Sequencing of these two overlapping fragments allowed for the design of two feline specific primers, 5' and 3', which amplified the entire coding sequence of feline IL-18 in one fragment.

5' primer: 5'-GCAGGAATAAAGATGGCTGC-3'

3' primer: 5'-GCTAATTCTTGTTTTGAACAG-3'

These primers were expected to amplify the full length feline IL-18 cDNA, approximately 595bp nucleotides in size, containing the complete protein coding sequence.

#### **3.2.4.2 Reaction conditions**

PCR reactions were set up and denatured for 5 minutes before thermal cycling, as described for feline IL-12, in section 3.2.3.3. To amplify the 5' end of IL-18 the thermal cycler was programmed to give 30 cycles of denaturation (94°C for 40 seconds), annealing (45°C for 55 seconds) and extension (72°C for 2 minutes), followed by a 7 minute extension step at 72°C. PCR reaction products were visualised by polyacrylamide gel electrophoresis as detailed in section 2.2.2.5.2, using five microlitres of reaction product per well. To amplify the 3' end of IL-18 the thermal cycler was programmed as above. However, a secondary PCR reaction, using the same cycling conditions and primers as for primary PCR, was required to amplify this fragment. PCR reaction products were visualised as previously described. Finally, PCR was performed with the feline specific primers,

5'-GCAGGAATAAAGATGGCTGC-3' and 5'-GCTAATTCTTGTTTTGAACAG-3' which amplified the entire coding sequence of feline IL-18 in one fragment. The following cycling conditions were employed; 30 cycles of denaturation (94°C for 45 seconds), annealing (58°C for 60 seconds) and extension (72°C for 2 minutes), followed by a final 7 minute extension step at 72°C and the 595bp fragment obtained was visualised using polyacrylamide gel electrophoresis.

### 3.2.5 CLONING OF FELINE IL-12 AND IL-18 PCR PRODUCTS

Products of PCR reactions using the p35 IL-12, p40 IL-12 and IL-18 primer pairs were cloned into the pCRII or pCR2.1 vectors. One microlitre of PCR product was added to 50 ng (two microlitres) vector, one microlitre 10 x ligation buffer, five microlitres dH<sub>2</sub>O and four units (one microlitre) T4 DNA ligase. The ligation reactions were incubated overnight at 14°C. The ligated vectors were then cloned into INVαF' *E.coli* cells as detailed in section 2.2.2.8. Colonies were selected on LB agar plates containing 50 µg/ml ampicillin. White colonies were picked and small scale DNA preparations made. Miniprep DNA was subjected to restriction endonuclease digestion for one hour at 37°C, using appropriate restriction enzymes to cut the cloned PCR products out of the pCRII or pCR2.1 vectors. Subsequently, products of digestion were run on 5% polyacrylamide gels and inserts of the desired size could be seen. Finally, bacterial glycerol stocks were made from the isolates that contained inserts of the appropriate size.

### 3.2.6 SEQUENCING OF FELINE IL-12 AND IL-18

DNA for cycle sequencing was prepared by the procedure detailed in section 2.2.2.2.3. Cycle sequencing reactions were carried out using the ThermoSequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP, as outlined in section 2.2.5.2. Cycling conditions were as described in section 2.2.5.2, with annealing for all reactions being performed at 55°C, except for those to cycle sequence the IL-12 p40 subunit, in which annealing was performed at 60°C. Sequencing reactions employed M13 universal IRD41 labelled primers with the following sequence:

M13 forward (-29) Primer: 5'-CACGACGTTGTAAAACGAC-3'

M13 reverse Primer: 5'-GGATAACAATTTACACAGG-3'

The reaction products were run on a Licor Model 4000 automated sequencer as described in section 2.2.5.2. The sequence data was collected and read automatically and any sequence ambiguities were checked by visual inspection of the gel image. For each cDNA, p40 IL-12, p35 IL-12 (both fragments), 3' and 5' fragments of IL-18 and full length IL-18, automated sequencing was carried out on at least six separate clones,

taken from three different PCR reactions. This was performed in order to check for the misincorporation of nucleotides, which may occur occasionally during DNA synthesis by *Taq* DNA polymerase. Using this sequence data, consensus sequences were obtained, as described in section 3.2.7., for all the cDNAs, with the exception of p40 IL-12, as described below. The consensus sequence of the feline p35 IL-12 subunit was obtained by aligning the cloned sequences of the overlapping 5' and 3' fragments and analysing the data using the GCG package.

Automated sequence analysis of the IL-12 p40 subunit clones yielded incomplete sequence data. The central portion of the p40 cDNA was found to be very GC rich, and it appeared that these GC rich areas were annealing with adjacent nucleotides, forming loops of internal secondary structure. Therefore, the thermostable DNA polymerase was unable to read through these compressions on both forward and reverse strands of the cDNA. Manual sequencing analysis was required to resolve these compressions. DNA for manual sequencing was prepared by the procedure detailed in 2.2.2.2.2. The DNA was sequenced using a Sequenase version 2.0 sequencing kit as outlined in section 2.2.5.1.1. After the acquisition of initial sequence data from the automated sequencer it was possible to design internal primers which spanned the compressed areas, allowing the complete sequence of both strands of the insert DNA to be accurately determined. Manual sequencing reactions used primers with the following sequence:

5' Primer      5'-GCTGACGGCAATCAGTACCG-3'

3' Primer      5'-AGTTTTTCGTA CTTGAGCTTG-3'

Primers were chosen to be stable at their 5' ends but less stable at their 3' ends to reduce false priming. Primers were 20 nucleotides in length and were chosen to anneal 30 - 60 bp upstream (5') to the sequence of interest. Completed sequencing reactions were run on six percent denaturing polyacrylamide gels and the gels were dried and exposed to autoradiography film as described in section 2.2.5.1.2. Finally, the sequences were read manually over a light box, and the data stored on a UNIX computer system and analysed as described below. This manual sequencing method did indeed resolve the areas of compression and a consensus sequence for the IL-12 p40 subunit was subsequently obtained.

### 3.2.7 SEQUENCE DATA ANALYSIS

Sequence data was stored and managed on a UNIX computer system using GCG software (University of Wisconsin). 'Raw' sequence data was handled and edited using the 'SeqEd' program. Consensus sequences were obtained using the 'Lineup', 'Pileup', 'Pretty' and 'Ugly', commands. The sequences of IL-12 p35, IL-12 p40 and IL-18 from other species were downloaded from the EMBL database using the 'Fetch' command. Sequence (nucleotide and amino acid) comparisons, to identify degrees of conservation between feline and other species, were performed using 'BestFit' which aligns sequences using the algorithm of Smith and Waterman. The amino acid sequences of feline IL-12 p35, IL-12 p40 and IL-18 were predicted using 'Translate'.

### 3.2.8 CREATION OF IL-12 EXPRESSION CONSTRUCTS

To create the IL-12 expression constructs the p35 and p40 subunits of feline IL-12 were cloned into the mammalian expression vector pCI-neo (Promega), as two separate plasmids. The IL-12 p40 cDNA was subcloned from the pCR2.1 vector into the pCI-neo vector as an *EcoRI* fragment. The p35 IL-12 expression construct was created by digesting the pCR2.1 (5'IL-12 p35) plasmid with *XhoI* and *RsaI*, and digesting the pCR2.1 (3'IL-12 p35) plasmid with *RsaI* and *NotI*. The digested 5' and 3' ends were subsequently gel purified and ligated into the pCI-neo vector, previously digested with *Xho I* and *Not I*. INV $\alpha$ F' *E.coli* bacteria were then transformed with the ligation reactions, colonies were picked and small scale DNA preparations were performed. The presence of inserts of the correct size was verified by subjecting a quantity of the DNA to restriction enzyme digestion with appropriate enzymes, followed by polyacrylamide gel electrophoresis. Subsequently, clones of the correct size were sequenced, as described previously, to ascertain that the nucleotide sequence was identical to that previously described. These plasmids were employed as the IL-12 adjuvant constructs in the FeLV DNA vaccination trial, described in chapter four. Due to time constraints the *in vitro* expression of these IL-12 constructs was not evaluated.





for each of the sets of IL-18 transfection flasks (cells only, no DNA) and a control transfection, performed alongside all test transfections, to assess transfection efficiency, (described in section 2.2.7.2.). Two separate flasks of 293T cells were transfected with 6µg of each construct, as mRNA was to be isolated from one flask of cells, and the cell lysates and supernatants harvested from the other flask, to assess IL-18 mRNA and protein expression, respectively, using Northern and western blot analysis. Cell lysates to be subject to Northern blot analysis were prepared as described in section 2.2.7.1., before proceeding with the isolation of mRNA, using the Quickprep mRNA purification kit (section 2.2.3.1.). Freshly isolated mRNA was stored at -70°C until required. Cell lysates to be subject to western blot analysis were resuspended in 250µl of 1X protein sample reducing buffer, scraped into 1.5 ml screw top eppendorfs, boiled for ten minutes, spun for 5 minutes at 13000rpm in a microcentrifuge, and finally the supernatants were aliquoted into fresh eppendorfs and stored at -20°C until required.

### **3.2.10.1 Identification of IL-18 mRNA by Northern Blot analysis**

In order to ascertain whether the IL-18 constructs had expressed IL-18 mRNA *in vitro*, mRNA synthesised from the transfected 293T cells was run on a 1% agarose gel containing 1 X MOPS and 2.2M formaldehyde. 3µg of polyA<sup>+</sup> mRNA from each sample was lyophilised (VR-1 Hetovac, Heto) then resuspended in 20µl of RNA loading buffer and denatured for 15 min at 65<sup>0</sup>C. Subsequently, 5µl of RNA running dye was added and the samples were electrophoresed for 3 hours at 100V. The gel was then washed and the RNA was transferred overnight onto Hybond-N in 10 X SSC and crosslinked to the membrane, as described in section 2.2.8.1. A feline IL-18 specific DNA probe was generated by PCR, using 50ng of pCR2.1(Pro-IL-18) as a template and the primers;

5' primer: 5'-GCAGGAATAAAGATGGCTGC-3'

3' primer: 5'-GCTAATTCTTGTGTTTTGAACAG-3'

The following cycling conditions were employed; 30 cycles of denaturation (94°C for 45 seconds), annealing (58°C for 60 seconds) and extension (72°C for 2 minutes).

The PCR product was subsequently gel-purified, using the QIAquick Gel Extraction kit (Qiagen), and radioactively labelled using a 'random prime' DNA labelling kit (High Prime, Boehringer Mannheim), as described in section 2.2.8.2. Standard methodologies for the hybridisation of labelled probes to membrane bound nucleic acids were used, as described in section 2.2.8.3. and hybridisation was carried out for 12 hours. High stringency washes were performed using 0.1% SSC and 0.5% SDS at 60°C and exposure to autoradiography films was performed at -70°C for three days. Subsequently, filters were stripped of the IL-18 probe and re-probed with a rat GAPDH probe to control for RNA loading and integrity. The GAPDH probe is a 750bp *EcoRI* fragment purified from the plasmid pGAPDH and was kindly supplied by Anne Terry, Molecular Oncology Laboratory, Department of Veterinary Pathology, University of Glasgow. Hybridisation and high stringency washes were performed as described for the IL-18 probe and exposure to autoradiography films was performed overnight at -70°C.

### **3.2.10.2 Identification of IL-18 protein by western blot analysis**

In order to ascertain whether the IL-18 constructs had expressed IL-18 protein *in vitro*, transfected 293T cell lysates, resuspended in 1X protein sample reducing buffer, were run on SDS-polyacrylamide gels, as described in section 2.2.6.1. 10µl of each sample and 10µl of Kaleidoscope Prestained SDS-PAGE standards (Biorad) were run on each gel. Subsequently, proteins were transferred from the gels to PVDF membrane by electroblotting, and immunodetection was performed as outlined in section 2.2.6.2., using ECL reagents (Amersham Life Science). The primary antibody used was a rabbit anti-equine IL-18 polyclonal antibody (kindly provided by Dr Lesley Nicolson and colleagues, Department of Veterinary Pathology, University of Glasgow), used at a 1:1000 dilution and the secondary antibody employed was an HRP conjugated goat anti-rabbit IgG monoclonal antibody (Sigma), used at a dilution of 1:5000. Following incubation with ECL reagents for one minute, the membranes were exposed to autoradiography film (Hyperfilm-ECL) for 30 seconds, one minute, five minutes and ten minutes, developing each film in an automated processor.

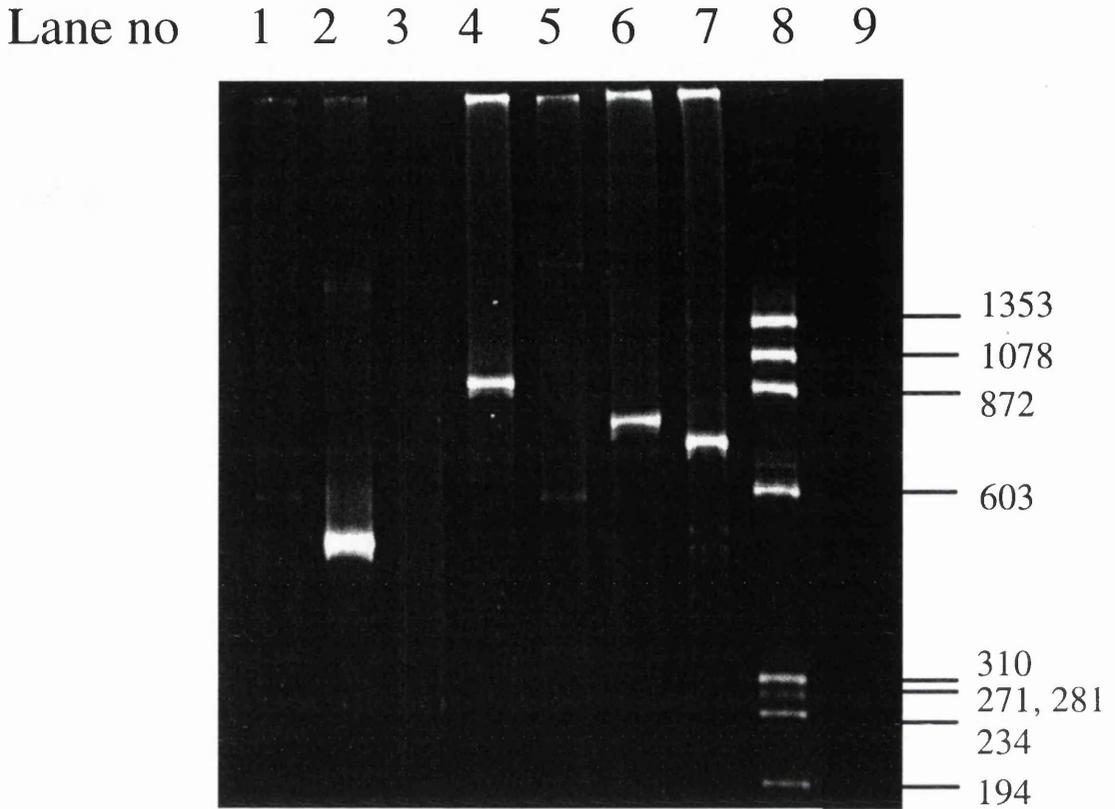
### 3.3 RESULTS

#### 3.3.1 RT-PCR AMPLIFICATION OF FELINE IL-12 AND IL-18 cDNA

The synthesis of mRNA gave yields of between six and ten micrograms per 162cm<sup>2</sup> flask and the purity of this mRNA, determined by the OD<sub>260</sub>/OD<sub>280</sub> reading, was generally very good; (OD<sub>260</sub>/OD<sub>280</sub> = 2.0). Figure 3.4 shows a polyacrylamide gel with the PCR products from the reactions used to amplify the p35 and p40 subunits of feline IL-12 and the appropriate controls. The product amplified using the p35 3' fragment primers (390bp, lane 2) is extremely strong, while the product amplified using the p35 5' fragment primers (550bp, lane 5) is very faint. Indeed, many PCR reactions, using different cycling conditions and reaction conditions, (such as varying MgCl<sub>2</sub> and primer concentrations) were performed before even this faint product was obtained (data not shown). Lanes 3 and 7 contain PCR reactions performed in an attempt to optimise the p35 5' fragment PCR; both these PCR reactions failed to generate a product of the correct size. The product amplified using the p40 IL-12 primers (lane 4) is very intense and is approximately 920bp in size, somewhat smaller than the predicted size of 1010bp. The lambda and β-actin positive control primers both generated products of the expected sizes (lane 1 500bp and lane 6 838bp, respectively), although the lambda PCR product was quite faint. Finally, no products were seen with the negative control (no DNA template, lane 9). φX174 RF DNA/Hae III fragments (size range 72-1,353 bp) were run in lane 8, to allow the approximate determination of the sizes of the PCR products.

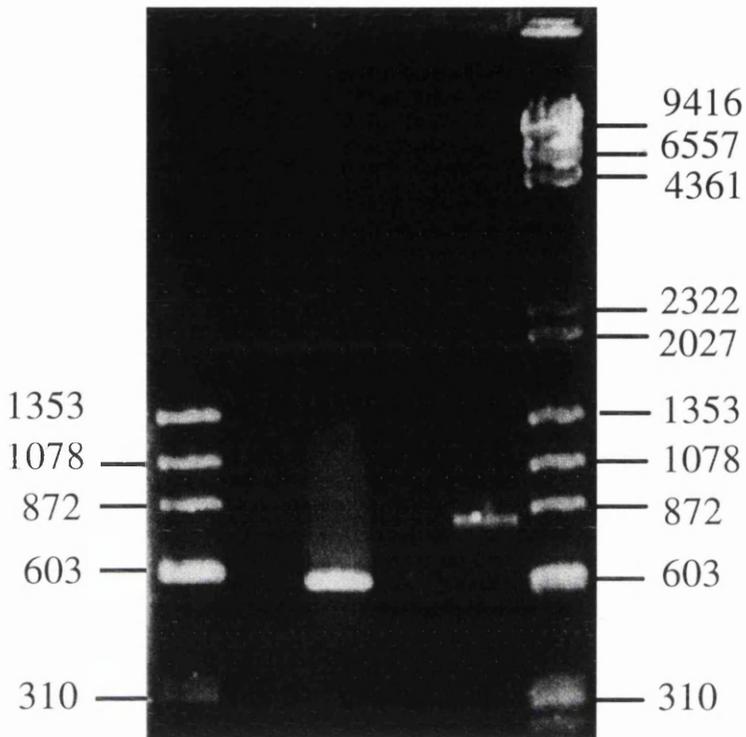
Meanwhile figure 3.5. shows a polyacrylamide gel with the PCR products from the reactions used to amplify feline IL-18 and the appropriate controls. Lanes 1 and 6 contain φX174 RF DNA/Hae III fragments (size range 72-1,353 bp), to allow the approximate determination of the sizes of the PCR products. The product amplified using the full length feline IL-18 primers (lane 3) is extremely strong, and is approximately 590bp, in good agreement with the predicted size of 595bp. The β-actin positive control primers generated a product of the expected size (lane 5

838bp), and no products were seen with the negative control (no DNA template, lane 2).



**Figure 3.4.** 5% polyacrylamide gel showing the reaction products of a typical PCR reaction used to amplify feline p35 and p40 IL-12 cDNAs and the control reactions carried out concurrently. Lane 8: molecular size markers ( $\phi$ X174 RF DNA/Hae III fragments); Lane 2: p35 3' fragment primers - feline alveolar macrophage cDNA template; Lane 5: p35 5' fragment primers - feline alveolar macrophage cDNA template; Lane 4: p40 IL-12 primers - feline alveolar macrophage cDNA template; Lane 6:  $\beta$ -actin primers - feline alveolar macrophage cDNA template; Lane 1: lambda primers - lambda DNA template; Lane 9: negative control (no added template). Lanes 3 and 7 contain PCR reactions performed in an attempt to optimise the p35 5' fragment PCR; both these PCR reactions failed to generate a product of the correct size (550bp).

Lane no    1   2   3   4   5   6



**Figure 3.5.** 5% polyacrylamide gel showing the reaction products of a typical PCR reaction used to amplify feline IL-18 cDNA and the control reactions carried out concurrently. Lane 1 and 6: molecular size markers ( $\phi$ X174 RF DNA/Hae III fragments; lane 6 also contains  $\lambda$  DNA/*Hind* III fragment markers); Lane 3: full length feline IL-18 primers - feline alveolar macrophage cDNA template; Lane 5:  $\beta$ -actin primers - feline alveolar macrophage cDNA template; Lane 2: negative control (no added template).

### 3.3.2 NUCLEOTIDE SEQUENCE OF FELINE IL-12 AND IL-18

For each cDNA, p40 IL-12, p35 IL-12 (both fragments), 3' and 5' fragments of IL-18 and full length IL-18, automated sequencing was performed on at least six separate clones, taken from three different PCR reactions. Additionally, manual sequencing analysis was required to resolve the compressions present in the p40 subunit of feline IL-12. Using this sequence data, consensus sequences were obtained, as described in section 3.2.7.; the consensus sequence of the feline p35 IL-12 subunit was obtained by aligning the cloned sequences of the overlapping 5' and 3' fragments and analysing the data using the GCG package. The consensus feline p35 IL-12, p40 IL-12 and IL-18 nucleotide sequences and deduced amino acid sequences are illustrated in figures 3.6, 3.7 and 3.8, respectively. The PCR primer sequences are underlined in each consensus sequence.

From the start to the stop codon the feline p35 IL-12 cDNA was 669bp in length and contained an open reading frame encoding a 222 amino acid predicted protein (figure 3.6.). Comparison of the full-length feline p35 IL-12 cDNA to published sequences in other species shows homology of 92.8% to canine, 89.7% to human and 70.8% to murine p35 IL-12 cDNAs. From the start to the stop codon the feline p40 IL-12 cDNA was 990bp in length and contained an open reading frame encoding a 329 amino acid predicted protein (figure 3.7.). Comparison of the full-length feline p40 IL-12 cDNA to published sequences in other species shows homology of 93.3% to canine, 87.8% to human and 76.4% to murine p40 IL-12 cDNAs.

From the start to the stop codon the feline IL-18 cDNA was 579bp in length and contained an open reading frame encoding a 192 amino acid predicted protein (figure 3.8.). The 192 amino acid protein was predicted to be the full length inactive precursor form of IL-18, pro-IL-18, cleaved by interleukin 1 $\beta$  converting enzyme, (ICE), or caspase-1, to yield the mature, active protein, 157 amino acids in length. The cleavage site was determined by comparing the feline IL-18 amino acid sequence with the human and murine sequences, illustrated in figure 3.11. Interleukin 1 $\beta$  converting enzyme (ICE, or caspase-1), an aspartic acid-specific protease, cleaves the

IL-18 molecule C-terminal to Asp<sup>36</sup> (human) or Asp<sup>35</sup> (mouse), to facilitate the removal of the pro sequence and liberate biologically active, mature IL-18. Therefore the cleavage site of interleukin 1 $\beta$  converting enzyme in the predicted feline IL-18 amino acid sequence was determined to be C-terminal to Asp<sup>35</sup>, yielding an active protein, 157 amino acids long. In figure 3.8. the nucleotides of the inactive precursor sequence are depicted in bold type. Comparison of the full-length feline IL-18 cDNA to published sequences in other species shows homology of 90% to canine, 86% to human and 74% to murine IL-18 cDNAs. The nucleotide sequence of feline IL-18 has been submitted to the Nucleotide Sequence Database, EMBL, Outstation EBI, Cambridge, England, and has been referenced under the accession number Y13923.

### **3.3.3 PREDICTED POLYPEPTIDE SEQUENCE OF FELINE IL-12 AND IL-18**

#### **3.3.3.1 Homology and predicted features of feline p35 IL-12 protein**

The predicted amino acid sequence of feline p35 IL-12 is shown in alignment with those of other species in Figure 3.9. The 222 amino acid derived protein shows identity of 90.5% to canine, 85.1% to human and 55.4% to murine homologues. Feline p35 IL-12 has five predicted *N*-glycosylation sites (NXT or NXS), at Asn, (asparagine), 42, 96, 110, 183 and 220. Proteins secreted via the endoplasmic reticulum are often glycosylated, that is modified by the addition of oligosaccharides, and the addition of these moieties generally occurs via asparagine (*N*-linked glycosylation) or may occur via serine, threonine or hydroxylysine residues (*O*-linked glycosylation). Feline p35 IL-12 also possesses eight cysteine residues, all of which are conserved in the canine, human and murine p35 IL-12 amino acid sequences, shown in figure 3.9. Cysteine residues are able to form intramolecular and intermolecular disulphide bonds, the formation of which determine the tertiary and quaternary structure of the particular protein.

### 3.3.3.2 Homology and predicted features of feline p40 IL-12 protein

The predicted amino acid sequence of feline p40 IL-12 is shown in alignment with those of other species in Figure 3.10. The 329 amino acid derived protein shows identity of 92.1% to canine, 84.2% to human and 68.1% to murine homologues. Feline p40 IL-12 has two predicted *N*-glycosylation sites (NXT or NXS), at Asn 135 and 223, and nine cysteine residues, all of which are conserved in the canine, human and murine p40 IL-12 amino acid sequences, shown in figure 3.10.

### 3.3.3.3 Homology and predicted features of feline IL-18 protein

The predicted amino acid sequence of feline IL-18 is shown in alignment with those of other species in Figure 3.11. The 192 amino acid derived full length protein shows identity of 89% to canine, 82% to human and 71% to murine homologues. The first 35 amino acids, shown in bold type in figure 3.11., indicate those belonging to the inactive precursor portion of feline IL-18, cleaved by interleukin 1 $\beta$  converting enzyme to yield the mature, active protein. The predicted amino acid sequence contains no hydrophobic signal peptide-like sites and no *N*-glycosylation sites. Four cysteine residues, three of which are conserved in the canine, human and murine IL-18 amino acid sequences, are found in the feline IL-18 predicted amino acid sequence, illustrated in figure 3.11. The IL-1 signature-like sequence, F-x(12)-F-x-S-x(6)-F-L, is also present in the feline predicted amino acid sequence, between amino acids 137 and 160, again highlighting the significant relationship between IL-18 and the IL-1 family.

5' FRAGMENT 5' PRIMER

1 ATG TGC CCG CCG CGT GGC CTC CTC CTT GTA ACC ATC CTG GTC CTG TTA AAC CAC CTG GAC 60  
1 M C P P R G L L L V T I L V L L N H L D 20

61 CAC CTC AGT TTG GCC AGG AAC CTC CCC ACA CCC ACA CCA AGC CCA GGA ATG TTC CAG TGC 120  
21 H L S L A R N L P T P T P S P G M F Q C 40

121 CTC AAC CAC TCC CAA ACC CTG CTG CGA GCC ATC AGC AAC ACG CTT CAG AAG GCC AGA CAA 180  
41 L N H S Q T L L R A I S N T L Q K A R Q 60

181 ACT CTA GAA TTT TAC TCC TGC ACT TCC GAA GAG ATT GAT CAT GAA GAT ATC ACA AAA GAT 240  
61 T L E F Y S C T S E E I D H E D I T K D 80

3' FRAGMENT 5' PRIMER

241 AAA ACC AGC ACA GTG GAG GCC TGC TTA CCA CTG GAA TTA ACC ATG AAT GAG AGT TGC CTG 300  
81 K T S T V E A C L P L E L T M N E S C L 100

301 GCT TCC AGA GAG ATC TCT CTG ATA ACT AAT GGG AGT TGC CTG GCC TCC AGA AAG ACC TCT 360  
101 A S R E I S L I T N G S C L A S R K T S 120

361 TTT ATG ACG ACC CTG TGC CTT AGC AGT ATC TAT GAG GAC TTG AAG ATG TAC CAG GTG GAG 420  
121 F M T T L C L S S I Y E D L K M Y Q V E 140

421 TTC AAG GCT ATG AAT GCA AAG CTG TTA ATG GAT CCT AAA AGG CAG ATC TTT CTG GAT CAA 480  
141 F K A M N A K L L M D P K R Q I F L D Q 160

481 AAC ATG CTG ACA GCT ATT GAT GAG CTG TTA CAG GCC CTG AAT GTC AAC AGT GTG ACT GTG 540  
161 N M L T A I D E L L Q A L N V N S V T V 180

5' FRAGMENT 3' PRIMER

541 CCA CAG AAC TCC TCC CTG GAA GAA CCA GAT TTT TAT AAA ACT AAA ATC AAG CTC TGC ATA 600  
181 P Q N S S L E E P D F Y K T K I K L C I 200

3' FRAGMENT 3' PRIMER

601 CTT CTT CAT GCT TTC AGA ATT CGT GCA GTG ACC ATC AAT AGA ATG ATG AGC TAT CTG AAT 660  
201 L L H A F R I R A V T I N R M M S Y L N 220

661 GCT TCC TAA 669  
221 A S \* 222

**Figure 3.6. Nucleotide sequence and deduced amino-acid sequence of feline p35 IL-12 cDNA. Primers used in the PCR to amplify the 5' and 3' fragments are underlined.**

```

1 ATG CAT CCT CAG CAG CTG GTC ATC GCC TGG TTT TCC CTG GTT TTG CTG GCA CCT CCC CTC 60
1 M H P Q Q L V I A W F S L V L L A P P L 20

61 ATG GCC ATA TGG GAA CTG GAG AAA AAC GTT TAT GTT GTA GAG TTG GAC TGG CAC CCT GAT 120
21 M A I W E L E K N V Y V V E L D W H P D 40

121 GCC CCC GGA GAA ATG GTG GTC CTC ACC TGC AAT ACT CCT GAA GAA GAT GAC ATC ACC TGG 180
41 A P G E M V V L T C N T P E E D D I T W 60

181 ACC TCT GAC CAG AGC AGT GAA GTC CTA GGC TCT GGT AAA ACT CTG ACC ATC CAA GTC AAA 240
61 T S D Q S S E V L G S G K T L T I Q V K 80

241 GAA TTT GCA GAT GCT GGC CAG TAT ACC TGT CAT AAA GGA GGC GAG GTT CTG AGC CAT TCG 300
81 E F A D A G Q Y T C H K G G E V L S H S 100

301 TTC CTC CTG ATA CAC AAA AAG GAA GAT GGA ATT TGG TCC ACT GAT ATC TTA AGG GAA CAG 360
101 F L L I H K K E D G I W S T D I L R E Q 120

361 AAA GAA TCC AAA AAT AAG ATC TTT CTA AAA TGT GAG GCA AAG AAT TAT TCT GGA CGT TTC 420
121 K E S K N K I F L K C E A K N Y S G R F 140

421 ACC TGC TGG TGG CTG ACG GCA ATC AGT ACC GAT TTG AAA TTC ACT GTC AAA AGC AGC AGA 480
141 T C W W L T A I S T D L K F T V K S S R 160

481 GGC TCC TCT GAC CCC CAA GGG GTG ACT TGT GGA GCA GCG ACA CTC TCA GCA GAG AAG GTC 540
161 G S S D P Q G V T C G A A T L S A E K V 180

541 AGA GTG GAC AAC AGG GAT TAT AAG AAG TAC ACA GTG GAG TGT CAG GAG GGC AGT GCC TGC 600
181 R V D N R D Y K K Y T V E C Q E G S A C 200

601 CCG GCT GCC GAG GAG AGC CTA CCC ATT GAA GTC GTG GTG GAC GCT ATT CAC AAG CTC AAG 660
201 P A A E E S L P I E V V V D A I H K L K 220

661 TAC GAA AAC TAC ACC AGC AGC TTC TTC ATC AGG GAC ATC ATC AAA CCG GAC CCA CCC AAG 720
221 Y E N Y T S S F F I R D I I K P D P P K 240

721 AAC CTG CAA CTG AAG CCA TTA AAA AAT TCT CGG CAT GTG GAA GTG AGC TGG GAA TAC CCT 780
241 N L Q L K P L K N S R H V E V S W E Y P 260

781 GAC ACC TGG AGC ACC CCA CAT TCC TAC TTC TCC TTA ACA TTT GGC GTA CAG GTC CAG GGC 840
261 D T W S T P H S Y F S L T F G V Q V Q G 280

841 AAG AAC AAC AGA GAA AAG AAA GAC AGA CTC TCC GTG GAC AAG ACC TCA GCC AAG GTC GTG 900
281 K N N R E K K D R L S V D K T S A K V V 300

901 TGC CAC AAG GAT GCC AAG ATC CGC GTG CAA GCC AGA GAC CGC TAC TAT AGC TCA TCC TGG 960
301 C H K D A K I R V Q A R D R Y Y S S S W 320

961 AGC AAC TGG GCA TCC GTG TCC TGC AGT TAG 990
321 S N W A S V S C S * 329

```

**Figure 3.7. Nucleotide sequence and deduced amino-acid sequence of feline p40 IL-12 cDNA.** 5' Primer used in the PCR to amplify the p40 subunit is underlined; 3' primer is outwith the 3' coding sequence.

**1** ATG GCT GCT **ATA CCA GTA GAT GAT TGC ATC AAC TTT GTG GGA ATG AAA TTT ATT GAC AAT** 60  
**1** **M A A I P V D D C I N F V G M K F I D N** 20  
  
**61** **ACA CTT TAC TTT GTA GCT GAC AGT GAT GAA AAC CTG GAA ACA GAT**↓**TAC TTT GGC AAG CTT** 120  
**21** **T L Y F V A D S D E N L E T D Y F G K L** 40  
  
**121** **GAA CAT AAA CTC TCA ATC TTA CGA AAC TTG AAC GAC CAA GTT CTC TTC ATT AAC CAG GGA** 180  
**41** **E H K L S I L R N L N D Q V L F I N Q G** 60  
  
**181** **GAT CAA CCT GTG TTT GAG GAT ATG CCT GAT TCT GAC TGT ACA GAT AAT GCA CCC CGG ACT** 240  
**61** **D Q P V F E D M P D S D C T D N A P R T** 80  
  
**241** **GAA TTT ATC ATA TAT ATG TAT AAA GAT AGC CTC ACT AGA GGT CTG GCA GTA ACC ATC TCT** 300  
**81** **E F I I Y M Y K D S L T R G L A V T I S** 100  
  
**301** **GTG AAT TAT AAG ACC ATG TCT ACT CTC TCC TGT GAG AAC AAA ATT ATT TCC TTT AAG GAA** 360  
**101** **V N Y K T M S T L S C E N K I I S F K E** 120  
  
**361** **ATG AGT CCT CCT GAG AGT ATC AAT GAT GAA GGA AAT GAC ATC ATA TTC TTT CAG AGA AGT** 420  
**121** **M S P P E S I N D E G N D I I F F Q R S** 140  
  
**421** **GTT CCA GGA CAT GAT GAT AAG ATA CAA TTT GAG TCT TCA TTG TAC AAG GGG TAC TTT CTA** 480  
**141** **V P G H D D K I Q F E S S L Y K G Y F L** 160  
  
**481** **GCT TGT GAA AAA GAG AAA GAT CTT TTC AAA CTC ATT TTG AAA AAA AAG GAT GAA AAT GGG** 540  
**161** **A C E K E K D L F K L I L K K K D E N G** 180  
  
**541** **GAT AAG TCC ATA ATG TTC ACT GTT CAA AAC AAG AAT TAG** 579  
**181** **D K S I M F T V Q N K N \* 192**

**Figure 3.8. Nucleotide sequence and deduced amino-acid sequence of full length feline IL-18 cDNA.** The nucleotides of the inactive precursor sequence are depicted in bold type and ↓ indicates the interleukin 1 $\beta$  converting enzyme, (ICE), or caspase-1, cleavage site. Primers used in the PCR to amplify full length IL-18 are underlined.

```

      •
1  MCPPRGLLLV  TILVLLNHL D HL SLARNLPT  PTPSPGMFQC  LNHSQTL LRA  50
1  -----S---  --TW--S---  AS-----I---  -----N----  50
1  ---A-S----  AT---...--  -----V  A--D----P-  -H---N-----  47
1  --QS-Y--FL  AT-A...-N  -----VI-V  SG-A...R-  -SQ-RN--KT  43

      •
51 ISNTLQKARQ  TLEFYSCTSE  EIDHEDITKD  KTSTVEACL P  LELTMNESCL  100
51 V-----  --DYIP----  -----  -----  -----  100
48 V--M-----  -----P----  -----  -----  ----K-----  97
44 TDDMVKT--E  K-KH----A-  D-----R-  Q---LKT---  ---HK-----  93

      N XS•
101 ASREISLITN  GSCLASRKTS  FMTTLCLSSI  YEDLKMYQVE  FKAMNAKLLM  150
101 -----  -----G-A-  ---V-----  -----M-  -----  150
98 N---T-F---  -----  --MA-----  -----  --T-----  147
94 -T--T-ST-R  ----PPQ---  L-M---G--  -----T-  -Q-I--A-QN  143

      NXS
151 DPKRQIFLDQ  NMLTAIDELL  QALNVNSVTV  PQNSSLEEPD  FYKTKIKLCI  200
151 -----  -----  ----F-----  --K-----  -----  200
148 -----  ---AV---M  ----F--E--  --K-----  -----  197
144 HNHQ--I--K  G--V-----M  -S--H-GE-L  R-KPPVG-A-  P-RV-M----  193

      N XS
201 LLHAFRIRAV  TINRMMSYLN  AS 222 FELINE
201 -----  --D-----  S- 222 CANINE
198 -----  --D-VT----  -- 219 HUMAN
194 -----ST-V-  ----V-G--S  SA 215 MURINE

```

**Figure 3.9. Alignment of the amino-acid sequences of feline, canine, human and murine p35 interleukin 12 polypeptides.** Identical amino-acids are indicated by dashes (-) and dots indicate gaps introduced to maintain optimal sequence alignment. Cysteine residues are depicted by • and potential N-glycosylation sites by NXS.

```

1 MHPQQLVIAW FSLVLLAPPL MAIWELEKNV YVVELDWHPD APGEMVVLTC 50
1 -----S- -----SS- -T-----D- ----- 50
1 -CH-----S- -----F--S-- V-----K-D- -----Y-- ----- 50
1 -C--K-T-S- -AI--VS-- --M-----D- -----V--T-- ----T-N-- 50

51 NTPEEDDITW TSDQSSEVLG SGKTLTIQVK EFADAGQYTC HKGGEVLSHS 100
51 H-----A----- ----- --G----- ----K--R- 100
51 D-----G--- -L----- ----- --G----- ----- 100
51 D----- --RHG-I- -----T-- --L----- ----T---- 100

• NXS •
101 FLLIHKKEDG IWSTDILREQ KESKNKIFLK CEAKNYSGRF TCWWLTAIST 150
101 L-----K-- ----- --G----- ----- 150
101 L--L----- -----KD- --P--T--R -----T-- 150
101 H--L----N- ----E--... -NF--T-- --P----- --S--VQRNM 147

151 DLKFTVKSSR GSSDPQGVTC GAATLSAEKV RVDNRDYKKY TVECQEGSAC 200
151 ----S----- -F----- --V-----R- ----- 200
151 --T-S----- -----R- -G--KE-.E- S-----D-- 199
148 ----NI---S S-P-SRA--- -M-S----- TL-Q---E-- S-S---DVT- 197

NXT
201 PAAEESLPIE VVVDAIHKLK YENYTSFFFI RDIKPDPPK NLQLKPLKNS 250
201 -S----- ----- -----T----- 250
200 -----M--V--- ----- ----- 249
198 -T--T---- LALE-RQON- ----ST---- -----M----- 247

251 RHVEVSWEYP DTWSTPHSYF SLTFGVQVQG KNNREK... ..KDRLSV 292
251 ----- -----C--A-- -----C- 292
250 -Q----- -----C----- -SK----- -VFT 291
248 .Q----- -S----- --K-F-RI-R -KEKM-ETEE GCNQ-GAFL- 296

293 DKTSAKVVCH KDAKIRVQAR DRYYSSWSN WASVSCS 329 FELINE
293 ----- -----D----- 329 CANINE
292 -----T-I-R -N-S-S-R-Q -----E ----P-- 328 HUMAN
297 E---TE-Q-. -GGNVC---Q ----N--C-K --C-P-RVRS 335 MURINE

```

**Figure 3.10. Alignment of the amino-acid sequences of feline, canine, human and murine p40 interleukin 12 polypeptides.** Identical amino-acids are indicated by dashes (-) and dots indicate gaps introduced to maintain optimal sequence alignment. Conserved cysteine residues are depicted by • and potential N-glycosylation sites by NXS or NXT.

```

      •
1  MAA. IPVDDC  INFVGMKFID  NTLYFVADSD  ENLETDYFGK  LEHKLSILRN  49
1  ---NLIE-N-  --L-K---VN  -----K-E--  -G--S-----  --P----I--  50
1  ---E.-E-N-  -S--E-----N  -----EN-  -D--S-----  --P----I--  49
1  ---EPVE-N-  ----A-----  -----I-ED-  ----S-----  --S---VI--  50
1  ----MSE-S-  V--KE-M---  -----IPEEN  GD--S-NF-R  -HCTTAVI--  49

      •
50  LNDQVLFINQ  GDQPVFEDMP  DSDCTDNAPR  TEFIIYMYKD  SLTRGLAVTI  99
51  -----V-E  -N-----  -----H  -I-----  -----  100
50  -----  -H-A-----  ----S---Q  -V-----  -----  99
51  -----D-  -NR-L----T  ----R-----  -I---S----  -QP--M----  100
50  I-----VDK  .R-----T  -I-QSASE-Q  -RL-----  -EV-----L  98

      •
100  SVNYKTMSTL  SCENKIIISFK  EMSPPEISIND  EGNDIIFQQR  SVPGHDDKIQ  149
101  --K-----  --K--T---Q  K----D-----  -----  -----  150
100  --QC-K----  --K--TL---  -----DN-D-  -----  -----  149
101  --KCEKI---  -----  --N--DN-K-  TKS-----  -----N-M-  150
99  --KDSK----  --K-----E  --D--N-D-  IQS-L---K  -V---.N-ME  147

      •
150  FESSLYKGYF  LACEKEKDLF  KLILKKKDEN  GDKSIMFTVQ  NKN  192  FELINE
151  -----H-  ---K--N---  -----D---  -----  --S  193  CANINE
150  -----  ---K--N---  -----E---C  -----  ---  192  PORCINE
151  ----S-E---  -----R---  -----E--L  --R-----  -ED  193  HUMAN
148  -----E-H-  ---Q--D-A-  -----  ----V---LT  -LHQ  192  MURINE

```

**Figure 3.11. Alignment of the amino-acid sequences of feline, canine, porcine, human and murine interleukin 18 polypeptides.** Identical amino-acids are indicated by dashes (-) and dots indicate gaps introduced to maintain optimal sequence alignment. Conserved cysteine residues are depicted by • and the IL-1 signature-like sequence F-x(12)-F-x-S-x(6)-F-L is underlined. The amino acids shown in bold type indicate those belonging to the inactive precursor portion of full length feline IL-18. The predicted amino acid sequence contained no hydrophobic signal peptide-like sites and no N-glycosylation sites.

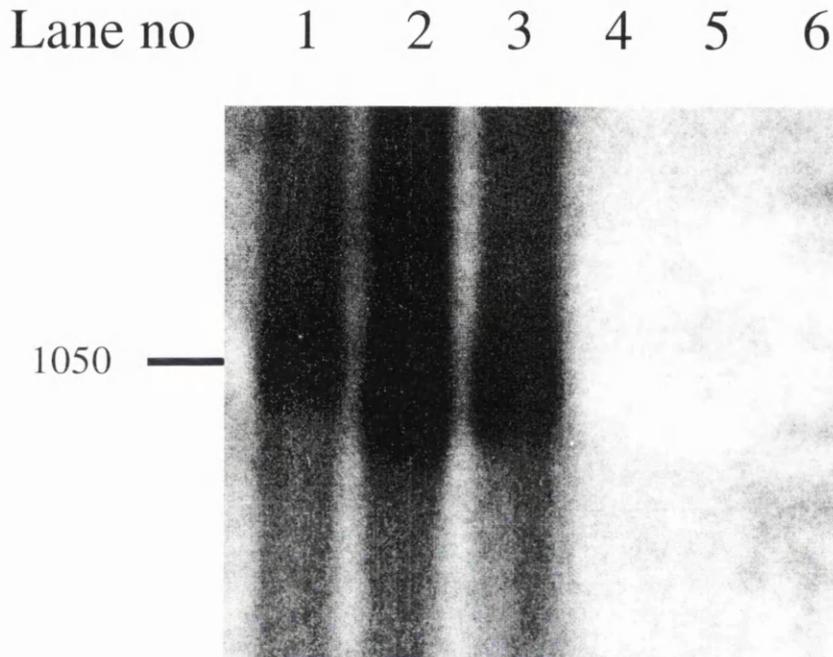
### **3.3.4 IDENTIFICATION OF *IN VITRO* EXPRESSION OF FELINE IL-18**

Feline IL-18 mRNA and protein expression were evaluated by performing Northern and western blot analysis, respectively, on 293T cell lysates. These 293T cells had been previously transfected with the IL-18 expression constructs. Six micrograms of each of the plasmids were co-transfected into 293T cells and transfection efficiency was monitored using a  $\beta$ -galactosidase construct control and was found to be in the order of 50%.

#### **3.3.4.1 Identification of IL-18 mRNA expression by Northern Blot analysis**

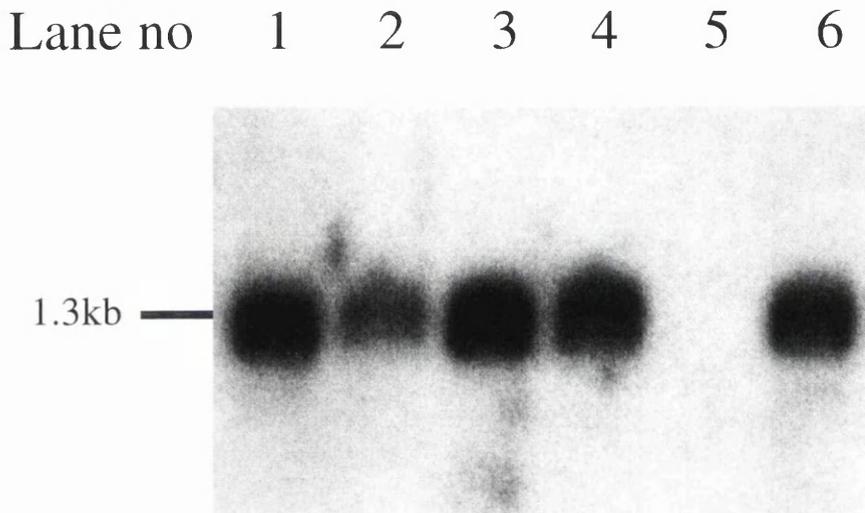
Northern blot analysis, using a feline IL-18 specific probe to assess *in vitro* mRNA expression of the constructs pCI-neo (pro-IL-18), pCI-neo (mature-IL-18) and PsecI (IL-18), revealed the presence of specific bands at approximately, 1050bp, 950bp and 1000bp, respectively, illustrated in figure 3.12. This was in good agreement with the expected sizes of pCI-neo derived pro-IL-18 mRNA transcripts, 1040bp; pCI-neo derived mature IL-18 mRNA transcripts, 930bp; and PsecI derived mature IL-18 mRNA transcripts, 980bp. Moreover, the IL-18 specific probe did not hybridise with mRNA made from the control transfection reactions; 293T cells transfected with pCI-neo only, and non-transfected 293T cells (no DNA). Therefore, it appeared that the feline IL-18 probe employed in this hybridisation technique specifically detected feline IL-18 mRNA and, therefore, that all three forms of feline IL-18 mRNA were expressed *in vitro* in transfected mammalian cells. Additionally, to control for RNA loading and integrity, filters were stripped of the IL-18 probe and re-probed with a rat GAPDH probe. This probe, a 750bp *EcoRI* fragment purified from the plasmid pGAPDH, hybridises to a 1.3kb GAPDH mRNA transcript, expressed at high levels in all cell types. Hybridisation with this probe revealed the presence of specific bands at 1.3kb, of approximately the same intensity, in each of the five lanes of mRNA; pCI-neo (pro-IL-18), pCI-neo (mature-IL-18), PsecI (IL-18), pCI-neo alone and non-transfected 293T cell mRNA, illustrated in figure 3.13. This indicated that all the

mRNAs were of good quality and integrity, and that similar amounts of mRNA had been loaded into each well of the 1% agarose, MOPS and formaldehyde gel.



**Figure 3.12.** An autoradiograph of a northern blot, after hybridisation with a feline IL-18 specific probe to assess *in vitro* mRNA expression of the constructs pCI-neo (pro-IL-18), lane 1; pCI-neo (mature-IL-18), lane 2; Psecl (IL-18), lane 3; pCI-neo only, lane 4 and non-transfected 293T cells, lane 6. The presence of specific bands at approximately, 1050bp, 950bp and 1000bp, in lanes 1, 2 and 3 respectively, was in good agreement with the expected sizes of the pCI-neo derived pro-IL-18 mRNA transcripts, 1040bp; pCI-neo derived mature IL-18 mRNA transcripts, 930bp; and Psecl derived mature IL-18 mRNA transcripts, 980bp. Lanes 4 and 6 are blank, indicating that the IL-18 specific probe did not hybridise with mRNA made from the negative control transfection reactions; 293T cells transfected with pCI-neo only, and non-transfected 293T cells (no DNA).

N.B. Due to the scale of this diagram only one RNA size marker (1050bp) is in the visible field.

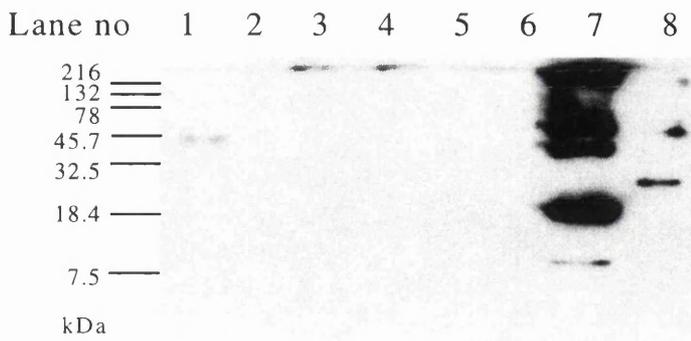


**Figure 3.13.** An autoradiograph of the northern blot illustrated in figure 3.12, after stripping and reprobing with a rat GAPDH probe, in order to assess *in vitro* expression of the GAPDH mRNA transcript. The GAPDH probe hybridised to a 1.3kb GAPDH mRNA transcript, expressed at high levels in all cell types. Hybridisation with this probe revealed the presence of specific bands at 1.3kb, of approximately the same intensity, in each of the five lanes of mRNA; pCI-neo (pro-IL-18), lane 1; pCI-neo (mature-IL-18), lane 2; PsecI (IL-18), lane 3; pCI-neo alone, lane 4 and non-transfected 293T cell mRNA, lane 6. This indicated that all the mRNAs were of good quality and integrity, and that similar amounts of mRNA had been loaded onto the gel.

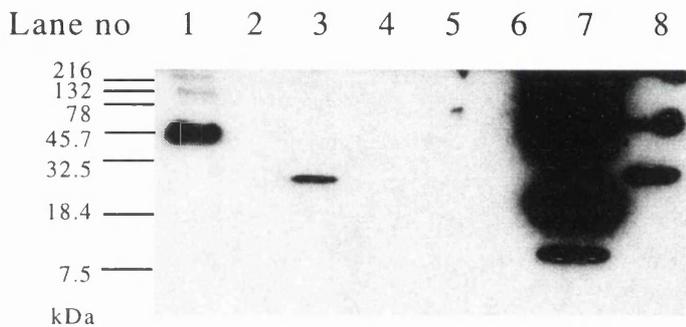
N.B. Due to the scale of this diagram only one RNA size marker (1300bp) is in the visible field.

### 3.3.4.2 Identification of IL-18 protein expression by western blot analysis

Western blot analysis and immunodetection, using a rabbit anti-equine IL-18 polyclonal antibody to assess *in vitro* protein expression of the pCI-neo (pro-IL-18), pCI-neo (mature-IL-18) and PsecI (IL-18) constructs, demonstrated the presence of a single 24kDa species, the predicted size of the feline pro-IL-18 protein, (illustrated in figure 3.14). However, specific signals were only observed in the lanes which contained the cell lysates from the 293T cells transfected with the feline pCI-neo (pro-IL-18) construct (lane 3), and the two positive controls, 1 $\mu$ g of recombinant equine mature and pro-IL-18 proteins, (lanes 7 and 8, respectively). The feline and equine pro-IL-18 proteins were estimated to be approximately 24kDa, while the equine mature IL-18 protein was estimated to be approximately 18kDa. No signal was observed in the lanes containing the cell lysates from the 293T cells transfected with pCI-neo only (lane 2) and cell lysates from non-transfected cells, (lane 6). Moreover, no signal was observed in the lanes containing the cell lysates from the 293T cells transfected with the feline pCI-neo (mature-IL-18) and PsecI (IL-18) constructs (lanes 4 and 5, respectively). Therefore, it appeared that either the rabbit anti-equine IL-18 polyclonal antibody did not recognise the mature form of the feline IL-18 protein (18kDa), or that the pCI-neo (mature-IL-18) and PsecI (IL-18) constructs did not express IL-18 protein in mammalian cells *in vitro*. However, the signal in the lane containing the cell lysates from 293T cells transfected with the feline pCI-neo (pro-IL-18) construct was strong and specific, indicating that feline pro-IL-18 protein was expressed in mammalian cells *in vitro*. Taken together, these experiments confirmed that feline IL-18 mRNA and protein were expressed *in vitro* in transfected mammalian cells.



Blot 1; 30 second exposure



Blot 1; 2 minute exposure

**Figure 3.14.** An autoradiograph of a western blot after immunodetection using a rabbit anti-equine IL-18 polyclonal antibody, to assess *in vitro* protein expression of the pCI-neo (pro-IL-18), pCI-neo (mature-IL-18) and PsecI (IL-18) constructs; lanes 3, 4 and 5, respectively. A single 24kDa species, the predicted size of the feline pro-IL-18 protein, was identified in lane 3, while no signals were detected in the lanes containing the cell lysates from 293T cells transfected with pCI-neo only, (lane 2), feline pCI-neo (mature-IL-18), (lane 4), PsecI (IL-18), (lane 5), or cell lysates from non-transfected cells (lane 6). However, specific signals of 18kDa and 24kDa were detected in the lanes which contained recombinant equine mature and pro-IL-18 proteins, (lanes 7 and 8, respectively), the predicted sizes of the equine IL-18 proteins. Two different exposures of the blot are illustrated; 30 seconds, where the positive control signals were detected, (lanes 7 and 8), but the pro-IL-18 transfection signal was not, (lane 3), and two minutes, where the 24kDa species was detected in lane 3, but the positive control signals were much too intense. Kaleidoscope Prestained SDS-PAGE standards, to allow the approximate estimation of protein size, are visible in lane 1.

## **3.4 DISCUSSION**

### **3.4.1 AMPLIFICATION OF FELINE IL-12 AND IL-18 BY PCR**

The polymerase chain reaction provides an attractive method of isolating novel genes, in cases where there is limited information regarding nucleotide sequence. Its utilisation in this project enabled the isolation of three feline Th1 type cytokine genes, p35 IL-12, p40 IL-12 and IL-18, without having to generate and screen a cDNA library, a time consuming and technically more difficult process. However, the use of PCR is not without potential disadvantages. The most significant of these disadvantages is the total failure to amplify the desired cDNA. This may occur despite attempts to optimise the PCR by varying conditions, including primer annealing temperature, primer concentration, number of PCR cycles and reaction conditions, such as  $Mg^{2+}$  concentration. A number of potential causes of PCR failure can be considered. Firstly, the mRNA of interest may simply not be present, or may be degraded, in the starting material. The correct choice of starting cells or tissue, additional mitogen stimulation and timing of mRNA harvest are absolutely essential in order to achieve optimal levels of mRNA expression of the gene of interest. Alternatively, there may be a failure of reverse transcription, in synthesising full length cDNA from mRNA, either due to the reaction conditions or because of secondary structure present in the mRNA. Treatment of mRNA with methylmercury hydroxide may help to remove secondary structure, in certain circumstances.

Another important consideration when attempting the isolation of cDNAs where the exact sequence is unknown, is the design of the oligonucleotide primers, for use in the PCR amplification. If there are mismatches between the primer and cDNA sequences, the primers may not anneal efficiently to the cDNA. In such circumstances, the design of alternative primers or the use of degenerate primers may prove successful. Another potential outcome of non-specific primer binding may be the generation of multiple PCR products, seen as multiple bands or smears on

polyacrylamide gels, or poor yield of specific product. The use of 'hot-start' protocols (e.g. using anti *Taq* antibody or wax beads) may improve the yield of a specific product by reducing the extension of primers bound non-specifically.

The full length feline p35 IL-12 cDNA sequence could not be amplified with any combination of primers or reaction conditions and, indeed, the full length sequence was only obtained when primers were designed to generate two overlapping fragments of the cDNA. In contrast, the p40 IL-12 cDNA was amplified by PCR with comparative ease. As discussed in section 3.1.2.4., stimulated PBMCs expressed p40 mRNA at a much higher level than p35 mRNA; the abundance of p40 transcriptions was up to 200 fold higher than that of the p35 transcripts (Cassatella et al. 1995). Therefore, the low levels of p35 mRNA compared to p40 mRNA, may help to explain the difficulties encountered in amplifying the former cytokine by PCR.

Furthermore, if isolation by RT-PCR is successful, another inherent disadvantage which must be examined is the misincorporation of nucleotides during DNA synthesis by *Taq* DNA polymerase. The most common error is that of single base substitutions, which generally occur at a low frequency (around one base substitution per  $10^6$  nucleotides). This error rate, however, may be increased when higher than optimal nucleotide and magnesium concentrations are employed, and may approach one per thousand nucleotides in unfavourable conditions. The nucleotide differences seen between different clones of p35 IL-12, p40 IL-12 and IL-18, were attributed to such errors. The sequencing of a number of cDNA clones, from different RT-PCR reactions enabled a consensus sequence to be determined with a relatively high degree of confidence. It has been suggested that sequencing of between three to six clones will generally suffice in the determination of a consensus sequence at a given allele (Ennis et al. 1996). The use of thermostable DNA polymerases which possess proof reading activity (e.g. *Pfu* polymerase or *Vent* polymerase) may result in an increase in fidelity over that seen with *Taq* polymerase. Therefore, the use of these alternative enzymes should be considered in future projects.

### **3.4.2 FEATURES OF THE PREDICTED FELINE p35 IL-12, p40 IL-12 AND IL-18 PROTEINS**

Feline p35 IL-12, p40 IL-12 and IL-18 proteins share a high degree of homology with the respective cytokines from other mammalian species; between 70 and 90% at the nucleotide level and between 55 and 90% at the amino acid level. Canine cDNA and amino acid sequences display the greatest degree of conservation with the feline sequences, and murine the least. This evolutionary conservation suggests a similar biological role for these Th1 type cytokines in the cat to that described for other species, such as mice and humans.

#### **3.4.2.1 Feline p35 IL-12 protein**

The p35 subunit of feline IL-12 is predicted to be a 222 amino acid derived protein, possessing identities of 90.5%, 85.15 and 55.4% to the canine, human and murine homologues, respectively. The predicted feline p35 amino acid sequence is the same size as the canine homologue, three amino acids longer than the human homologue and seven amino acids longer than the murine homologue. Feline p35 IL-12 has five predicted *N*-glycosylation sites (NXT or NXS), at Asn, (asparagine), 42, 96, 110, 183 and 220, and eight cysteine residues, the latter of which are conserved in the canine, human and murine p35 IL-12 amino acid sequences. These cysteine residues may be important in determining the tertiary and quaternary structure of the p35 protein and IL-12 heterodimer.

The human p35 protein possesses a hydrophobic signal-like sequence, 22 amino acids in length, yielding a mature protein composed of 197 amino acids (Podlaski et al. 1992). A signal peptide acts to direct ribosomal protein synthesis to the endoplasmic reticulum; the signal sequence is then removed on the luminal side of the endoplasmic reticulum. Signal sequences typically have an N-terminal basic region, a hydrophobic core and a polar C-terminal region followed by a proteolytic cleavage site (von Heijne, 1983). Interestingly the predicted feline p35 IL-12 protein may possess a similar signal sequence to the human homologue; 18 of the first 25

amino acids in the feline sequence are hydrophobic in nature ( the amino acids 16, 17 and 18 in the feline sequence are deleted in the human sequence).

### **3.4.2.2 Feline p40 IL-12 protein**

The feline p40 IL-12 subunit is predicted to be a 329 amino acid derived protein, possessing identities of 92.1%, 84.2% and 68.1% to the canine, human and murine homologues, respectively. The predicted feline p40 amino acid sequence is the same size as the canine homologue, one amino acid longer than the human homologue, and six amino acids shorter than the murine homologue. The feline p40 IL-12 protein has two predicted *N*-glycosylation sites (NXT or NXS), at Asn 135 and 223, and nine cysteine residues, all of which are conserved in the canine, human and murine p40 IL-12 amino acid sequences. Similar to the p35 subunit protein, these cysteine residues may be important in determining the tertiary and quaternary structure of the p40 protein and the IL-12 heterodimer. The human p40 protein possesses a hydrophobic signal-like sequence, 22 amino acids in length, yielding a mature protein composed of 306 amino acids (Trinchieri, 1994). Interestingly, the predicted feline p40 IL-12 protein may possess a similar signal sequence to the human homologue; 18 of the first 22 amino acids in the feline p40 sequence are hydrophobic in nature and the feline p40 signal-like sequence also possesses an N-terminal basic region, (the second residue is histidine, a basic amino acid).

While experimental work to isolate the feline IL-12 gene was ongoing, two independent research groups cloned and sequenced the feline p35 and p40 IL-12 cDNAs (Schijns et al. 1997), (Fehr et al. 1997). Both p40 nucleotide sequences were submitted to the Nucleotide Sequence Database, EMBL, and were referenced under the accession numbers Y07762 (Schijns) and U83184 (Fehr). Interestingly, differences between these published sequences and the feline p40 nucleic acid sequence obtained during the course of this project were discovered.

Three individual base pair changes were discovered when comparing the p40 cDNA sequence illustrated in this thesis (figure 3.7.) with the published p40 cDNA sequence Y07762; at positions 16, 31 and 500. The nucleotide change at position 16

was from a C to a T (Y07762). However, this nucleotide change did not initiate an amino acid change; the amino acid at position 6 was leucine in both polypeptide sequences. The nucleotide change at position 31 was from a T to a C (Y07762) and this nucleotide change did initiate an amino acid change at position 11; from phenylalanine, a neutral, very hydrophobic amino acid, to leucine, also a neutral, hydrophobic amino acid (Y07762). Therefore, although an amino acid change was evident at position 11, both amino acids possessed similar properties and this substitution was predicted to have little effect on the structure or function of the p40 protein. Moreover, the amino acid at position 11 in the canine, human and murine p40 amino acid sequences was also found to be phenylalanine.

The nucleotide change at position 500 was from a G to an A (Y07762) and this nucleotide change did initiate an amino acid change at position 167; from glycine, a neutral, hydrophobic amino acid, to glutamic acid, an acidic, hydrophilic amino acid (Y07762). These two amino acids obviously possessed very different properties. Significantly, the amino acid at position 167 in the canine and human p40 amino acid sequences was also found to be glycine, conserved with the author's p40 sequence. Interestingly, this area of the p40 nucleotide sequence was very GC rich and additional manual sequencing was required to resolve compressed loops of secondary structure; automated sequence analysis alone was not effective. Although the amino acid substitution at position 167 may reflect allelic variation between different gene pools of cats, the evidence above may imply that Schijns and colleagues did not effectively sequence through the compressed areas of the p40 cDNA and therefore did not obtain the correct feline p40 IL-12 cDNA sequence.

Four individual base pair changes were discovered when comparing the p40 cDNA sequence illustrated in this thesis with the published p40 cDNA sequence U83184; at positions 150, 151, 153 and 156. Significantly, the author's p40 cDNA sequence and the sequence U83184 were conserved at positions 16, 31 and 500. The nucleotide change at position 150 was from a C to a T (U83184). However, this nucleotide change did not initiate an amino acid change; the amino acid at position 50 was cysteine in both the published (U83184) and the author's p40 polypeptide sequences. The nucleotide changes at position 151 and 153 were from an A to a G (U83184) and

a T to a C (U83184), respectively. These nucleotide changes did initiate an amino acid change at position 51; from asparagine, a neutral, hydrophilic amino acid, to aspartic acid, an acidic hydrophilic amino acid (U83184). Therefore, although an amino acid change was evident at position 51, both amino acids possessed similar properties and this substitution was predicted to have little effect on the structure or function of the p40 protein. Interestingly, the amino acid at position 51 in the canine p40 amino acid sequence was found to be histidine, a basic hydrophilic amino acid; while the amino acid at position 51 in the human and murine p40 amino acid sequence was found to be aspartic acid, conserved with the published p40 amino acid sequence U83184.

Finally, the nucleotide change at position 156 was from a T to a G (U83184). However, this nucleotide change did not initiate an amino acid change; the amino acid at position 52 was threonine in both the published (U83184) and the author's p40 polypeptide sequences. It is likely that the majority of these amino acid substitutions reflect allelic variation between different gene pools of cats. It should be noted that two of the observed base pair changes between the author's p40 sequence and the published p40 sequence Y07762 are found in the hydrophobic signal-like sequence. The signal-like sequence is probably only necessary for the transport of the protein out of cells; it is cleaved from the mature form of the p40 protein and is therefore not important in terms of the structure or biological activity of the active protein. Therefore, there is perhaps less evolutionary pressure for the signal-like amino acid sequence to remain conserved.

### **3.4.2.3 Feline IL-18 protein**

The full length feline IL-18 species is predicted to be a 192 amino acid protein, possessing identities of 89% to canine, 82% to human and 71% to murine homologues. The predicted feline IL-18 amino acid sequence is one amino acid shorter than the canine and human homologues and the same size as the porcine and murine homologues. Four cysteine residues, three of which are conserved in the canine, human and murine IL-18 amino acid sequences, are found in the feline IL-18 predicted amino acid sequence. Interestingly, the size of the recombinant human IL-

18 protein did not change after treatment with the reducing agent DTT (Ushio et al. 1996). This suggested that no disulphide bonds existed in the protein structure, although the human IL-18 protein contains four cysteine residues. Similar to the human and murine sequences (Ushio et al. 1996), the feline IL-18 protein has no predicted *N*-glycosylation sites. This is in contrast to the canine IL-18 predicted amino acid sequence, which contains three predicted *N*-glycosylation sites (Argyle et al. 1999), (Okano et al. 1999). Significantly, the IL-1 signature-like sequence, F-x(12)-F-x-S-x(6)-F-L, identified in the human, murine and canine IL-18 amino acid sequences (Ushio et al. 1996), (Argyle et al. 1999), (Okano et al. 1999), is also present in the feline IL-18 predicted amino acid sequence, between amino acids 137 and 160. Again, this highlights the significant relationship between IL-18 and the IL-1 family.

The predicted feline IL-18 amino acid sequence contains no hydrophobic signal peptide-like sites, similar to the human, murine and canine IL-18 amino acid sequences (Ushio et al. 1996), (Argyle et al. 1999), (Okano et al. 1999). As discussed in the introduction, Gu *et al* have demonstrated that the activation of human IL-18 occurs via the cleavage of the inactive leader sequence by the enzyme interleukin 1 $\beta$  converting enzyme (ICE, or caspase-1) (Gu et al. 1997). The N-terminus of the human mature protein occurs after Asp<sup>36</sup> (Asp<sup>35</sup> in the murine sequence) and it has been demonstrated that ICE, an aspartic acid-specific protease, cleaves the human IL-18 precursor molecule C-terminal to Asp<sup>36</sup> (human) or Asp<sup>35</sup> (mouse). This facilitates the removal of the inactive pro sequence and liberates the biologically active, mature IL-18 protein. The similarity between the feline and the human and murine full length IL-18 amino acid sequences, regarding the lack of a conventional hydrophobic signal sequence, suggests that the cellular handling of full length feline IL-18 may occur in a similar fashion to the human and murine homologues. The first 35 amino acids, shown in bold type in figure 3.11., indicate those predicted to belong to the inactive precursor portion of feline IL-18. ICE may cleave the feline IL-18 precursor molecule C-terminal to Asp<sup>35</sup> to facilitate the removal of the inactive pro sequence and liberate the biologically active, mature feline IL-18 protein, 157 amino acids in length.

### 3.4.3 IN VITRO EXPRESSION OF FELINE IL-18

All three forms of feline IL-18 mRNA, expressed from the pCI-neo (pro-IL-18), pCI-neo (mature-IL-18) and PsecI (IL-18) constructs, were expressed *in vitro* in transfected mammalian cells, as detected by Northern blot analysis. However, western blot analysis, performing immunodetection with a rabbit anti-equine IL-18 polyclonal antibody, demonstrated protein expression from only one of the constructs, pCI-neo (pro-IL-18). It is likely that the failure to demonstrate protein expression from the constructs pCI-neo (mature-IL-18) and PsecI (IL-18) was associated with lack of antibody specificity (using an anti-equine antibody to detect a feline protein), rather than a complete failure of these constructs to express mature IL-18 protein. Perhaps the anti-equine IL-18 antibody predominantly recognised epitopes present in the full length, pro-IL-18 protein, which were absent in the mature feline IL-18 protein. To facilitate more specific and sensitive detection of feline mature and pro-IL-18 proteins, future work should concentrate on the generation of polyclonal and monoclonal anti-feline IL-18 antibodies, to be used in feline specific assays.

A biological assay for human IL-18 has been developed, using the human myelomonocytic cell line, KG-1, which produces IFN- $\gamma$  in response to human IL-18 (Konishi et al. 1997). However, attempts to utilise feline IL-18 in the human KG-1/IFN- $\gamma$  assay have proved unsuccessful. Obviously the development of a feline-specific bioassay for this molecule would be extremely useful, especially when determining which constructs would be the most effective for *in vivo* usage. Evaluation of which constructs expressed biologically active feline IL-18 most potently *in vitro* could quickly and easily be determined. Future studies should attempt to create a specific bioassay for feline IL-18.

Furthermore, future studies should evaluate the *in vitro* expression of the feline IL-12 expression constructs described in this chapter, using Northern and western blotting techniques and a suitable bioassay. A recent paper describing the cloning and

expression of canine IL-12 cDNA described a lymphocyte proliferation assay, used to evaluate the biological activity of IL-12 in this species (Okano et al. 1997). The supernatants of COS-1 cells co-transfected with the vectors expressing each subunit of canine IL-12, was demonstrated to stimulate the proliferation of canine lymphocytes activated with PHA. Perhaps a similar lymphocyte proliferation assay would be suitable to evaluate the biological activity of feline IL-12.

#### **3.4.4 CONCLUSION**

The isolation of feline IL-12 and IL-18 sequences provides pivotal information which will enable investigation into the role of these feline Th1 type cytokines in immune-regulation and immunotherapy. This information will also allow the expression of feline IL-12 and IL-18 *in vitro* using a number of potential techniques. Ultimately these cytokines may form part of a new array of therapeutic agents to treat feline disease. IL-12 and IL-18 DNA constructs and recombinant proteins may be utilised in cancer treatment, immunotherapy and as vaccine adjuvants against retroviral pathogens such as FeLV, as discussed in chapter four, and FIV.

## **4. CHAPTER FOUR; FeLV DNA VACCINATION TRIAL**

## 4.1 INTRODUCTION

### 4.1.1 DNA VACCINATION AGAINST RETROVIRUSES

The principles of DNA vaccination are described in detail in Chapter 1. The success of DNA vaccination technology has led many researchers to investigate the potential of this approach in providing protection against retroviral pathogens, such as FIV, SIV and, most importantly, HIV (Hosie et al. 1998), (Cuisinier et al. 1997), (Letvin et al. 1997), (Boyer et al. 1997), (MacGregor et al. 1998). A recent report regarding the first human trial of a DNA vaccine, expressing HIV-1 *env* and *rev* genes, in the treatment of HIV-1 infection, demonstrated the generation of specific anti-HIV immune responses. Increases in anti-gp120 antibody levels, increases in cytotoxic T lymphocyte activity against gp160-bearing targets and increases in lymphocyte proliferative activity, were observed in some patients (MacGregor et al. 1998). However, no consistent changes were observed in CD4 or CD8 lymphocyte counts, or in plasma HIV levels. Further trials using expression plasmids encoding a wider range of HIV-1 antigens, and in concert with highly active antiretroviral therapy, HAART, are now in progress.

A number of reports have shown that DNA vaccines can generate HIV- and SIV-specific CTL activity, helper T cells, and antibodies in mice and non-human primates (Wang et al. 1993), (Ugen et al. 1997), (Boyer et al. 1997), (Fuller et al. 1997) and FIV specific CTL activity in cats (Hosie et al. 1998). Several studies in primates have also demonstrated protective immunity following HIV-1 or chimaeric HIV/SIV virus challenge (Boyer et al. 1997), (Letvin et al. 1997), although the viruses used are largely non-pathogenic, or replicate only at low levels, in the primate species described. It is therefore difficult to determine whether DNA vaccination will be as successful in inducing protection against retroviral infection in the natural host species. However, a recent FIV DNA vaccination trial demonstrated significant protection against homologous FIV challenge, in cats vaccinated with defective mutant provirus DNA (Hosie et al. 1998). Interestingly, a previous study

demonstrated immunoenhancement of FIV infection, following immunisation with a DNA vaccine expressing a limited range of FIV gene products (Richardson et al. 1997). The former study provides the first evidence that DNA vaccination can protect against lentiviral infection, a subfamily of the retroviridae, in its natural host species.

#### **4.1.2 FELINE LEUKAEMIA VIRUS DNA VACCINE CONSTRUCTION**

In the light of the success of DNA vaccination to date, and its promising development as a prophylactic agent for use against retroviral disease, a novel DNA FeLV vaccine was designed and constructed. The single cycle FeLV vector vaccine consisted of two separate pUSE1-1 series plasmids, one expressing FeLV *gag/pol* genes, and the other expressing FeLV subgroup A *env* gene, both under control of the CMV-IE promoter. The construction of this vaccine is described in section 4.2.5.1. *In vitro* and *in vivo* studies have demonstrated the potency of the Th1 type cytokines, IL-18, IFN- $\gamma$ , and IL-12, in enhancing cellular immunity and altering the magnitude and nature of the immune response elicited by DNA vaccination, (Micallef et al. 1996), (Kohno et al. 1997), (Chow et al. 1998), (Tsuji et al. 1997). It was considered that these Th1 cytokines might augment the immune response elicited by the FeLV DNA vaccine, and thus induce better protection against FeLV challenge. Therefore, the development of plasmids expressing these feline cytokine genes, to be used as vaccine adjuvants, was undertaken, as described in sections 3.2.8., 3.2.9. and 4.2.6.2.

#### **4.1.3 OBJECTIVES OF THE FELINE LEUKAEMIA VIRUS DNA VACCINE TRIAL**

There were three main objectives of the trial. Firstly, to determine if the novel FeLV DNA vaccine could protect cats against FeLV challenge. Secondly, to establish if plasmids encoding feline Th1 type cytokines could produce an adjuvant effect, when co-inoculated with the vaccine. Thirdly, to establish the presence or absence of DNA constructs in blood samples collected 48 hours after each immunisation and at

specific timepoints after viral challenge. The last objective is considered in detail in Chapter 5. Figure 4.1. provides an overview of the vaccine trial schedule.

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## 4.2 MATERIALS AND METHODS

### 4.2.1 FELINE LEUKAEMIA VIRUS DNA VACCINE TRIAL DESIGN

#### 4.2.1.1 Immunisation Groups

Twenty nine SPF kittens, aged 10-12 weeks, were randomly arranged (using a random assortment programme), into four groups of six, and one of five, as detailed in Table 4.1. One kitten died prior to the start of the trial, explaining why group B consisted of only five animals. The kittens were allowed three weeks to adjust to their environment and social groups, before beginning the trial. Throughout the trial, the animals were housed in two separate rooms and each individual group of six was evenly split between the two rooms. Susceptibility to FeLV persistent infection decreases with age (Hoover et al. 1976). Therefore, the immunisation schedule was designed so that viral challenge was completed before the unvaccinated control kittens were old enough to mount a protective immune response naturally, and thus be resistant to infection.

GROUPS	CONSTRUCTS	NUMBER OF CATS
A L1-6	CMV-gag-pol + CMV-env A	6
B L7-12	CMV-gag-pol + CMV-env A + IFN- $\gamma$	5
C L13-18	CMV-gag-pol + CMV-env A + IL-12	6
D L25-30	CMV-gag-pol + CMV-env A + IL-12 + IL-18	6
E L19-24	Empty pCI-neo plasmid	6

**Table 4.1. Immunisation groups**

Table 4.1. lists the DNA constructs which each group of cats received. The six cats in group A were immunised with the FeLV DNA vaccine, without an adjuvant. The construction of the FeLV vaccine antigen plasmids is described and illustrated in section 4.2.5.1. The cats in groups B, C and D were inoculated with the FeLV DNA vaccine and pCI-neo vector constructs expressing cytokine genes, to act as adjuvants. Interferon gamma, interleukin 12 and a combination of interleukin 12 (IL-12) and interleukin 18 (IL-18) DNA constructs, respectively, were administered to cats in these three groups. The IL-18 DNA construct consisted of the mature form of IL-18, fused to a 5' synthetic signal peptide sequence, as described in section 3.2.9. Therefore, following expression of this construct, the biologically active form of the IL-18 protein should be transported out of cells. The IL-12 construct consisted of two separate plasmids, encoding the p35 and p40 subunits. Finally, the cats in Group E, the positive control group, were immunised with pCI-neo plasmid only.

#### **4.2.1.2 Immunisation Schedule**

Kittens received three consecutive immunisations with high quality endotoxin-free DNA, two weeks apart, at the times indicated in Table 4.2. The preparation and analysis of this DNA is described in section 4.2.5.3. The cats were inoculated intramuscularly in one site, the quadriceps femoris muscle, using the left hindlimb for the first and third immunisations and the right hindlimb for the second immunisation. They received a total of 100µg of each DNA construct, as detailed in Table 4.1, diluted with endotoxin-free PBS to a volume of 200µl per inoculation. The kittens were carefully observed by cattery staff for the twenty-four hours, following each treatment. Twenty-four hours after each immunisation the temperature, pulse, respiration rate and general demeanour of every kitten was recorded and the vaccination site examined. No local or systemic adverse reactions were observed after vaccination or viral challenge.

Immunisation Number	Week	Age of cats
1	1	13-15 weeks
2	3	15-17 weeks
3	5	17-19 weeks
VIRAL CHALLENGE	8	20-22 weeks

**Table 4.2. Immunisation Schedule**

#### **4.2.1.3 Viral Challenge**

Viral challenge was performed by the intraperitoneal route three weeks after the last immunisation when the cats were between 20-22 weeks of age (Table 4.2.).  $2 \times 10^5$  ffu (focus forming units), of FeLV-A/Glasgow-1 were administered to each cat, diluted with endotoxin-free PBS to a volume of one ml. The results of the assay of the virus used for challenge is described in section 4.2.6. As the different groups of cats were housed together after viral challenge, the persistently infected cats, excreting virus, became a source of infection. This effectively meant that the cats that were capable of mounting a protective immune response against the intraperitoneal viral challenge were subjected to a second, more prolonged and perhaps more rigorous natural viral challenge, more analogous to the situation in the field, beginning 3-4 weeks after intraperitoneal challenge.

#### **4.2.1.4 Blood Sampling Schedule**

Sampling procedures were constrained by the weight of the kittens. A maximum volume of 0.5ml per 100g of body weight can be obtained at two to three week intervals, according to Home Office guidelines. The expected average weight of the kittens on Day 1 of the trial was 1000g, although there was a significant difference between individual animals at this stage. The sampling schedule was conveniently divided into three parts.

#### 4.2.1.4.1 Pre-Immunisation Blood Sample

One pre-immunisation blood sample was taken immediately before the first immunisation. This was to ascertain FeLV status of the cats pre-trial and to establish normal haematological parameters. The blood was screened for the presence of FeLV p27 antigen, infectious virus, and virus neutralising antibodies. These tests are described more fully in section 4.2.4. DNA extracted from plasma and PBMCs, collected forty-eight hours after each immunisation, was to be screened by PCR for the presence of DNA constructs. Therefore, to confirm the absence of the DNA constructs pre-trial, this pre-immunisation sample was screened by the same technique. Table 4.3. indicates how these samples were analysed.

Blood Sample Volume = 2.0ml Total	Analysis
1.5 ml Heparin	a) plasma for VN Ab assay, p27 ELISA, VI on QN10 cells.
	b) Plasma for PCR; to confirm absence of DNA constructs pre-trial.
	c) Buffy coat (PBMCs) from total sample volume for PCR; to confirm absence of DNA constructs pre-trial.
0.5ml EDTA	Routine Haematology

**Table 4.3. Pre-immunisation blood sample analysis**

#### 4.2.1.4.2 Immunisation Period blood samples

Blood samples were obtained during the immunisation period, in the seven weeks prior to challenge. These samples were taken every two weeks, 48 hours after each immunisation, then on the day of challenge (immediately before challenge). Table 4.4. provides an overview of how sample analysis was conducted. Serum was analysed for the presence of FeLV specific non-neutralising antibodies, elicited by vaccination, by western blot analysis against complete viral lysate and an anti-gp70

antibody ELISA. Residual serum was stored in aliquots, for future cytokine analysis. Heparinised blood and PBMCs were also isolated and the former was used to determine levels of plasma infectious virus, FeLV p27 antigen and FeLV specific virus neutralising antibodies. PCR was also performed on DNA extracted from plasma and PBMCs in an attempt to detect the presence of DNA constructs, in either of these compartments. Lastly EDTA blood was taken on the day of viral challenge only, for haematological analysis. The timing of blood sample collection is outlined in Table 4.5.

Blood Sample Volume = 2.0ml	Analysis
1.5ml Heparin	a) Plasma for VN Ab assay, VI on QN10s and p27 ELISA
	b) Plasma for PCR; to isolate DNA constructs.
	c) PBMCs from total sample volume; PCR to isolate DNA constructs.
0.5ml Plain	a) Serum for detection of non-neutralising antibody; gp70 ELISA, western blot analysis of viral lysates
	b) Remaining serum stored for future cytokine analysis.

**Table 4.4.. Immunisation Period Blood Sample Analysis**

N.B. An extra 0.5ml EDTA sample was taken on day of challenge, for haematological analysis; therefore total sample volume = 2.5ml.

TIMING	WEEK
48hrs post first immunisation	Week 1
48hrs post second immunisation	Week 3
48hrs post third immunisation	Week 5
day of challenge	Week 8

**Table 4.5. Timing of Immunisation Period Blood Samples**

*4.2.1.4.3 Post-Challenge Period Blood Samples*

Sampling began three weeks after viral challenge, and was repeated on three occasions, approximately three weeks apart, until week 21 ( weeks 11, 14, 17 and 21). Virus isolation (VI) on QN10 cells, to determine levels of plasma infectious virus, FeLV p27 antigen ELISA and FeLV specific virus neutralising antibody assays were performed on plasma from heparinised blood samples. Haematological analysis of blood was conducted to establish if the development of FeLV infection produced any changes in normal haematological parameters. Lastly, DNA extracted from PBMCs and plasma was again screened by the PCR technique, to see if the DNA vaccine constructs had persisted in either of these compartments. Table 4.6. highlights how sample analysis was conducted and the timing of blood sample collection is outlined in Table 4.7.

Blood Sample Volume = 3.0ml	Analysis
2.0ml Heparin	VN Ab assay, p27 ELISA, VI on QN10s. Store remaining plasma for future analysis.
0.5ml EDTA	Haematology
0.5ml Plain	Store serum for future analysis

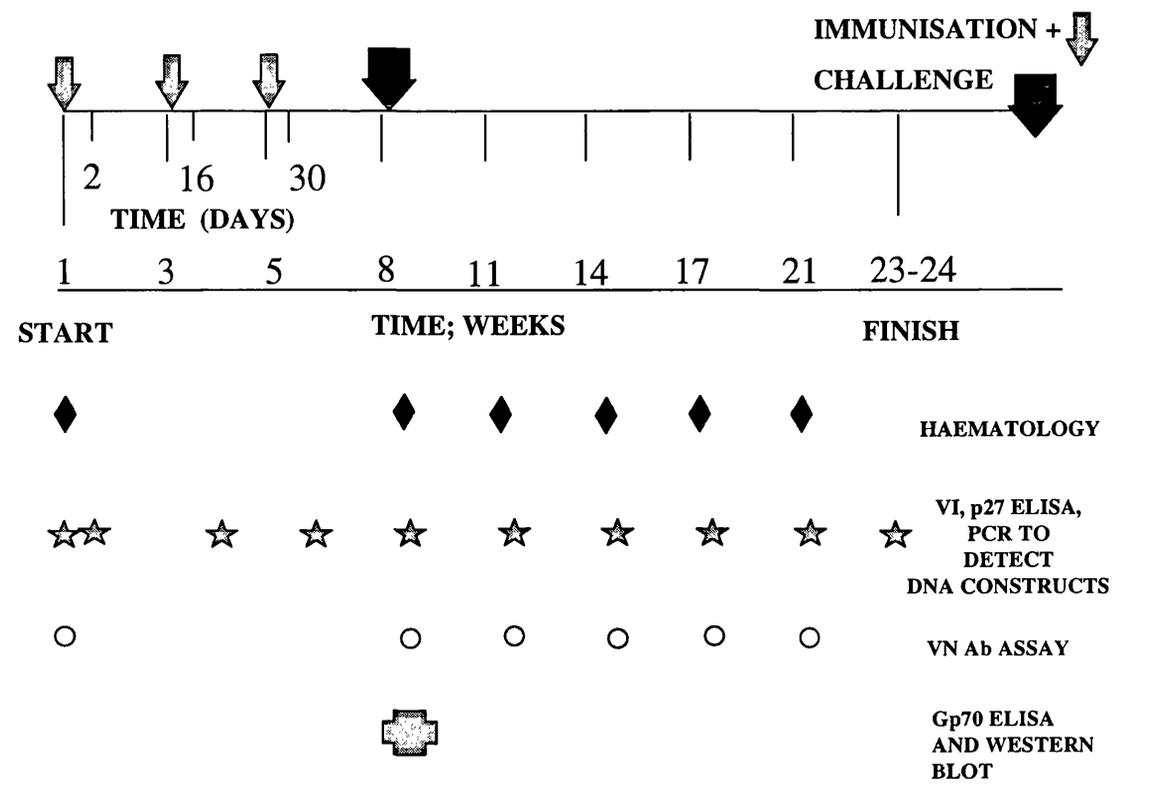
**Table 4.6. Post challenge Period Blood Sample Analysis**

TIMING	WEEK
first post challenge blood sample	Week 11
second post challenge blood sample	Week 14
third post challenge blood sample	Week 17
fourth post challenge blood sample	Week 21

**Table 4.7. Timing of Post challenge Period Blood Samples**

#### **4.2.1.5 Post-trial tissue analysis**

All cats, with the exception of L21, L23, L24 and L28, were euthanased at the termination of the experiment, at weeks 23 and 24, (between two and three weeks after the last post-challenge blood sample, at week 21). Blood samples were collected and screened by the virus isolation technique, to determine FeLV status of the cats immediately prior to euthanasia. Bone marrow biopsies were obtained from all non-viraemic cats and from several viraemic cats, to act as positive controls. These biopsies were cultured and then screened for the presence of FeLV, by virus isolation and p27 ELISA, to determine if ostensibly immune, non-viraemic cats, harboured latent FeLV infection in bone marrow. These collection and culturing techniques are described in sections 4.2.2.2. and 4.2.3. Tissues and blood from each cat were also collected and stored under liquid nitrogen and in  $-70^{\circ}\text{C}$  freezers, respectively, for future analysis.



**Figure 4.1. Overview of FeLV Vaccine Trial Schedule**

The timing of immunisations and challenge are represented by small and large arrows, respectively. Haematological analysis, VI, p27 ELISA and PCR to detect DNA constructs, and VN antibodies assays were performed at the times indicated by the diamond, star and circle symbols, respectively. Gp70 ELISA and western blot analysis were performed on day of challenge, as indicated by the large cross symbol. VI, virus isolation; ELISA, enzyme linked immunosorbent assay; VN Ab assay, virus neutralising antibodies assay.

## **4.2.2 EXPERIMENTAL ANIMALS**

Twenty-nine specific pathogen free (SPF) cats were obtained from a commercial breeding cattery and housed at Glasgow University. The kittens were between seven and nine weeks of age, on arrival, and were fed a commercial diet. After a initial settling in period of three weeks, the kittens were randomly arranged into four groups of six and one group of five and received their first immunisation at between 13 and 15 weeks old. All procedures were carried out in accordance with Home Office regulations. All cats, with the exception of L21, L23, L24 and L28, were euthanased at the termination of the procedure, between weeks 23 and 24, using intracardiac injections of pentobarbitone (Euthatal) when anaesthetised.

## **4.2.3 COLLECTION AND ANALYSIS OF SAMPLES**

### **4.2.3.1 Blood collected for analysis**

Animals were restrained manually and blood was collected from either the cephalic or the jugular veins. Pre-euthanasia samples (week 23-24) were collected by intracardiac puncture, while the animals were under general anaesthesia. A combination of ketamine (Ketaset - Willows Francis Veterinary, Crawley, UK) and xylazine (Rompun - Bayer plc, Bury St Edmunds, UK), administered by intramuscular injection, was used to anaesthetise the cats. Blood for haematology was collected into 1.3ml tubes, containing potassium-EDTA (Bibby Sterlin Ltd., Stone, UK). Cell counts were obtained using an automated cell counter (ABX Minos Vet - Roche Products Limited) and differential counts were performed manually using May-Grunwald-Giemsa stained smears.

Blood for detection of plasma infectious virus, p27 viral antigen, virus neutralising antibody and DNA constructs, was collected in 2ml heparin coated tubes. Blood for serological tests, the detection of non-neutralising antibody, by means of anti-gp70

ELISA and western blot analysis of viral lysates, was collected into plain two millilitre tubes. Plasma and PBMCs obtained from these samples were also screened by PCR, to identify DNA vaccine constructs. Plasma, serum and PBMCs were separated by centrifugation of heparin and plain blood samples at 2000rpm, for ten minutes. Residual plasma and serum were aliquoted and stored at  $-70^{\circ}\text{C}$ , for future analysis.

#### **4.2.3.2 Bone Marrow collected for culture and viral isolation**

Bone marrow biopsies were obtained from all non-viraemic cats and from several viraemic cats to act as positive controls. Cats were anaesthetised using alphaxelone/alphadolone acetate (Saffan, Pitman-Moore Ltd., Crewe, UK), given by intravenous injection. The left gluteal region was clipped and prepared aseptically, and aspirates of marrow were then collected from the femur via the inter-trochanteric fossa, using sterile disposable 18G Illinois Sternal/Iliac bone marrow aspiration needles (Pharmaseal, Allegiance healthcare Corporation, USA.) and 10ml sterile disposable syringes. Aspirates were transferred into small sterile universal containers containing 3ml of bone marrow collection medium, comprising Alpha Minimal Essential Medium (Gibco), supplemented with 10%FCS, 2mM L-glutamine, 400 units/ml penicillin/streptomycin,  $10^{-6}\text{M}$  hydrocortisone succinate (AFC10) and 50iu/ml preservative-free heparin (Pularin). Bone marrow aspirates were also collected from cats immediately after euthanasia, with the femur being removed and opened with bone cutting forceps.

#### **4.2.3.3 Tissues collected for analysis**

Samples of liver, lungs, spleen, lymph nodes, (mesenteric, popliteal, prescapular and iliac), brain, thymus, kidney, heart, muscle (two samples; one sample from the left quadriceps injection site and one control sample from the triceps), ovaries, serum, plasma, PBMCs and skin were collected at post-mortem, from each of the euthanased cats. Testicles were removed when the male cats were castrated, at five to six months old. All tissue samples were snap frozen and stored in liquid nitrogen, for future analysis. This will include isolation of DNA and RNA from every tissue sample.

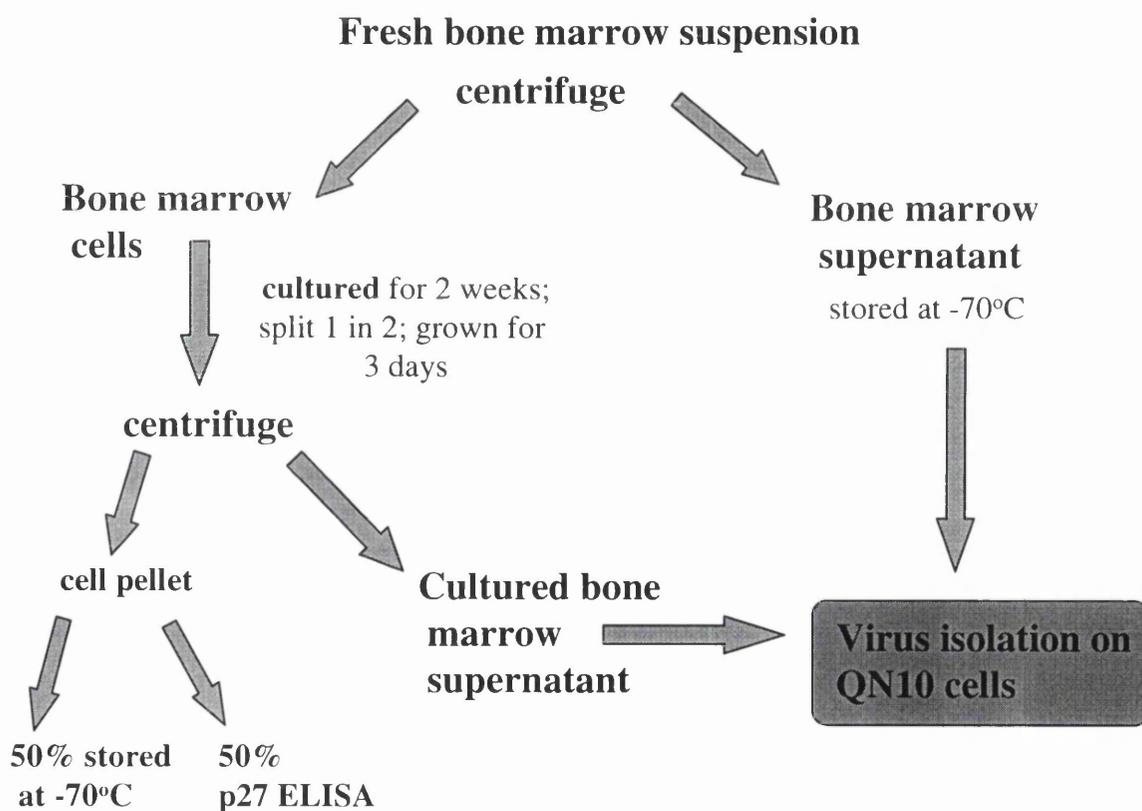
PCR and RT-PCR techniques will be used to identify the presence of DNA constructs and mRNA expression, respectively, in all samples. Plasma and PBMCs were collected in an equal volume of Alsever's solution (20ml of blood), while EDTA and heparin samples (2ml of blood) were also obtained for plasma harvest. Serum was collected in plain universal containers (50ml of blood) and all of the above blood fractions were stored in aliquots at -70°C.

#### **4.2.4 BONE MARROW CULTURE TECHNIQUE AND SCREENING FOR FeLV**

Single cell suspensions of bone marrow cells were prepared in 10ml of bone marrow collection medium (section 4.2.3.2.), by passing the cells through a 21 gauge needle and then through a 23 gauge needle. The resulting suspensions were centrifuged at 2000rpm for ten minutes. The bone marrow supernatants (BMSN) were decanted and stored at -70 °C, for future virus isolation on QN10 cells. The cell pellets were resuspended in 10ml of 0.83% ammonium chloride and incubated on ice for five minutes in order to lyse the red blood cells. Following a second centrifugation at 2000rpm for ten minutes, the supernatants were discarded and the cell pellets were resuspended in 10ml Alpha Minimal Essential Medium supplemented with 20%FCS, 2mM L-glutamine, 400 units/ml penicillin/streptomycin and  $10^6$  M hydrocortisone succinate (AFC20). The cells were then counted and were cultured in 10ml AFC20 at a concentration of  $2 \times 10^6$ /ml in 25cm<sup>2</sup> plastic flasks. Duplicate cultures were prepared for each biopsy and were incubated at 37°C in 5 per cent carbon dioxide in air.

The bone marrow cells grew as an adherent cell monolayer. After one week in culture small colonies of fibroblasts developed, which gradually coalesced, so that by two weeks in culture the fibroblasts were confluent. Also, by this time, large granulated cells were present and areas of more mature myeloid cells could be identified. After two weeks of growth, the bone marrow cells were split 1 to 2, using AFC20 medium, and grown for three more days. Cell suspensions were then spun at 2000rpm for ten minutes. The bone marrow culture supernatants were decanted and virus isolation,

using the method described in section 4.2.4., was performed on the culture supernatants. After the centrifugation, cell pellets were also obtained. Half of these pellets were stored at  $-70^{\circ}\text{C}$ , for future analysis, while the other half of these pellets were analysed for the presence of FeLV p27 antigen, using the p27 ELISA assay, described in section 4.2.4. At this point, the bone marrow supernatants (BMSN) stored at  $-70^{\circ}\text{C}$ , described above, were thawed for virus isolation. Figure 4.2. provides an overview of the whole process. These virus isolation and p27 ELISA techniques were employed to determine if ostensibly immune, non-viraemic cats, harboured latent FeLV infection in bone marrow.



**Figure 4.2. Overview of bone marrow culture and FeLV diagnostic tests**

## **4.2.5 FELINE LEUKAEMIA VIRUS DIAGNOSTIC TESTS**

### **4.2.5.1 Virus isolation protocol**

Infectious FeLV in the plasma and bone marrow cultures of cats was detected by the virus isolation technique described by Jarrett (Jarrett and Ganiere, 1996). Twelve-well cluster plates were used in this technique; each well was 22mm in diameter. On day one  $5 \times 10^4$  QN10 cells in 1 ml of growth medium (2.2.1.3.), supplemented with 4µg/ml polybrene, were added to each well. The plates were incubated overnight at 37°C in a humid incubator, with 5% CO<sub>2</sub>. On day two, 0.2ml of each sample to be screened was added to the individual wells and allowed to absorb for two hours at 37°C. This was then removed and replaced with 1.5ml fresh growth medium. The plates were then incubated as before. Separate pipettes were used for each individual sample, to avoid cross-contamination. As a positive virus control a ten-fold dilution series of FeLV-A was included in the plates and also a negative control, such as a serum sample from a FeLV-free SPF cat. The plates were incubated until between day five and day seven, when they were examined for evidence of morphological transformation.

Samples were scored positive or negative at this stage. All positive samples were defined as definitely positive, while all negative samples were subcultured by removing the medium from each well and adding 250µl of trypsin/versene mixture to the cells. This was left in place until the cells detached. Cells were then resuspended with 1 ml of growth medium and transferred to a 5 cm plate with 4 ml of growth medium. The positive and negative controls were also subcultured. These plates were then incubated for a further three to four days before they were finally scored positive or negative, depending on the presence or absence of transformation. The subculturing technique is performed because if a very low titre of FeLV is present in the test sample, the week-long initial incubation period may not be long enough to allow the virus to reach sufficient levels to cause visible cellular transformation. FeLV replicates only in actively dividing cells, so subculturing to five centimetre plates allows the QN10 cells to grow and divide at a faster rate, thus speeding up viral replication and the appearance of transformation.

#### 4.2.5.2 Feline leukaemia virus p27 ELISA

FeLV p27 antigen was detected in the plasma of cats using a commercial enzyme-linked immunosorbant assay (ELISA) kit (Innochem C. Lutz, Switzerland). All p27 ELISAs performed were based on the method described by Lutz (Lutz et al. 1983). Briefly, the technique employed was a double antibody sandwich ELISA. Test wells were pre-coated with a monoclonal antibody, directed against an epitope on the species-specific portion of the FeLV p27 molecule. Fifteen microlitres of plasma to be screened, 80µl conjugate, and 1 µl of normal mouse serum, to block any anti-mouse antibody in the sample, were added to each well. Positive and negative controls were included and the plates were incubated at room temperature for one hour, with shaking.

The conjugate was a second antibody raised to a different epitope on the species-specific portion of the FeLV p27 molecule, conjugated to horseradish peroxidase, HRP. This labelled antibody should bind to any p27 antigen present in the samples, captured by the first antibody. The plates were then removed and washed vigorously with PBS/0.05% Tween 20. KPL's tetramethylbenzidine hydrochloride peroxidase substrate was then added to each well, and left for between five and ten minutes at room temperature, on a plate shaker, to allow colour development. The enzymatic action of HRP on TMP caused a visible blue colour change and the optical density, O.D., of each sample was measured on the ELISA reader, at a wavelength of 650nm, after stopping the reaction using 0.12% hydrofluoric acid. The O.D. values were noted and the ELISA S/P, the sample to positive (S/P) ratio, was calculated for all samples, using the formula below:

$$\text{S/P Ratio} = \frac{\text{Sample OD} - \text{Negative Control OD}}{\text{Positive Control OD} - \text{Negative Control OD}}$$

The positive and negative control OD values were obtained from standard positive and negative serum samples. These internal controls were tested alongside the experimental samples and were included in each ELISA. The S/P ratio is a value

which takes into account both positive and negative control readings, and therefore corrects for variations between individual ELISA batches. It is generally accepted that a S/P ratio of greater than 0.1 represents a positive ELISA result; i.e., the sample contains p27 antigen. However, a sample which possesses an ELISA OD reading greater than twice the value of the negative control ELISA OD, may also be defined as positive. The latter method of defining a positive is considered more sensitive and has been employed when analysing the data obtained during the course of this trial.

#### **4.2.5.3 Feline leukaemia virus neutralising antibodies assay**

Virus neutralising antibodies (VNAs) to FeLV-A in the plasma of cats were measured in a focus reduction assay, using the basic method described by Jarrett (Jarrett and Ganiere, 1996). Briefly, QN10 cells were plated at  $4 \times 10^4$ /well, in 12-well plates, in 1 ml of Dulbecco's MEM, containing HEPES buffer, 10% FCS and four microlitres/ml of polybrene. The plates were incubated at 37°C overnight. Four wells of cells were used for each plasma sample to be tested. The next day, plasma samples were heat inactivated at 56 °C for thirty minutes and were then diluted in 96-well plates from 1:4, to 1:256, using 50µl volumes of plasma and Leibovitz medium. An equal volume (50µl) of FeLV-A at  $4 \times 10^2$  FFU/ml was added to each plasma sample, and incubated at 37°C for six hours. Twenty-five microlitres of 1:4, 1:8, 1:16 and 1:32 dilutions were then plated onto the QN10 cells and incubated at 37°C for three to four days. The remainder of the dilutions were stored at -70 °C. Positive and negative controls were included with each test. On day five, the QN10 cells were examined for the presence of foci of transformation, and in this way levels of infectious virus were quantified. The titre of antibody present in each plasma sample was taken as the reciprocal of the plasma dilution that reduced the focus count of FeLV by 75%, compared with a virus control incubated without plasma.

#### **4.2.5.4 Detection of feline leukaemia virus non-neutralising antibodies.**

Day of challenge samples of sera were isolated and analysed for the presence of FeLV specific non-neutralising antibodies, elicited by vaccination. This was

performed by western blot analysis against complete viral lysate, and an anti-gp70 ELISA assay, to detect antibodies raised to this FeLV antigen.

#### *4.2.5.4.1 ELISA for feline antibodies to FeLV gp70*

FeLV surface glycoprotein gp70 was captured by an immobilised monoclonal antibody coated onto a 96-well plate. Cat serum or plasma were then applied to the plate and any anti-gp70 antibody present in the serum or plasma was subsequently bound. Capture of the cat antibody was detected by the subsequent binding of goat anti-cat IgG conjugated with alkaline phosphatase enzyme, and addition of substrate (phosphate substrate). Dilutions of sera were assayed and the titre of antibody was calculated as the reciprocal of the highest dilution which gave a pre-determined absorbance; an ELISA S/P ratio of greater than 0.1 (as explained in section 4.2.5.2.), represented a positive ELISA result. The materials used in this protocol are described in section 2.1.11.3.

Purified FeLV was disrupted, before use, in the following manner. The virus was thawed rapidly, with gentle shaking, in a 37 °C water bath. Virus was then diluted in virus disruption buffer (2.1.11.3.), in an Eppendorf tube, and incubated in a 37°C water bath, for 30 minutes. Centrifugation at 6000rpm for five minutes followed this incubation, and the supernatant was removed for use. Monoclonal antibody 3-17 was diluted 1:1000 in coating buffer and 100µl of this dilution was pipetted into each well of a 96 well plate. Working concentrations of virus and antibody were determined previously by cross-titrating FeLV and 3-17 antibody in the ELISA, using positive cat anti-FeLV control serum, P-11, diluted 1:100. In the ELISA described above, this antibody had a titre of 10000. A second positive control serum sample, P-4, of low titre (500), and negative control serum from SPF cats were also included in the ELISA. The plate was then incubated overnight at 4 °C. Next morning, the plate was washed four times with TBT wash buffer. The TBT was then removed, and 200µl of blocking buffer was pipetted into each well. The plate was incubated at room temperature, 25 °C, for 60 minutes, and after this, washed three times with TBT wash buffer. The virus supernatant, as prepared above, was then diluted 1:200 in TBT wash buffer, with 20% w/v goat serum. 100µl of this mixture was then pipetted into

each well of the plate and incubated for 60 minutes at room temperature, 25 °C. The plate was again washed three times with TBT.

Cat serum to be tested was then diluted in TBT wash buffer, with 20% w/v goat serum. A volume of 100µl of these dilutions were pipetted into the wells and, again, the plate was incubated for 60 minutes at room temperature, 25 °C. After four washes with TBT wash buffer, 100µl of diluted conjugate was pipetted into each well of the plate and a 60 minute incubation at room temperature followed. The plate was then washed six times with TBT wash buffer and 50µl substrate was pipetted into each well. The plate was incubated for 20 minutes at room temperature, and the reaction was stopped by pipetting 50µl of the stop solution, 0.4M NaOH, into each well. The optical density of the solutions in the wells was then read immediately, in a Titertek plate reader, at a wavelength of 405nm.

#### *4.2.5.4.2 Western blot analysis of serum against complete viral lysate*

The techniques of SDS - polyacrylamide gel electrophoresis of proteins and detection of proteins by immunoblotting, are described in sections 2.2.6.1. and 2.2.6.2., respectively. Briefly, complete FeLV lysate, and protein molecular weight standards, were run on an SDS-polyacrylamide gel, to separate the viral proteins on the basis of molecular size. Fifty microlitres of purified FeLV of a known protein concentration, was added to 25µl of 3 x protein sample loading buffer. The p27 concentration of this viral lysate had been estimated previously, by comparing the intensity of the Coomassie blue stained p27 protein, with the intensity of a Coomassie blue stained ovalbumin control, of a known concentration. The p27 concentration of this preparation of virus, was estimated to be 1mg/ml. As p27 protein accounts for approximately 30% of the total viral protein, approximately 170µg of viral protein was loaded onto the gel. These proteins were then transferred to a nitrocellulose membrane, using electroblotting apparatus. Vertical strips were cut from the blot, approximately 1mm thick. In this case, strips were cut for the twenty-nine cats involved in the trial and for positive and negative control serum samples. Serum samples from individual cats were incubated with the nitrocellulose membrane strips, in an attempt to determine if sera contained FeLV-specific antibodies. The detection

of proteins by immunoblotting exploits the inherent specificity of antigen recognition by antibodies (Towbin et al. 1979). The method of detection used to visualise this specific interaction of antibody and antigen was the streptavidin peroxidase method, which is described below.

Proteins were transferred to the membrane using the electroblotting system, run at 100V, for one hour, after which time the nitrocellulose membrane was removed and rinsed in TBS. Non-specific binding sites were blocked by immersing the membrane in a TBS + 2% Marvel solution and placing on the orbital shaker, for 3-4 hours. The membrane was then washed with PBS + 0.05% Tween 20, (500ml PBS + 0.25ml Tween 20), for two hours. Lastly, the membrane was air dried at room temperature, on 3MM paper, inserted into folded parafilm, and stored at -20°C in a sealed freezer bag, until required.

Solution A was made by adding 0.8g of Marvel to 20ml TBS and 100µl Tween 20. Nitrocellulose strips were incubated in small individual compartments of large multi-compartmented dishes, so that each individual strip was separate from all the others. Serum from each of the twenty-nine cats, and positive and negative serum controls, was added to each individual nitrocellulose strip, (20µl serum per 200µl solution A, as above, with EDTA; 2µl of 500mM EDTA was added per ml of solution A), and incubated for two hours at room temperature, or at 4 °C overnight. Three washes with PBS/Tween (0.05% Tween in PBS), were then performed and 200µl of biotinylated protein A (Amersham), in solution A (10µl of biotinylated protein A per 2.5ml of solution A), was added. The strips were then incubated for one hour at room temperature, with gentle rocking. The strips were washed three times as before, with PBS/Tween, and 200µl streptavidin peroxidase (Sapu), diluted 1:500 in solution A, was added. The strips were again incubated for one hour at room temperature, with gentle rocking. After this incubation step, three washes with PBS/Tween were performed and 200µl substrate, prepared immediately before use, was added. The substrate was prepared as detailed below.

Ten millilitres of methanol was decanted into a universal container, which was capped tightly and submerged into an ice box. An aliquot of H<sub>2</sub>O<sub>2</sub> was also submerged in ice. 25ml of NT buffer was then measured into a 50ml centrifuge tube, and kept on the bench until required. Thirty milligrams of 4-chloro-1-naphthol was weighed out into an universal container and also kept on ice. After the final PBS/Tween wash, the methanol was tipped into the 4-chloro-1-naphthol, to give reagent A, and 15µl H<sub>2</sub>O<sub>2</sub> was added to the NT buffer, to give reagent B. Finally, 5ml of reagent A was added to reagent B to give the active developer. As detailed above, 200µl of this substrate was added to all the strips after the final wash. The reaction was then stopped by pouring off the substrate and washing with water. If specific binding of FeLV specific antibodies to FeLV antigens has occurred the active developer will initiate a colour change on the nitrocellulose strips, which will be clearly visible. Thus, this method of detecting proteins by immunoblotting, the streptavidin peroxidase method, allows the visualisation of specific antibody and antigen interactions.

#### **4.2.6 CONSTRUCTION OF FeLV VACCINE ANTIGEN AND ADJUVANT CONSTRUCTS**

##### **4.2.6.1 Construction of FeLV antigen DNA constructs**

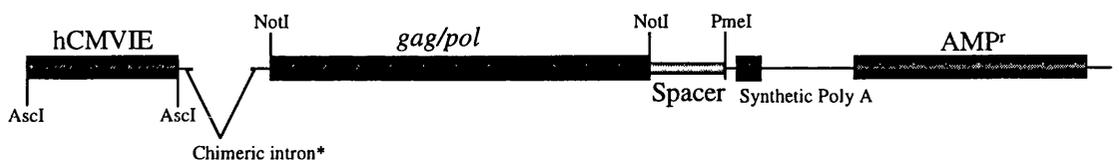
A mammalian expression vector series, comprising pUSE, pUSE1<sup>-</sup> and pUSE2<sup>-</sup>, was constructed by Dr Derek Bain from a commercial plasmid available from Promega; pCI-neo. PCR and standard recombinant DNA technology was used to construct these bicistronic vectors, incorporating unique restriction sites for the rare cutting enzymes, *Asc* I, *Pac* I, *Pme* I and *Not* I. The use of rare cutting restriction enzymes allowed the rapid cloning of promoters, reporter genes, or *gag/pol* and *env*, without the need to return to an empty plasmid backbone.

The antigen-encoding component of the FeLV DNA vaccine consisted of two separate pUSE1<sup>-</sup> series constructs, one expressing FeLV *gag/pol* genes, and the other expressing FeLV subgroup A *env* gene, both under control of the CMV-IE

promoter. The construction of these FeLV antigen DNA constructs is described below and illustrated in figure 4.3 and 4.4.

#### 4.2.6.1.1 Construction of pUSE1<sup>-</sup>CMVT(*gag/pol*)

The CMV promoter was cloned into the *Asc* I site in pUSE1<sup>-</sup> to create pUSE1<sup>-</sup>CMVT. *Gag/pol* from the FeLV A/Glasgow-1 molecular clone was then subcloned from the vector pCDNA3 into pUSE1<sup>-</sup>CMVT. A *Not* I restriction digest and ligation were performed to create pUSE1<sup>-</sup>CMVT(*gag/pol*).

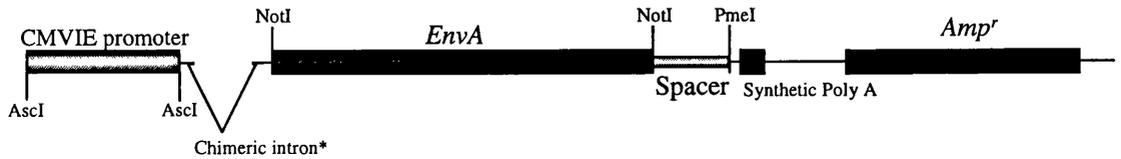


\*= 5'- donor site from the first intron of the human beta-globin gene and the branch and 3'-acceptor of an immunoglobulin gene heavy chain variable region.

**Figure 4.3.** pUSE1<sup>-</sup>CMVT(*gag/pol*)

#### 4.2.6.1.2 Construction of pUSE1<sup>-</sup>CMVT(*envA*)

*Env A* from the FeLV A/Glasgow-1 molecular clone was subcloned from pCDNA3 into pUSE1<sup>-</sup>CMVT. *Env A* was first excised from pCDNA3 by *Pst* I/*Bam* HI digestion, and then blunt ended. pUSE1<sup>-</sup>CMVT(*gagpol*) was then *Not* I digested, gel purified and blunt ended to create pUSE1<sup>-</sup>CMVT. Finally, *envA* was blunt end ligated into pUSE1<sup>-</sup>CMVT to create pUSE1<sup>-</sup>CMVT(*envA*).



\*= 5'- donor site from the first intron of the human beta-globin gene and the branch and 3'-acceptor of an immunoglobulin gene heavy chain variable region.

**Figure 4.4.** pUSE1<sup>-</sup>CMVT(*EnvA*)

#### 4.2.6.1.3 Transient co-transfection of 293 cells to establish that FeLV antigen constructs are able to produce virions *in vitro*

To ascertain that the FeLV constructs described above were capable of producing virions, a transient production of recombinant retrovirus containing the reporter gene LacZ was performed. Three plasmids were used for this purpose: pUSE1<sup>-</sup>CMVT(*gagpol*) and pUSE1<sup>-</sup>CMVT(*EnvA*), as described above, and pHIT111. PHIT111 was kindly donated by Dr Alan Kingsman and comprised the recombinant MoMLV vector genome, containing the LacZ gene driven by the CMV promoter. The Lac Z gene encodes the enzyme  $\beta$ -galactosidase, the enzymatic action of which initiates a blue colour change when  $\beta$ -galactosidase assay solution is added to cells.

293 cells were split the day before the transfection experiment and transferred to a 25cm<sup>2</sup> flask, so that they would be approximately 30% confluent for the transfection. Five micrograms of each of the three plasmids were co-transfected into the 293 cells,

using the calcium phosphate precipitation transfection method. Sixteen hours later the media was changed and 5ml of fresh media was added to the cells. Two days post-transfection the supernatant was harvested from the cells and filtered through a 0.45µm syringe filter. Polybrene to a final concentration of 8µg/ml was added to 1ml of this supernatant which was then added to a flask of FEA cells, a fibroblast cell line derived from whole feline embryos, susceptible to infection with FeLV. The supernatant was placed on the FEA cells at 37°C for two hours, then fresh medium was added to a volume of 5ml. Three days post-infection the cells were labelled for LacZ expression, using the β-galactosidase assay method, detailed in section 2.2.2.2. Finally, the total number of blue cells in the flask was counted. Approximately 1150 cells were stained blue indicating that recombinant retrovirus containing the reporter gene LacZ (expressing the enzyme β-galactosidase) had been produced. Therefore, it was established that the FeLV constructs were able to produce virions *in vitro*.

#### **4.2.6.2 Construction of feline cytokine DNA constructs**

The construction of the plasmids encoding p35 IL-12, p40 IL-12 and IL-18 cDNA is described in sections 3.2.8. and 3.2.9 and the expression *in vitro* of the IL-18 constructs is described in section 3.2.10. The cloning, characterisation and expression of feline interferon gamma, IFN-γ, have been described previously (Argyle et al. 1995), (Argyle et al. 1998). For use as an adjuvant gene in the FeLV DNA vaccine trial, the IFN-γ cDNA was cut out of the pRC-RSV vector using *EcoR* I and *Not* I restriction enzymes and subcloned into the pCI-neo vector as a *EcoR* I-*Not* I fragment.

#### **4.2.6.3 Preparation, quantification, sequencing and endotoxin testing of DNA for vaccine trial**

##### *4.2.6.3.1 Preparation of DNA for vaccine trial*

The ultrapure, endotoxin-free DNA required for the trial was prepared using Qiagen's Endofree Plasmid Giga kits (Qiagen, UK), following the manufacturer's

protocol. All seven DNA constructs, pUSE1<sup>-</sup>CMVT(EnvA), pUSE1<sup>-</sup>CMVT(gagpol), pCI-neo + IFN- $\gamma$ , pCI-neo + IL-12 p35, pCI-neo + IL-12 p40, pCI-neo + IL-18, (mature form bolted to a 5' synthetic signal peptide sequence), and empty pCI-neo plasmid alone, were prepared using this method. The manufacturers claim that yields of up to 10mg of high-copy plasmid DNA, with endotoxin levels of less than 0.1EU/ $\mu$ g of DNA may be obtained from a 2.5 litre culture volume and that the DNA produced is suitable for gene therapy research and genetic vaccination (Qiagen, 1998).

Endotoxin-free plastic pipette tips and tubes were used for elution and all subsequent steps. A single bacterial colony, from an ampicillin selective plate was picked and inoculated into a starter culture of 5 ml LB medium, supplemented with 50 - 100  $\mu$ g/ml ampicillin, in a sterile universal container. The culture was incubated for 8 hours at 37°C in an orbital incubator with vigorous shaking (250rpm). The starter culture was then diluted 1/500 into 2.5 litres of LB medium supplemented with 50 - 100  $\mu$ g/ml ampicillin and incubated for 12 - 16 hours at 37°C in an orbital incubator with vigorous shaking (250rpm). A 10 litre baffle-bottomed flask was used for this purpose. The culture reached a cell density of  $1 \times 10^9$  cells per ml and the bacterial cells were harvested by centrifugation at 4000rpm for 15 minutes at 4°C using a Omnigene 2.0RS centrifuge (Heraeus Sepatech, Germany). After centrifugation, the supernatant was carefully poured out of the centrifuge bottle without disturbing the cell pellet. All traces of supernatant were removed by inverting the open centrifuge bottle until all residual medium had been drained.

A QIAfilter Giga cartridge was screwed onto a 45 mm neck glass bottle and connected to a vacuum source. The bacterial pellet was completely resuspended, leaving no cell clumps, in 125 ml of Buffer P1 (resuspension buffer). Rnase A had been added to the bottle of Buffer P1 previously, to give a final concentration of 100 $\mu$ g/ml. A volume of 125 ml of Buffer P2 (lysis buffer) was then added and mixed gently but thoroughly, to avoid the shearing of genomic DNA, by inverting the bottle four - six times. The lysate was then incubated for exactly five minutes, after which time it appeared viscous. The next step was the addition of 125 ml of chilled Buffer P3 (neutralisation buffer) followed immediately by thorough mixing until a fluffy

white material had formed and the lysate was no longer viscous. Precipitation was enhanced by the use of chilled Buffer P3. The cloudy precipitate contained genomic DNA, cell debris, proteins and SDS. Thorough mixing ensured that the QIAfilter Giga cartridge would not become clogged. The lysate was then poured into the QIAfilter Giga cartridge and incubated at room temperature for 10 minutes. A precipitate containing proteins, genomic DNA and detergent floated and formed a layer on top of the solution.

By applying a vacuum all of the liquid was pulled through the QIAfilter Giga cartridge. The vacuum source was then switched off and 50 ml of Buffer FWB (QIAfilter wash buffer), was added to the cartridge and stirred gently with a sterile spatula, to enhance the flow of liquid through the filter unit. The vacuum source was again switched on until all the liquid had been completely pulled through the unit. The glass bottle then contained the filtered lysate containing the plasmid DNA. A volume of 30 ml of Buffer ER (endotoxin removal buffer) was then added to the filtered lysate, mixed by inverting the bottle approximately 10 times, and incubated on ice for 30 minutes. The endotoxin removal buffer, consisting of sodium chloride, isopropanol and endotoxin removal agent, bound the endotoxins, cell membrane components of Gram-negative bacteria, and formed a fine precipitate which was subsequently captured by the QIAGEN-tip 10000. A QIAGEN-tip 10000 was equilibrated by applying 75 ml of Buffer QBT (equilibration buffer), and allowing the column to empty by gravity flow. The lysate was then applied to the QIAGEN-tip and allowed to enter the resin in the tip by gravity flow. The QIAGEN-tip was then washed twice with 300 ml of Buffer QC (wash buffer), to remove all contaminants. Finally, the DNA was eluted from the QIAGEN-tip using 75 ml of Buffer QN (elution buffer).

The eluted DNA was then precipitated by adding 52.5 ml isopropanol (0.7 volumes) at room temperature. The solution was mixed and centrifuged immediately at 4000rpm for 30 minutes at 4°C, using a Omnigene 2.0RS centrifuge. The supernatant was carefully decanted and the DNA pellet washed with 10 ml of endotoxin-free 70% ethanol at room temperature and then centrifuged as before for 10 minutes. The supernatant was again carefully decanted without disturbing the DNA pellet. The

pellet was air dried for approximately 30 minutes to evaporate residual traces of ethanol and was then resuspended in 2 ml of endotoxin-free Buffer TE. The DNA was incubated overnight in a 37°C water bath to aid resuspension and was mixed on a rolling device for two hours to ensure complete dissolution of the DNA pellet. The DNA solution was then quantified, sequenced and tested for endotoxin, as described below, before inoculation into cats.

#### 4.2.6.3.2 *Quantification and sequencing of DNA for vaccine trial*

The concentration and purity of the DNA prepared by the above method was determined by spectrophotometry and by estimation of double stranded DNA concentration via agarose gel electrophoresis, as described in chapter two, section 2.2.2.3. Uncut and restriction enzyme digested DNA samples were run out on agarose gels and the DNA concentration was determined. Test digests were performed on every DNA sample, to ensure that the correct restriction enzyme sites were present and that the original DNA sequence was intact. Finally, the four cytokine DNA constructs and the empty pCI-neo DNA prepared by the above method were sequenced by the Licor automated sequencing method described in section 2.2.5.2. Small scale preparations of pUSE1<sup>-</sup>CMVT(EnvA) and pUSE1<sup>-</sup>CMVT(gagpol) DNA had been sequenced by Dr Derek Bain previously.

#### 4.2.6.3.3 *Endotoxin testing of DNA for vaccine trial*

Samples of all seven DNA constructs required for the trial were submitted to Q1 Biotech Ltd. (Glasgow, U.K.) for assessment of the level of endotoxin contamination. A sample of 10µg of DNA in 10µl endotoxin free water was submitted for each construct. Endotoxin levels were measured using a chromogenic *Limulus amoebocyte lysate* (LAL) assay. The LAL assay quantifies endotoxin through its capacity to initiate clotting of *Limulus polyphemus* haemolymph.

The simplest LAL endotoxin assay is the gel-clot test which semi-quantitatively measures the endotoxin level in a test sample by comparison of the ability of the sample to form a solid clot, when mixed with the LAL enzyme and substrate,

compared to known standards. The chromogenic method replaces the clotting protein with a synthetic peptide that forms a substrate for the enzyme. The synthetic peptide is covalently attached to a chromophore, para-nitroanilide (p-NA); following pre-incubation with endotoxin, the activated enzyme cleaves p-NA to form a coloured product which can be quantified by spectrophotometry, at 405nm. The colour change is proportional to the amount of active enzyme and hence the endotoxin level of the test substance (Novitsky, 1983).

#### **4.2.7 VIRUS USED FOR CHALLENGE**

The virus used for challenge was derived from a molecular clone of FeLV-A/Glasgow-1 (Stewart et al. 1986) and was grown in feline embryo fibroblasts of the FEA cell line (Jarrett et al. 1973). The batch used, batch 53, was assayed on QN10 cells before and after inoculation of cats, as detailed in section 4.2.4.1. The virus was kept at -80 °C and thawed immediately before use. Ten-fold dilutions of frozen and thawed virus were tested in duplicate, from  $10^{-4}$  to  $10^{-6}$  and the number of foci was counted for each dilution. The titre of the virus before and after use was calculated to be  $2.2 \times 10^6$  focus forming units (ffu)/ml.

#### **4.2.8 STATISTICAL ANALYSIS**

##### **4.2.8.1 Dataset analysed**

The data consisted of five treatment groups (A to E) and 29 cats (groups A, C-E: n=6, group B: n=5). Measurements were taken at four timepoints after viral challenge. Statistical analysis was performed on three separate variables: the ELISA S/P ratio, the ELISA result (positive if ELISA optical density exceeds twice that of negative control) and the virus isolation result (positive or negative). The small number of cats in each treatment group limited the analyses, particularly for the binary variables where the tests performed had low power to detect differences

between treatment groups. Therefore, where no significant results were detected at the 5% level of significance, the 10% level of significance was also investigated.

#### **4.2.8.2 Statistical analyses: ELISA S/P ratio**

Repeated measures analysis of variance, including factors for treatment, cat (treatment), time and treatment-by-time interaction, were performed. The test for a treatment-by-time interaction indicates whether the profile of treatment mean S/P across time follows a different shape for any treatment group. This may, for example, indicate if one group showed a transient elevation not seen in other groups. Additionally, one-way analysis of variance was performed individually using the data reported for each timepoint. Pairwise comparisons were performed between all pairs of treatments using Fisher's protected LSD test: i.e. if the overall F-test was significant, t-tests between treatment means were performed at the same level of significance.

#### **4.2.8.3 Statistical analyses: ELISA result and virus isolation result**

Fisher's exact test was performed at each timepoint on the 2 x 5 contingency tables. When a significant overall test was detected, pairwise Fisher's exact tests were performed between all pairs of treatments. In addition, for the virus isolation data, profiles were summarised as negative, transient or positive. "Negative" was defined as those profiles with a negative result at all four timepoints, "transient" as a positive result that disappears at later timepoints, and "positive" as a result that becomes and remains positive by a certain timepoint. These data were analysed using an additional Fisher's exact test.

## **4.3 RESULTS**

### **4.3.1 THE YIELD, PURITY AND ENDOTOXIN LEVELS OF PLASMID DNA USED FOR VACCINE TRIAL**

The yields, purity and endotoxin levels of the DNA prepared using Qiagen's Endofree plasmid Giga kits (Qiagen, UK), were determined by the methods outlined in sections 2.2.2.3. and 4.2.5.3. The yields were calculated using spectrophotometry, and by estimation of double stranded DNA concentration via agarose gel electrophoresis. The results obtained by each method were in good agreement. The majority of the DNA yields obtained were greater than the maximum 10mg stated in the manufacturer's protocol.

The purity of all the DNA samples was estimated using the ratio of the OD readings at 260nm and 280nm ( $OD_{260}/OD_{280}$ ). Pure preparations of DNA are known to have an  $OD_{260}/OD_{280}$  of 1.8; a lower value suggests possible protein or phenol contamination. The DNA prepared for the trial had  $OD_{260}/OD_{280}$  readings of between 1.88 and 1.91 indicating that the DNA was of high purity. Endotoxin levels in all samples of DNA were almost undetectable; less than 0.5 EU/ml of DNA in endotoxin free water, at a concentration of 1mg DNA/ml. Therefore, less than 0.5EU were present in each milligram of plasmid DNA. No local or systemic reactions were observed in any of the kittens, following immunisation with this ultrapure, endotoxin free DNA. Table 4.8. illustrates the results.

CONSTRUCT	YIELD OF DNA	ESTIMATE OF PURITY OF DNA OD <sub>260</sub> /OD <sub>280</sub>	ENDOTOXIN LEVELS
pUSE1 <sup>-</sup> CMVT(EnvA)	11.2mg in 2ml TE	1.9	<0.5 EU/ml
pUSE1 <sup>-</sup> CMVT(gagpol)	13.3mg in 2ml TE	1.91	<0.5 EU/ml
pCI-neo + IFN- $\gamma$	14.8mg in 2ml TE	1.91	<0.5 EU/ml
pCI-neo + IL-12 p35	17.6mg in 2ml TE	1.88	<0.5 EU/ml
pCI-neo + IL-12 p40	19.1mg in 2ml TE	1.90	<0.5 EU/ml
pCI-neo + IL-18	18.8mg in 2ml TE	1.90	<0.5 EU/ml
Empty pCI-neo	8.5mg in 2ml TE	1.90	<0.5 EU/ml

**Table 4.8. Purity, yield and endotoxin levels of the DNA prepared for the vaccine trial**

#### **4.3.2 INFECTIOUS VIRUS IS NOT PRODUCED AS A RESULT OF HOMOLOGOUS RECOMBINATION BETWEEN FeLV VACCINE ANTIGENS AND ENDOGENOUS RETROVIRAL SEQUENCES**

From the pre-trial, post-vaccination and day of challenge heparinised blood samples, plasma and PBMCs were isolated and the former was used to determine levels of plasma infectious virus and FeLV p27 antigen. These samples were analysed for the presence of virus and p27 antigen during the immunisation period mainly because there was a concern that the FeLV antigen component of the vaccine might be able to recombine with exogenous non-pathogenic or endogenous feline retroviral sequences, enFeLV, described in section 1.1.4 and 1.1.5. In theory, replication competent virions could then be produced, and FeLV infection established in the cats.

As detailed in section 4.2.5.2., a positive is defined as an ELISA OD reading greater than twice the value of the negative control ELISA OD and none of the pre-challenge ELISA OD readings fell into this category. Virus isolation and p27 ELISA results were consistently negative for all blood samples collected at all timepoints, pre-viral challenge, as illustrated in table 4.9. This indicated that cats were free from FeLV infection and suggested that a recombination event, between vaccine antigens and exogenous non-pathogenic or endogenous retroviral sequences, generating replication competent virus, had not occurred. Moreover, no local or systemic reactions were observed in any of the kittens after immunisation.

Cat No.	Pre-trial bleed 09-Jun			2 days post 1st vaccination 11-Jun		2 days post 2nd vaccination 25-Jun		2 days post 3rd vaccination 09-Jul		Day of viral challenge; three weeks after last vaccination 28-Jul				
	ELISA O.D	V.I	VNA	ELISA O.D	V.I	ELISA O.D	V.I	ELISA O.D	V.I	ELISA O.D	V.I	VNA	W.B	GP70
L 1	0.048	-	0	0.051	-	0.054	-	0.053	-	0.053	-	0	-	-
L 2	0.047	-	0	0.052	-	0.057	-	0.049	-	0.050	-	0	-	-
L 3	0.048	-	0	0.050	-	0.052	-	0.051	-	0.055	-	0	-	-
L 4	0.046	-	0	0.052	-	0.055	-	0.051	-	0.054	-	0	-	-
L 5	0.049	-	0	0.056	-	0.053	-	0.052	-	0.052	-	0	-	-
L 6	0.055	-	0	0.060	-	0.056	-	0.054	-	0.055	-	0	-	-
L 8	0.053	-	0	0.053	-	0.055	-	0.056	-	0.054	-	0	-	-
L 9	0.052	-	0	0.062	-	0.053	-	0.053	-	0.064	-	0	-	-
L 10	0.054	-	0	0.076	-	0.056	-	0.055	-	0.049	-	0	-	-
L 11	0.053	-	0	0.056	-	0.052	-	0.050	-	0.045	-	0	-	-
L 12	0.054	-	0	0.061	-	0.055	-	0.051	-	0.047	-	0	-	-
L 13	0.056	-	0	0.054	-	0.050	-	0.051	-	0.047	-	0	-	-
L 14	0.054	-	0	0.055	-	0.050	-	0.051	-	0.048	-	0	-	-
L 15	0.057	-	0	0.063	-	0.049	-	0.055	-	0.049	-	0	-	-
L 16	0.047	-	0	0.061	-	0.050	-	0.057	-	0.052	-	0	-	-
L 17	0.047	-	0	0.055	-	0.050	-	0.053	-	0.058	-	0	-	-
L 18	0.046	-	0	0.052	-	0.048	-	0.052	-	0.052	-	0	-	-
L 19	0.048	-	0	0.055	-	0.049	-	0.052	-	0.052	-	0	-	-
L 20	0.050	-	0	0.054	-	0.054	-	0.059	-	0.049	-	0	-	-
L 21	0.047	-	0	0.057	-	0.050	-	0.056	-	0.051	-	0	-	-
L 22	0.050	-	0	0.059	-	0.050	-	0.060	-	0.052	-	0	-	-
L 23	0.050	-	0	0.064	-	0.050	-	0.058	-	0.054	-	0	-	-
L 24	0.053	-	0	0.054	-	0.048	-	0.053	-	0.054	-	0	-	-
L 25	0.050	-	0	0.055	-	0.051	-	0.050	-	0.058	-	0	-	-
L 26	0.051	-	0	0.065	-	0.060	-	0.051	-	0.052	-	0	-	-
L 27	0.052	-	0	0.053	-	0.052	-	0.051	-	0.051	-	0	-	-
L 28	0.053	-	0	0.053	-	0.057	-	0.054	-	0.049	-	0	-	-
L 29	0.053	-	0	0.052	-	0.055	-	0.050	-	0.062	-	0	-	-
L 30	0.052	-	0	0.058	-	0.057	-	0.053	-	0.069	-	0	-	-
+	1.514			1.562		1.533		1.775		1.670				
-	0.068			0.114		0.079		0.077		0.054				

**Table 4.9. Table of pre-challenge, (pre-trial and post-immunisation), p27 ELISA and VI results, and day of challenge p27 ELISA, VI, anti-gp70 ELISA, VN Ab assay and western blot analysis of serum against complete viral lysate results.**

ELISA O.D, p27 ELISA OD reading; VI, virus isolation result; VNA, virus neutralising antibody titre; GP70, anti-gp70 antibody ELISA result; W.B, western blot analysis of serum against complete viral lysate result.

### 4.3.3 PROTECTION AGAINST TRANSIENT, PERSISTENT AND LATENT FeLV INFECTION

#### 4.3.3.1 The FeLV DNA vaccine does not provide protection against persistent FeLV infection

Sampling began three weeks after viral challenge (week eight), and was repeated on three occasions, approximately three weeks apart, until week 21 (weeks 11, 14, 17 and 21). A final pre-euthanasia sample, at week 23-24, was also collected (cats L21, 23, 24 and 28 were not euthanased). The main objective was to analyse the blood samples for evidence of FeLV infection to determine if vaccination had protected the cats from either transient or persistent viraemia. This was done by means of p27 ELISA and virus isolation (VI) on QN10 cells (section 4.2.4.1. and 4.2.4.2.); positive VI and p27 results indicated FeLV infection. Virus neutralising antibody titres were also measured at each timepoint as a significant post-challenge VN titre usually correlates with protection (Hoover et al. 1976). Table 4.10 lists the DNA constructs which each group of cats received. Table 4.11 illustrates the virus isolation, p27 ELISA and VN Ab assay results of the first and second post-challenge bleeds, for each individual cat (three and six weeks post-challenge, respectively), while table 4.12 illustrates results of the third and fourth post-challenge bleeds, for each individual cat (nine and thirteen weeks post-challenge, respectively). Table 4.13. and 4.14, meanwhile, summarise the virus isolation and p27 ELISA results, respectively, for each group of cats at defined timepoints, post-viral challenge.

GROUPS	CONSTRUCTS	NUMBER OF CATS
A L1-6	CMV-gag-pol + CMV-env A	6
B L7-12	CMV-gag-pol + CMV-env A + IFN- $\gamma$	5
C L13-18	CMV-gag-pol + CMV-env A + IL-12	6
D L25-30	CMV-gag-pol + CMV-env A + IL-12 + IL-18	6
E L19-24	Empty pCI-neo plasmid	6

**Table 4.10. Immunisation groups**

	Cat	Group	Week 11; 3 weeks P/C					Week 14; 6 weeks P/C				
			18-Aug		ELISA		08-Sep		ELISA		V.I	VNA
			ELISA O.D	ELISA S.P	RESULT	V.I	VNA	ELISA O.D	ELISA S.P	RESULT		
L 1	A	0.056	-0.007	-	-	8	0.054	-0.013	-	-	>64	
L 2	A	0.587	0.350	+	-	0	0.766	0.358	+	-	0	
L 3	A	0.059	-0.005	-	-	0	0.064	-0.008	-	-	0	
L 4	A	0.206	0.094	+	-	0	0.057	-0.011	-	-	0	
L 5	A	1.538	0.991	+	+	0	1.640	0.813	+	+	0	
L 6	A	0.329	0.177	+	+	0	0.074	-0.003	-	-	0	
L 8	B	0.067	0.000	-	-	0	0.067	-0.006	-	-	0	
L 9	B	1.275	0.814	+	+	0	1.063	0.513	+	+	0	
L 10	B	1.192	0.758	+	+	0	1.273	0.622	+	+	0	
L 11	B	0.205	0.093	+	-	0	0.067	-0.006	-	-	0	
L 12	B	0.069	0.001	-	-	0	0.070	-0.005	-	-	0	
L 13	C	0.217	0.101	+	-	0	0.069	-0.005	-	-	16	
L 14	C	1.556	1.003	+	+	0	1.564	0.774	+	+	0	
L 15	C	0.681	0.414	+	+	0	1.527	0.755	+	+	0	
L 16	C	0.084	0.011	-	-	0	0.076	-0.002	-	-	8	
L 17	C	1.614	1.042	+	+	0	1.419	0.698	+	+	0	
L 18	C	0.940	0.588	+	+	0	1.284	0.628	+	+	0	
L 19	E	0.161	0.063	+	-	0	0.094	0.008	-	-	0	
L 20	E	0.794	0.490	+	+	0	0.089	0.005	-	-	16	
L 21	E	1.291	0.825	+	+	0	1.608	0.797	+	+	0	
L 22	E	1.369	0.877	+	+	0	0.863	0.409	+	-	0	
L 23	E	1.337	0.856	+	+	0	1.175	0.571	+	+	0	
L 24	E	1.317	0.842	+	+	0	1.711	0.850	+	+	0	
L 25	D	0.129	0.042	-	-	16	0.065	-0.007	-	-	16	
L 26	D	0.066	-0.001	-	-	0	0.070	-0.005	-	-	0	
L 27	D	0.099	0.022	-	-	0	0.071	-0.004	-	-	0	
L 28	D	0.104	0.025	-	-	0	0.069	-0.005	-	-	0	
L 29	D	0.087	0.013	-	-	0	0.077	-0.001	-	-	0	
L 30	D	0.280	0.144	+	-	0	0.080	0.001	-	-	8	
	+	control	1.551				1.998					
	-	control	0.067				0.079					

**Table 4.11. Table of VI, p27 ELISA and VN Ab assay results for first and second post-challenge bleeds; three and six weeks post-challenge, respectively**

**ELISA O.D**, p27 ELISA OD reading; **ELISA S.P**, p27 ELISA sample to positive ratio; **VI**, virus isolation result; **VNA**, virus neutralising antibody titre.

		Week 17; 9 weeks P/C						Week 21; 13 weeks P/C						Week
	Cat		02-Oct		ELISA			27-Oct		ELISA			23-24	
	No	Group	ELISA O.D	ELISA S.P	RESULT	V.I	VNA	ELISA O.D	ELISA S.P	RESULT	V.I	VNA	VI	
L	1	A	0.053	-0.001	-	-	>64	0.05	-0.003	-	-	1024	-	
L	2	A	1.515	0.831	+	+	0	1.163	0.728	+	+	0	+	
L	3	A	0.070	0.009	-	-	16	0.05	-0.003	-	-	32	-	
L	4	A	0.063	0.005	-	-	16	0.05	-0.003	-	-	128	-	
L	5	A	1.455	0.797	+	+	0	0.971	0.602	+	+	0	+	
L	6	A	0.062	0.004	-	-	32	0.054	-0.001	-	-	128	-	
L	8	B	0.055	0.000	-	-	0	0.056	0.001	-	-	16	-	
L	9	B	1.231	0.669	+	+	0	0.968	0.600	+	+	0	+	
L	10	B	1.476	0.809	+	+	0	0.942	0.583	+	+	0	+	
L	11	B	0.062	0.004	-	-	0	0.056	0.001	-	-	0	-	
L	12	B	0.062	0.004	-	-	0	0.058	0.002	-	-	0	-	
L	13	C	0.065	0.006	-	-	>64	0.058	0.002	-	-	512	-	
L	14	C	1.567	0.861	+	+	0	1.198	0.751	+	+	0	+	
L	15	C	1.683	0.927	+	+	0	1.065	0.664	+	+	0	+	
L	16	C	0.052	-0.002	-	-	>64	0.053	-0.001	-	-	1024	-	
L	17	C	0.893	0.477	+	-	0	0.597	0.356	+	-	0	+	
L	18	C	1.384	0.756	+	+	0	0.975	0.605	+	+	0	+	
L	19	E	0.057	0.001	-	-	32	0.054	-0.001	-	-	128	-	
L	20	E	0.074	0.011	-	-	>64	0.057	0.001	-	-	512	-	
L	21	E	1.760	0.970	+	+	0	1.345	0.848	+	+	0	+	
L	22	E	0.162	0.061	+	-	8	0.104	0.032	-	-	128	-	
L	23	E	1.475	0.808	+	+	0	1.005	0.625	+	+	0	+	
L	24	E	1.503	0.824	+	+	0	1.205	0.756	+	+	0	+	
L	25	D	0.056	0.001	-	-	>64	0.056	0.001	-	-	1024	-	
L	26	D	0.056	0.001	-	-	16	0.053	-0.001	-	-	64	-	
L	27	D	0.058	0.002	-	-	32	0.055	0.000	-	-	128	-	
L	28	D	0.061	0.003	-	-	16	0.056	0.001	-	-	64	-	
L	29	D	0.066	0.006	-	-	8	0.058	0.002	-	-	32	-	
L	30	D	0.086	0.018	-	-	32	0.063	0.005	-	-	64	-	
	+	control	1.812					1.576						
	-	control	0.055					0.055						

**Table 4.12. Table of VI, p27 ELISA and VN Ab assay results for third and fourth post-challenge bleeds; nine and thirteen weeks post-challenge, respectively.**

**ELISA O.D**, p27 ELISA OD reading; **ELISA S.P**, p27 ELISA sample to positive ratio; **VI**, virus isolation result; **VNA**, virus neutralising antibody titre.

Group	No.of cats	Number of cats VI positive at week post-challenge				
		3	6	9	13	15-16
A	6	2/6	1/6	2/6	2/6	2/6
B	5	2/5	2/5	2/5	2/5	2/5
C	6	4/6	4/6	3/6	3/6	4/6
D	6	0/6	0/6	0/6	0/6	0/6
E	6	5/6	3/6	3/6	3/6	3/6

**Table 4.13. Virus isolation results for each group of cats at defined timepoints, post-viral challenge. N.B. viral challenge was performed at week 8 of the trial.**

Group	No.of cats	Number of cats p27 antigen positive at week post-challenge			
		3	6	9	13
A	6	4/6	2/6	2/6	2/6
B	5	3/5	2/5	2/5	2/5
C	6	5/6	4/6	4/6	4/6
D	6	1/6	0/6	0/6	0/6
E	6	6/6	4/6	4/6	3/6

**Table 4.14. p27 ELISA results for each group of cats at defined timepoints, post-viral challenge. N.B. viral challenge was performed at week 8.**

Firstly, it should be noted that the small numbers of cats in each group and the results obtained precluded definitive statistical analyses. Analysing ELISA S/P data,

the test for a treatment-by-time interaction indicated that group E, the control group, had the greatest proportion of transient infections at the first post-challenge bleed (3 weeks post-challenge) and the sharpest decline between the first and second post-challenge bleeds (between week three and week six).

Virus isolation (VI) is regarded as the definitive method of diagnosing FeLV infection. When comparing the number of cats that were virus isolation positive in group A, the FeLV DNA vaccine alone group, with group E, the control group, (table 4.13.), it appeared that, initially, the vaccine was providing some degree of protection against FeLV infection. At the first timepoint, three weeks post viral challenge, week 11, only two cats in group A were VI positive, while five in group E were VI positive. There was not, however, a statistically significant difference between these two groups, in terms of p27 ELISA and virus isolation results (positive or negative), at this or any other timepoint during the trial. By the end of the trial, week 23-24, the number of cats that were VI positive in each group was almost identical; two cats in group A were VI positive, while three in group E were VI positive. The p27 ELISA results (table 4.14), displayed a similar trend. Therefore, it was concluded that the FeLV DNA vaccine alone did not provide significant protection against persistent or transient FeLV infection.

#### **4.3.3.2 Cytokine DNA constructs act as vaccine adjuvants, enhancing protection against persistent FeLV infection**

Firstly, an analysis of the mean p27 ELISA S/P profiles indicated that group D had consistently the lowest S/P values which were close to zero. Pairwise comparisons between mean p27 ELISA S/P values for each group indicated that the mean of group D (vaccine + IL-12 + IL-18) was lower than that of group C (vaccine + IL-12) or group E (controls);  $p < 0.1$ . Statistical analysis of p27 ELISA results (positive or negative, table 4.14.), using Fisher's exact test, and performing pairwise comparisons between groups, revealed that group D possessed significantly fewer positives than groups C and E at weeks 11, 14 and 17 (3, 6 and 9 weeks post-challenge);  $p < 0.1$ . Group D also possessed significantly fewer positives than group E, at week 11;  $p < 0.05$ .

Similarly, analysis of virus isolation results (table 4.13.) revealed that at week 11 group D possessed significantly fewer positives than groups C and E ( $p < 0.1$ ) and significantly fewer positives than group E ( $p < 0.05$ ). In fact, no cat in group D ever tested VI positive (table 4.11., 4.12. and 4.13) and only one cat in this group, L30, tested weakly p27 ELISA positive at the first timepoint (table 4.11. and 4.14.). Therefore the combination of the FeLV DNA vaccine and IL-12 and IL-18 DNA constructs protected cats against the development of transient and persistent viraemia. Due to the small number of cats in each group, a statistically significant difference was not identified between the number of cats virus isolation positive in group A, the vaccine alone group, and group D, the vaccine and IL-12 and IL-18 group. However, at the end of the trial, 2/6 cats in group A were virus isolation positive, as opposed to 0/6 virus isolation positive in group D. Therefore, it was concluded that the IL-12 and IL-18 combination was acting as a potent adjuvant providing protection against the development of both transient and persistent viraemia.

The cats in group B were immunised with the FeLV DNA vaccine and the IFN- $\gamma$  construct. At all four post-challenge samples and the pre-euthanasia bleed, 2/5 cats in this group tested virus isolation and p27 ELISA positive. Statistical analysis as above revealed no significant difference between the number of cats p27 ELISA or virus isolation positive in this group and the control group E. Similarly, no significant difference was detected between the number of cats p27 ELISA and virus isolation positive in group B and group A, the FeLV DNA vaccine alone group. Therefore, it was concluded that the cats in group B were not protected from the development of transient and persistent viraemia and that IFN- $\gamma$  was not an effective vaccine adjuvant in this system.

The cats in group C were immunised with the novel FeLV DNA vaccine and the IL-12 construct. As detailed above, a statistically significant difference was detected between the number of cats p27 ELISA and virus isolation positive in this group and group D (vaccine + IL-12 + IL-18), in that group D had significantly fewer positive virus isolation results than groups C and E, at week 11 ( $p < 0.1$ ). In fact, throughout

the trial the number of cats virus isolation and p27 ELISA positive in group C was similar to that of group E (the control group) and was consistently, although not statistically significantly, higher than that of group A (vaccine alone group), especially at the first two post-challenge sampling timepoints. These results indicate that IL-12 was not an effective vaccine adjuvant in this system and suggest that the inclusion of IL-12 DNA constructs with the novel FeLV DNA vaccine may actually reduce vaccine efficacy. Interestingly, the VI status of one of the cats in group C, L17, changed from negative to positive, in the time between the fourth post-challenge bleed and the pre-euthanasia bleed, which was between two and three weeks. L17 had been VI and p27 ELISA positive at the first and second post-challenge bleeds, had been discordant (p27 ELISA positive and VI negative) at the third and fourth post-challenge bleeds and had never possessed detectable virus neutralising antibodies. The reasons for the changes in p27 ELISA and VI status in this cat are considered in the discussion.

#### **4.3.3.3 The FeLV DNA vaccine does not provide protection against the development of latent FeLV infection**

Bone marrow biopsies were obtained from all non-viraemic cats and from several viraemic cats, to act as positive controls. The cells obtained from these biopsies were cultured and the resulting cultures were screened for the presence of FeLV by virus isolation and p27 ELISA to determine if ostensibly immune, non-viraemic cats, harboured latent FeLV infection in bone marrow. Table 4.15. illustrates the number of cats in each group which were persistently viraemic or latently infected at the termination of the trial (15-16 weeks post viral challenge) while table 4.16. illustrates the results of p27 ELISA and VI performed on bone marrow supernatants and cultured bone marrow supernatants and cell lysates.

Group	Number of cats	Positive VI on plasma; persistent viraemia	Positive VI on uncultured bone marrow supernatants	Positive VI on cultured bone marrow from non-viraemic cats; latent infection	Combined total of persistent and latent infection
A	6	2\6	2\6	2\6	4\6
B	5	2\5	2\5	0\5	2\5
C	6	4\6	4\6	0\6	4\6
D	6	0\6	0\6	1\6	1\6
E	6	3\6	3\6	2\6	5\6

**Table 4.15. Number of cats persistently viraemic and latently infected in each group at the termination of trial; 15-16 weeks post viral challenge.**

**Key: VI, virus isolation.**

		ELISA test results on BM cell lysates				VI on QN10 cells after					
		Following 1 passage of BM fibroblasts				1 passage of BM fibroblasts				Total clinical	
		Lutz ELISA									
Group	Date	ELISA O.D.	ELISA S/P ratio	ELISA result	BMSN VI-1	BMSN VI-2	TCF VI-1	TCF VI-2	Clinical Score	Out of 7	score for each group
L 1	A	11/11/98	0.049	<0	-	-	-	-	-	0	20
L 2	A	18/11/98	1.21	1.04	+	+	+	+	+	7	
L 3	A	11/11/98	0.046	<0	-	-	-	-	-	0	
L 4	A	11/11/98	1.32	1.11	+	-	-	+	+	3	
L 5	A	VI +	N/D	N/D	N/D	N/D	N/D	N/D	N/D	7	
L 6	A	18/11/98	1.292	1.11	+	-	-	+	+	3	
L 8	B	20/11/98	0.049	<0	-	-	-	-	-	0	14
L 9	B	VI +	N/D	N/D	N/D	N/D	N/D	N/D	N/D	7	
L 10	B	VI +	N/D	N/D	N/D	N/D	N/D	N/D	N/D	7	
L 11	B	20/11/98	0.05	<0	-	-	-	-	-	0	
L 12	B	20/11/98	0.054	0.003	-	-	-	-	-	0	
L 13	C	20/11/98	0.056	0.004	-	-	-	-	-	0	28
L 14	C	VI +	N/D	N/D	N/D	N/D	N/D	N/D	N/D	7	
L 15	C	VI +	N/D	N/D	N/D	N/D	N/D	N/D	N/D	7	
L 16	C	20/11/98	0.054	0.003	-	-	-	-	-	0	
L 17	C	20/11/98	1.233	1.06	+	+	+	+	+	7	
L 18	C	17/11/98	1.388	1.2	+	+	+	+	+	7	
L 19	E	11/11/98	0.107	0.05	-	-	-	-	-	0	27
L 20	E	11/11/98	0.947	0.78	+	-	-	+	+	3	
L 21	E	11/11/98	1.062	0.89	+	+	+	+	+	7	
L 22	E	11/11/98	1.291	1.09	+	-	-	+	+	3	
L 23	E	11/11/98	1.451	1.23	+	+	+	+	+	7	
L 24	E	11/11/98	1.248	1.05	+	+	+	+	+	7	
L 25	D	18/11/98	0.061	0.01	-	-	-	-	-	0	1
L 26	D	18/11/98	0.062	0.01	-	-	-	-	-	0	
L 27	D	18/11/98	0.072	0.02	-	-	-	-	-	0	
L 28	D	11/11/98	0.084	0.03	-	-	-	-	+	1	
L 29	D	18/11/98	0.074	0.02	-	-	-	-	-	0	
L 30	D	18/11/98	0.085	0.03	-	-	-	-	-	0	

**Table 4.16. Results of p27 ELISA and VI performed on bone marrow supernatants and cultured bone marrow supernatants and cell lysates.**

**Key:** N/D, not done as confirmed plasma VI positive; **BM**, bone marrow; **BMSN**, **uncultured** bone marrow supernatant; **TCF**, tissue culture fluid (supernatant) of **cultured** bone marrow cells.

VI is performed on **uncultured** bone marrow supernatants and **cultured** bone marrow supernatants, the latter procedure in an attempt to identify the presence of latent FeLV infection. **Cultured** bone marrow cell lysates are also tested for the presence of p27 antigen by Lutz' commercial ELISA, to further confirm the presence of latent infection.

Cats with latent FeLV infections are by definition non-viraemic but their bone marrow cells release infectious virus when cultured for a short period *in vitro* (Rojko et al. 1982). There were insufficient non-viraemic cats in each group to consider whether the vaccine alone protected against the development of latent FeLV infection. More relevant in determining vaccine efficacy was the combined total of cats persistently and latently infected at the end of the trial in group A, the vaccine alone group, compared with the group E, the control group. Considering the results in table 4.15. and 4.16., it appeared that the vaccine alone did not protect against the development of persistent or latent FeLV infection, as the combined total of cats persistently and latently infected with FeLV at the end of the trial was almost the same in groups A and E; 4\6 cats compared with 5\6 cats, respectively. Two of six cats in each of these groups harboured latent FeLV infection in bone marrow.

#### **4.3.3.4 Cytokine DNA constructs act as vaccine adjuvants, enhancing protection against latent FeLV infection**

Again, there were insufficient non-viraemic cats in each group to consider whether the vaccine and cytokine DNA constructs protected against latent FeLV infection alone. More relevant in determining how effective the cytokines were in enhancing vaccine efficacy, and thus protecting against FeLV challenge, was the combined total of cats persistently and latently infected at the end of the trial in groups D (the vaccine and IL-12 and IL-18 combination group), B (vaccine and IFN- $\gamma$  group), and C (vaccine and IL-12 group), compared with group E, (the control group), and group A, (the vaccine alone group).

Considering the results displayed in tables 4.15. and 4.16., it appeared that no cats in group C were latently infected, although four out of six were persistently viraemic, at the end of the trial. The combined total of cats persistently and latently infected, at trial termination, in group C was almost identical to that of group A (vaccine alone) and group E (the control group); 4\6, 4\6 and 5\6, respectively. Thus, it appeared that the IL-12 DNA constructs were not effective vaccine adjuvants in this system and that the IL-12 and FeLV DNA vaccine combination did not protect cats against the development of persistent or latent FeLV infection. Similarly the cats receiving

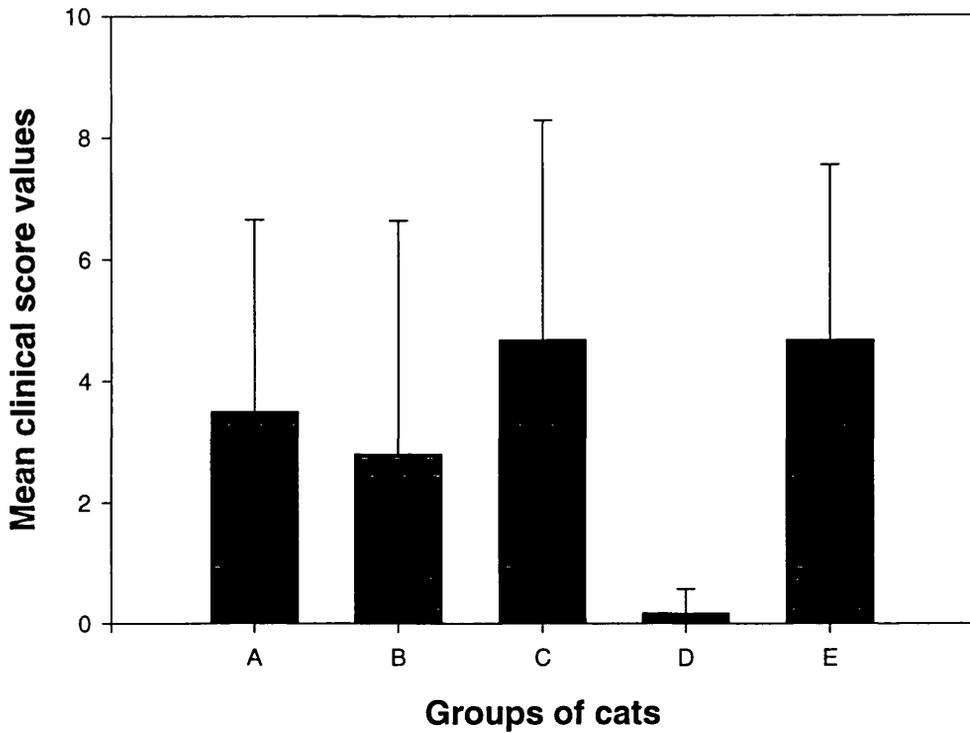
vaccine and IFN- $\gamma$  were not protected against the development of persistent or latent FeLV infection; at the end of the trial two cats in this group were persistently viraemic, while none were latently infected. The combined total of cats persistently and latently infected in this group, 2\5, was not statistically significantly different to the totals in groups A, C and E, as detailed above. Thus, IFN- $\gamma$  was not an effective vaccine adjuvant in this system.

In contrast, analysing the results displayed in tables 4.15. and 4.16., it appeared that only one cat in group D was latently infected (L28) and no cats were persistently viraemic, at the end of the trial. Moreover, the cultured bone marrow supernatant of L28 tested virus isolation positive only after subculture. The combined total of cats persistently and latently infected, at trial termination, in group D was much lower than either group A (vaccine alone), or group E (control group); 1\6, 4\6 and 5\6 respectively. Thus, co-inoculated IL-12 and IL-18 DNA constructs were acting as potent vaccine adjuvants, and the vaccine, IL-12 and IL-18 combination was protecting against the development of transient and persistent viraemia and latent FeLV infection.

#### **4.3.3.5 Scoring system to represent the FeLV status of cats at trial termination**

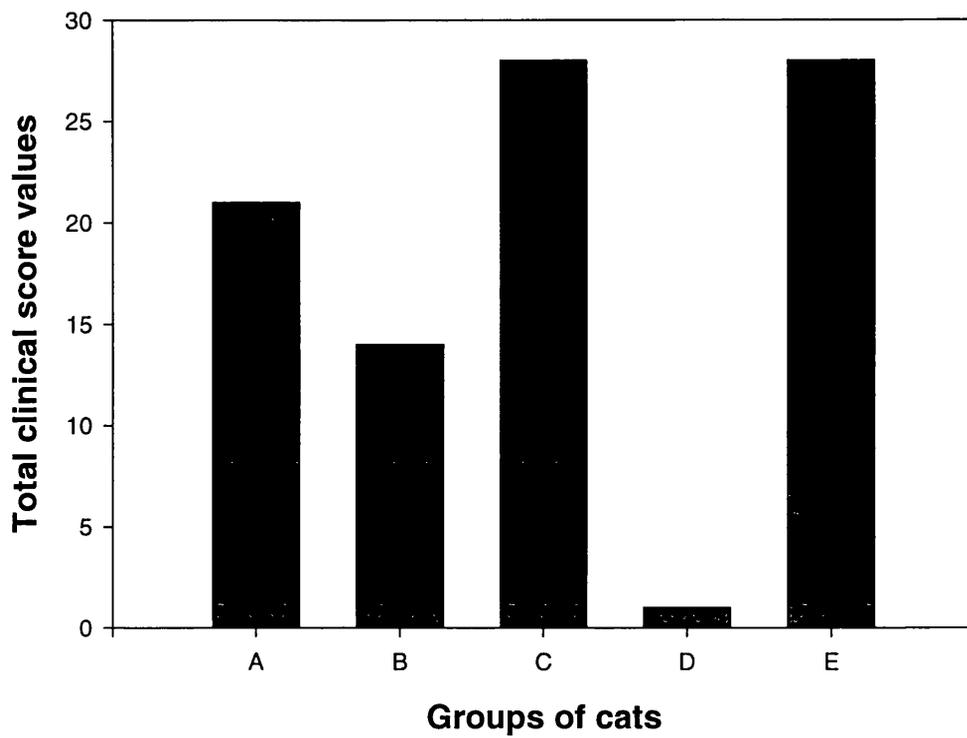
A scoring system was developed, to rank the groups in terms of overall vaccine efficiency. One point was assigned to each of seven individual parameters, to give a maximum possible total of seven points per cat. A positive result in any of the seven individual parameters counted as one point and plasma and bone marrow samples were collected for analysis at 15-16 weeks post-challenge. The seven parameters were; p27 ELISA results on cultured bone marrow cell lysates (Lutz's ELISA), primary virus isolation (VI-1) and sub-culture (VI-2) results on plasma, primary virus isolation (VI-1) and sub-culture (VI-2) results on uncultured bone marrow supernatants and primary virus isolation (VI-1) and sub-culture (VI-2) results on cultured bone marrow supernatants. (VI-1 represents the virus isolation result on QN10 cells, while VI-2 represents the virus isolation result after subculture, following one passage of QN10 cells).

This system, then, is an indicator of how well each group of cats was protected from persistent and latent FeLV infection, at the end of the trial. Two graphs, illustrated in figures 4.5. and 4.6., have been created to show, respectively, the mean clinical score values and the combined total clinical score values, for each group of cats. The mean clinical score value for each group was obtained by adding together the clinical score values of all the cats in a group and simply calculating the mean, while the total clinical score values were obtained by adding together the clinical score values of all the cats in a group, and calculating the total. The graph of mean clinical score values for each group of cats has standard deviation bars, to illustrate the degree of variance in scores within individual groups. Correlating well with the results described in previous sections, mean and total clinical score values for groups C (vaccine and IL-12) and E (the control group), were very similar, again suggesting that the inclusion of IL-12 DNA constructs with the novel FeLV DNA vaccine may actually reduce vaccine efficacy. These graphs clearly show that the cats in group D, receiving the FeLV DNA vaccine and IL-12 and IL-18 constructs, had much lower mean and total clinical score values, than the other four groups, and were therefore better protected from persistent and latent FeLV infection.



**Figure 4.5. Graph of mean clinical score values for each group of cats**

**Key: A = vaccine alone, B = vaccine + IFN- $\gamma$ , C = vaccine + IL-12,  
D = vaccine + IL-12 + IL-18 and E = empty pCI-neo plasmid.**



**Figure 4.6. Graph of total clinical score values for each group of cats**

**Key: A = vaccine alone, B = vaccine + IFN- $\gamma$ , C = vaccine + IL-12,  
D = vaccine + IL-12 + IL-18 and E = empty pCI-neo plasmid.**

#### **4.3.4 IMMUNISATION WITH DNA DOES NOT ELICIT VIRUS NEUTRALISING OR NON-NEUTRALISING ANTIBODIES; DEVELOPMENT OF PRECHALLENGE ANTIBODIES DOES NOT CORRELATE WITH PROTECTION AGAINST FeLV CHALLENGE**

From the day of challenge heparinised blood samples, plasma and PBMCs were isolated and the former was used to determine levels of FeLV specific virus neutralising antibodies. From a plain tube blood sample, serum was isolated and tested for anti-FeLV gp70 antibodies by western blotting against complete viral lysate and an anti-gp70 ELISA assay. VN and non-VN antibody titres were measured to determine if the vaccine could generate an antibody response that would correlate with protection. It was decided to test the day of challenge plasma and serum samples first and if detectable levels of antibody were identified, to test the three post-immunisation samples, retrospectively

Table 4.9. illustrates that neither virus neutralising nor non-neutralising antibodies were elicited in any of the cats by FeLV DNA immunisation. All day of challenge plasma and serum samples were negative for presence of VN antibodies, anti-gp70 antibodies and antibodies to other viral proteins. However, obviously some cats were protected against viral challenge, so development of pre-challenge antibodies did not correlate with protection against persistent FeLV infection. In particular the FeLV antigen and IL-12 and IL-18 combination of DNA constructs was able to provide protection against FeLV challenge, in the absence of a detectable pre-challenge VN or non-VN antibody response. Significant VN antibody titres only developed three to six weeks after viral challenge. Protection may therefore have involved the induction of cytotoxic T cells and cellular immunity, or alternatively, the vaccine may have primed the immune response to produce protective VN antibody titres. However, the possibility that, anti-DNA or anti-cytokine antibodies may have overwhelmed the FeLV specific humoral immune response, cannot be discounted and will be considered in the discussion.

#### **4.3.5 DEVELOPMENT OF POST-CHALLENGE VN ANTIBODIES APPEARS TO CORRELATE WITH PROTECTION AGAINST FeLV INFECTION**

There was a definite correlation between the development of post-challenge virus neutralising antibodies and protection against FeLV infection, as expected (Hoover et al. 1976). Tables 4.11 and 4.12 clearly illustrate that cats protected from persistent viraemia, defined as cats which were virus isolation negative at week 21 (13 weeks P/C), possessed a significant virus neutralising antibody titre. Similarly, cats which tested virus isolation positive at this time did not possess a detectable virus neutralising antibody titre. The only exceptions were cats L8, L11 and L12 (all group B, vaccine and IFN- $\gamma$  group), and cat L17, (group C, vaccine and IL-12 group); although these cats tested virus isolation negative at week 21, they possessed either a very low (cat L8) or undetectable VN antibody titre (cat L11, L12 and L17). The latter cat, L17, was discordant at week 17 and week 21 and became virus isolation positive at week 23 (pre-euthanasia bleed). However, cats L8, L11 and L12 consistently tested virus isolation negative throughout the trial and at the pre-euthanasia bleed.

#### **4.3.6 FeLV INFECTION PRODUCES HAEMATOLOGICAL CHANGES**

Haematological analysis of blood was conducted, at the timepoints described in section 4.1.3., (FeLV DNA vaccine trial design), to establish if the development of FeLV infection produced any changes in normal haematological parameters. The haematology results are displayed in the appendix.

The main change observed was a profound neutropenia in four persistently infected cats, L10 (group B, vaccine and IFN- $\gamma$ ), L18 (group C, vaccine and IL-12), L2 (group A, vaccine alone) and L9 (group B, vaccine and IFN- $\gamma$ ), six weeks after viral challenge, at the second post-challenge bleed. Additionally, cat L9 also exhibited a low total white blood cell and neutrophil count three weeks after viral challenge, at the first post-challenge bleed. L9, L10 and L18 tested virus isolation positive at the

first post-challenge bleed, and remained so, while L2 was discordant (p27 ELISA positive, but virus isolation negative), at the first and second post-challenge bleeds, then became virus isolation positive for the remainder of the trial.

Cat L26 (group D, vaccine and IL-12 and IL18), a vaccinated, protected cat, which never tested virus isolation positive, also exhibited a slight neutropenia at the second, third and fourth post-challenge bleeds. However, the neutropenia bordered on the low range of normal, and it was observed that this cat had possessed a borderline neutrophil count even before viral challenge was performed (day of viral challenge bleed). By the third post-challenge bleed, nine weeks after viral challenge, neutrophil counts had risen in the neutropenic cats and by the fourth blood sample, thirteen weeks after viral challenge, they had all returned to within the low end of the normal range. The one exception to the above was cat L26, which remained very slightly neutropenic. The normal range of neutrophil counts in cats is  $2.5-12.5 \times 10^9/L$ ; cat L26 possessed a count of  $2.400 \times 10^9/L$  at the fourth post-challenge bleed.

## 4.4 DISCUSSION

### 4.4.1 DISCORDANT CATS

p27 ELISA and feline leukaemia virus isolation techniques identify FeLV p27 antigen and infectious virus, respectively, in plasma or serum. A positive p27 ELISA result usually indicates the presence of widespread, disseminated FeLV infection in the body, from which viral antigen is being produced (Jarrett et al. 1991). Therefore, cats that are p27 ELISA positive are normally also virus isolation positive, as p27 protein is produced by active FeLV replication. However, this is not always the case. In about 10% of blood samples tested at the Glasgow Veterinary School Feline Virus Unit, p27 ELISA results were positive, while virus isolation (VI), results were negative (Jarrett et al. 1991). As virus isolation is a reliable method of detecting active virus in blood, and the p27 ELISA results were consistently positive, using six different ELISA kits, it was concluded that these cats were “discordant” cats.

There are several theories as to why this discordant state exists; that is, a cat which is p27 ELISA positive and VI negative. The first explanation for the discordant state, is that the cat is at the beginning or the end of FeLV infection, when antigen but not virus can be detected in the blood (Jarrett et al. 1991). Thus discordancy is only a temporary state and a later blood sample would probably yield a consistent result between the two tests. Another explanation is that a focus of viral infection exists in the body, perhaps sequestered in a tissue such as the bone marrow, so that viral infection is not disseminated, but controlled and limited to one or two specific areas. This viral focus produces p27 antigen which reaches the blood and can be detected, but the low levels of virus produced cannot reach the periphery, and thus the cat is VI negative (Jarrett et al. 1991). Cats can remain in this discordant state for up to two years, but these cases generally progress to persistent viraemia and clinical FeLV infection.

It is generally regarded that virus isolation is the most definitive method of establishing the FeLV status of a cat, as p27 ELISA test kits can be unreliable. However, p27 ELISA and VI results should be considered together when assessing how well a vaccine protects against viral challenge, for example. On examination of the data from the FeLV DNA vaccine trial, it was observed that there were some discordant p27 ELISA and VI results. However, these mainly occurred just after viral challenge and were not consistent for the remainder of the trial. This related to the theory that discordant results may occur at the end or the beginning of FeLV infection; either when virus is being cleared and immunity established, (resulting in a negative VI and p27 ELISA result, as in the case of cat L13), or when virus is replicating but is not yet at high enough levels to be detected in peripheral blood (resulting in a positive VI and p27 ELISA result, as in the case of cat L2).

The one exception to the above was provided by cat L17. This cat became discordant nine weeks post-challenge, and remained so at the fourth post-challenge bleed (thirteen weeks post-challenge), being both p27 ELISA and VI positive before this time. It was considered that this cat may have developed partial immunity, resisting widespread disseminated viral infection, but rather than completely clearing virus and developing complete immunity, a focus of sequestered infection developed, capable of producing p27 antigenaemia. Interestingly, virus neutralising antibodies were never detected in the sera of this cat, illustrating that an incomplete immune response was mounted. Moreover, at the subsequent pre-euthanasia bleed, fifteen weeks post-challenge, the virus isolation result became positive. This late recrudescence may have arisen due to the combination of inadequate immunity and increased exposure to high doses of infective virus from persistently infected FeLV excreting cats.

## **4.4.2 FeLV DNA VACCINE AND ADJUVANTS; POSTULATED MECHANISMS OF ACTION**

### **4.4.2.1 Basic antigen component**

The single cycle FeLV vector vaccine consisted of two separate pUSE1<sup>-</sup>CMVT series plasmids, one expressing FeLV *gag/pol* genes, and the other expressing FeLV subgroup A *env* gene, both under control of the CMV-IE promoter. If a single host target cell is transfected with both constructs, structural and non-structural FeLV proteins should be expressed in a proportion of transfected cells, allowing the assembly of “empty” FeLV virions. These virions may bud from transfected cells, initiating an immune response similar to that raised against native FeLV viral particles, and will be able to enter adjacent B and T cells, but due to the absence of a functional viral genome, will not be capable of replication, proviral integration or establishing productive infection.

However, if only one construct transfects a target cell, the protein encoded by the DNA may be expressed in that cell, and presented to cytotoxic T lymphocytes in the context of the target cell’s endogenous MHC encoded class I molecules. Alternatively, the protein produced within the cell may be transported to the cell surface and allowed to accumulate there, as in the case of the FeLV-A gp70 protein. Antibodies may then be elicited to this foreign protein. The protein may also be secreted, engulfed by antigen presenting cells (APC), processed and presented to T helper cells in the context of the APC’s MHC class II molecules. The secreted antigenic protein may also be recognised by specific, antibody bearing B lymphocytes, which, upon antigen recognition and binding, differentiate into antibody-forming plasma cells. Thus, both cellular and humoral immune responses may be elicited. It was hoped that the FeLV DNA vaccine would elicit FeLV specific immune responses, which protect against persistent FeLV infection following viral challenge, by one or all of the above mechanisms.

Although the role cell-mediated immunity plays in FeLV infection is undefined and the generation of post-challenge virus neutralising antibodies is generally thought to

correlate with protection (Hoover et al. 1976), the exact mechanism by which FeLV vaccines protect cats from infection remains unknown. Indeed, this FeLV DNA vaccine and most of the experimental and commercial FeLV vaccines failed to induce significant titres of virus neutralising antibodies prior to viral challenge, so protection could involve either a priming of the immune system to produce these antibodies, or, alternatively, the induction of FeLV specific cytotoxic T cells (Jarrett, 1996). Interestingly, studies have documented cell mediated responses, (cytotoxic T cells and natural killer cells), to FeLV transformed cells, *in vitro* (Tompkins and Tompkins, 1985). Moreover, some cats resistant to infection do not develop VN antibodies, and occasionally detectable VN antibodies titres are found in persistently viraemic cats (Charreyre and Pedersen, 1991), although the titres are very low. While the development of virus-neutralising antibodies in an infected cat may block further spread of virus to uninfected cells, elimination of cells already infected may be due to other immune mechanisms, such as cell mediated immunity (Charreyre and Pedersen, 1991). Therefore, cell mediated immunity may be extremely important in the generation of an effective immune response to FeLV, especially in protecting against the development of latent infection.

The T helper cell type 1, Th1, response, characterised by IFN- $\gamma$  synthesis, controls cellular immunity. As mentioned earlier, in section 1.1.3., plasmid DNA itself may stimulate the development of cellular immunity, due to the presence of unmethylated CpG motifs. CpG motifs, 6-base DNA motifs consisting of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines, are present at high frequencies in bacterial plasmid DNA. These CpG motifs have been shown to possess T-helper type 1 immunostimulatory activity (Pisetsky, 1996), inducing the rapid and coordinated secretion of IL-6, IL-12, and IFN- $\gamma$  *in vivo* and *in vitro* (Klinman et al. 1996). Moreover, many reports have demonstrated that saline DNA inoculations, whether intradermal or intramuscular, as used in this trial, stimulate predominantly Th1 immune responses (Feltquate et al. 1997). Therefore, the endogenous non-specific adjuvant activity possessed by DNA vaccines and the delivery method employed, have the potential to enhance the development of the cellular immune response elicited in vaccinated cats.

Initially, p27 ELISA and VI results indicated that the FeLV DNA vaccine alone provided some degree of protection against viral challenge. Statistical analysis of the ELISA S/P results indicated that group E, the control group, possessed the largest transient infection at the first post-challenge bleed (3 weeks post-challenge), while virus isolation results revealed that only two cats in group A were VI positive, while five cats in group E were VI positive. However, the latter result was not statistically significant;  $p > 0.1$ . Interestingly, the control group E displayed the largest drop in transient infection between the first and second post-challenge bleeds (between week three and week six). Moreover, two cats which were VI positive in the control group E became VI negative between these two timepoints, so only three cats in the control group E tested VI positive at the second post-challenge bleed.

Cats in the control group E were immunised with empty pCI-neo plasmid alone, to establish whether the presence of CpG motifs could non-specifically enhance the immune response raised to challenge virus. Not all cats became persistently viraemic. However, the failure to establish persistent viraemia in all of these cats was most likely due to the innate, partly age-related resistance to FeLV infection possessed by a significant proportion of cats (Hoover and Mullins, 1991), rather than the endogenous adjuvant activity of CpG motifs. Obviously it is difficult to assess the efficacy of a vaccine if a significant proportion of the control cats develop natural immunity to the challenge virus. This is, in fact, a recurrent and consistent problem in FeLV vaccine efficacy studies and reflects host resistance factors, such as age at viral challenge (Hoover et al. 1976), immune system functional status and known virus virulence factors, such as magnitude of exposure and virus genotype. Intraperitoneal viral challenge was performed at 20-22 weeks of age in this trial, and this may have influenced the proportion of cats in the control group E that became persistently viraemic. Although intraperitoneal viral challenge has been performed effectively in cats much older than those involved in this trial (Hofmann-Lehmann et al. 1994), an experimental challenge study, employing intraperitoneal viral inoculation, has documented that persistent viraemia and FeLV-related disease developed in 100% of cats inoculated as newborns, in 85% of cats inoculated at 2 weeks to 2 months of age, and in 15% of cats inoculated at 4 months or 1 year of age (Hoover et al. 1976).

Therefore, the age of cats at viral challenge is of crucial importance in determining the outcome of infection.

Due in part to the above and the small numbers of cats in each group, a statistically significant difference was not detected between these two groups, (A and E), in terms of p27 ELISA and virus isolation results (positive or negative), at any timepoint during the trial. However, by the end of the trial, at week 23-24, the combined total of cats persistently and latently infected with FeLV was almost identical in groups A and E; four out of six cats compared with five out of six cats, respectively. Clearly, administration of the vaccine to the cats in group A did not confer any resistance to infection more potent than that acquired by natural immunity. Therefore, it appeared that CpG motifs in bacterial plasmid DNA were not acting as non-specific enhancers of the immune response to challenge virus, and it was concluded that the FeLV DNA vaccine alone was not effective in preventing the development of transient or persistent viraemia or latent FeLV infection.

At the termination of the trial, two cats in each of groups A and E were found to be latently infected with FeLV. In group A, cat L4 had tested p27 ELISA positive at the first post-challenge bleed only (12-13 weeks earlier) while cat L6 had tested p27 ELISA and VI positive at the first post-challenge bleed only. In group E, cat L20 had tested p27 ELISA and VI positive at the first post-challenge bleed only and cat L22 had tested p27 ELISA positive at the first, second and third post-challenge bleeds, but virus isolation positive at the first post-challenge bleed only. At the fourth post-challenge bleed, 13 weeks after viral challenge, all four cats possessed a significant VN antibody titre. In most exposed cats, immunity is established before or during bone marrow infection, so a marrow-associated viraemia does not develop. However, in a subset of exposed cats, perhaps exemplified by cats such as L4, L6, L20 and L22, immunity does not develop until after bone marrow-origin viraemia is established (Sparkes, 1997) so viraemia and/or antigenaemia are observed. Although this viraemia and/or antigenaemia are usually short-lived, lasting only days to weeks, they indicate that bone marrow infection with FeLV has occurred. Therefore, cats which have tested p27 ELISA or VI positive during the trial are more likely to become latently infected with FeLV. This may explain why these cats, L4, L6, L20 and L22,

testing p27 ELISA and VI positive early in the trial, later were found to be latently infected with FeLV. Following viral exposure bone marrow infection must have been established before a complete and effective immune response was raised. Similarly, cats which had not tested p27 ELISA or VI positive at any point during the trial, indicating that immunity had been established before bone marrow infection had occurred, tended not to develop latent FeLV infection.

#### **4.4.2.2 Adjuvant component**

##### *4.4.2.2.1 Overview*

*In vitro* studies have demonstrated that T cells exposed to the cytokines IL-18 and IL-12, exhibit a synergistic increase in interferon gamma production (Micallef et al. 1996), (Kohno et al. 1997). The latter cytokine is known to be integral to the development of a functional cellular immune response. Moreover, several *in vivo* studies in mice and cats have shown that the magnitude and nature of an immune response elicited by a DNA vaccine, can be engineered by the co-inoculation of cytokine, or other immunostimulant, plasmid DNA constructs (Chow et al. 1998), (Chow et al. 1998), (Kim et al. 1997), (Tsuji et al. 1997), (Hosie et al. 1998).

Codelivery of IL-12 DNA with vaccine antigens has been shown to significantly enhance the development of Th1 cell populations and the production of Th1 cytokines, essential in the generation of a functional cellular immune response (Chow et al. 1998). The development of Th2 cells, usually associated with a humoral immune response, was markedly inhibited. A dramatic increase in virus-specific CTL responses has also been demonstrated, following combined antigen and IL-12 inoculation (Tsuji et al. 1997), (Kim et al. 1997). As detailed in section 4.4.2.1., cell mediated immunity may be extremely important in the generation of an effective immune response against FeLV. Therefore, the coadministration of these Th1 type cytokine genes, IL-12, IL-18 and IFN- $\gamma$ , with FeLV vaccine antigens may have steered the immune response further towards the cellular arm, thus enhancing the efficacy of the FeLV DNA vaccine.

#### 4.4.2.2.2 *IFN- $\gamma$ as a vaccine adjuvant*

IFN- $\gamma$  is an extremely pleiotropic cytokine that has a major physiological role in regulating immune and inflammatory processes. The various roles that interferon plays in host defence mechanisms include a role in innate immunity (macrophages/neutrophils), lymphocyte migration, antigen recognition by T cells and effector T cell development (De Maeyer and De Maeyer-Guignard, 1992). This cytokine is known to be the principal agent responsible for macrophage activation, and therefore is the primary cytokine responsible for inducing non-specific cell-mediated mechanisms of host defence. IFN- $\gamma$  also plays an important role in the enhancement of MHC I expression and the induction of MHC II expression on antigen presenting cells, and may also induce NK cell proliferation and activation. In addition to enhancing non-specific cell-mediated cytotoxic activities, IFN- $\gamma$  also enhances the ability of macrophages to participate in other immune response effector functions. DNA plasmids encoding IFN- $\gamma$  protein have been found to increase the efficacy of DNA vaccines (Pasquini et al. 1997). It is thought that IFN- $\gamma$  may mediate its adjuvant effects by enhancing MHC II expression on antigen presenting cells, and, thus, antigen presentation.

The cats which received vaccine and IFN- $\gamma$ , in group B, were not protected against the development of persistent or latent FeLV infection. At the termination of the trial the number of cats persistently and latently infected in this group (2/5), was not statistically significantly different to that of group A (4/6), the vaccine alone group, group C (4/6), the vaccine and IL-12 group, or group E (5/6), the control group, although there were obviously fewer infected cats in the vaccine and IFN- $\gamma$  group. Thus, IFN- $\gamma$  was not an effective vaccine adjuvant in this system. However, the small number of cats in each group made definitive statistical analysis of the results very difficult.

Interestingly, a recent report regarding an FIV DNA vaccination trial, immunising with a defective mutant provirus of FIV, FIVDeltaRT, demonstrated that a DNA plasmid encoding feline IFN- $\gamma$  could act as an adjuvant (Hosie et al. 1998). In this

trial, immunisation with FIVDeltaRT in conjunction with IFN- $\gamma$  gave the highest proportion of protected cats, with only two of five vaccinates showing evidence of infection following challenge. However, direct comparisons cannot be made between these trials. Although FIV and FeLV are both retroviruses, they follow very different pathogenesis courses and mediate different pathologies in their hosts. Therefore, a specific adjuvant may have very different effects in one disease model as opposed to another. Moreover, little is known regarding the levels of IFN- $\gamma$  protein expression achieved following inoculation with IFN- $\gamma$  expressing DNA plasmids. Perhaps the expression achieved was at too low a level, or too localised, to act as an adjuvant, stimulating the immune system and potentiating the effect of the vaccine.

#### *4.4.2.2.3 IL-12 as a vaccine adjuvant*

IL-12 is a potent cytokine, able to enhance proliferation and cytolytic activity of activated T lymphocyte and natural killer cells, induce T helper cell type 1 immune responses and stimulate the differentiation of CD8<sup>+</sup> T lymphocytes (Brunda, 1994). The cats which received vaccine and IL-12, in group C, were not protected against the development of persistent or latent FeLV infection. The number of cats persistently infected in group C was similar throughout the trial to that of group E (the control group), was consistently, although not statistically significantly, higher than that of group A (the vaccine alone group) and was statistically significantly higher than that of group D (the vaccine and IL-12 and IL-18 group). Moreover, the late recrudescence of viral infection in cat L17, as detailed in the results section 4.3.3.2., again suggested that the FeLV DNA vaccine and IL-12 adjuvant combination did not provide solid protection against viral challenge. Together, these results indicated that IL-12 alone was not an effective vaccine adjuvant in this system and that the inclusion of IL-12 DNA constructs with the novel FeLV DNA vaccine may actually have reduced vaccine efficacy.

Interestingly, the adjuvant properties of IL-12 have been well documented in other DNA vaccination studies, using different disease models (Chow et al. 1998). The CTL activity induced by hepatitis B virus (HBV) DNA vaccination was significantly enhanced by codelivery of the IL-12 gene, and when challenged with HBV surface

antigen (HBsAg)-expressing syngeneic tumours, significant reduction of tumour growth was observed in mice that were co-inoculated with the IL-12 gene. However, HBV and FeLV are very different viruses, interacting with the immune system in different ways, and therefore direct comparisons cannot be made between the effects IL-12 expressing DNA plasmids mediate in these and other disease models. Alternatively, the levels of IL-12 protein expression achieved following inoculation with IL-12 expressing DNA plasmids may have been too low or too localised to stimulate the immune system.

A criticism of this work might be that *in vitro* experiments were not performed with the IL-12 expressing DNA constructs, to confirm their biological activity *in vitro*, before they were used *in vivo* in the FeLV DNA vaccination trial. Therefore, a simple explanation for the apparent lack of adjuvant activity of the IL-12 constructs, was that the protein they expressed was not biologically active and was thus not able to mediate the effects of endogenous IL-12. *In vitro* experiments, such as T lymphocyte proliferation assays, should be performed in the future to rule out this possibility. However, it is interesting to consider why the coinoculation of IL-12 DNA constructs with the FeLV DNA vaccine actually appeared to reduce vaccine efficacy. Indeed, the number of cats virus isolation and p27 ELISA positive in group C (vaccine and IL-12 group) was consistently, although not statistically significantly, higher than that of group A (vaccine alone group), throughout the trial, especially at the first two post-challenge sampling timepoints.

An investigation of the role of the human p40 subunit in ligand binding and signal transduction, by expressing this subunit alone in COS cells (Ling et al. 1995) suggested that the IL-12 p40 subunit contained the essential epitopes for receptor binding, although the proper conformation required for high affinity binding was achieved only when the p40 subunit was associated with a p35 subunit or another p40 subunit. When the p40 subunit was associated with a p35 subunit, the heterodimer acted as an agonist, mediating biologic activity. By contrast, when the p40 subunit associated with another p40 subunit, the homodimer behaved as a receptor antagonist *in vitro*, inhibiting IL-12-induced T cell proliferation. Similarly, it has been reported that recombinant murine p40 is able to form a homodimer, which

acts as a potent IL-12 antagonist, without biological activity *in vitro* (Gillesen et al. 1995). Perhaps the p40 homodimer plays a physiological role in the regulation of the biological activity of endogenous IL-12 in immune responses. However, administration of exogenous IL-12, as in the case of DNA vaccination, might lead to over-expression of the p40 subunit protein, resulting in the blockade of the IL-12 receptor, such that endogenous IL-12 cannot mediate its effects.

The IL-12 construct utilised in the FeLV DNA vaccination trial consisted of two separate plasmids, one expressing the p35 subunit and the other expressing the p40 subunit, both under the control of the CMV promoter. Although equal amounts of each plasmid were administered to each cat, it was impossible to determine if expression levels were similar, or even if both constructs transfected the same target cells, essential for the generation of the biologically active IL-12 heterodimer. Therefore, the p40 subunit may have been over-expressed relative to the p35 subunit, resulting in the formation of p40 homodimers, able to act as potent IL-12 receptor antagonists. Thus, the immunostimulatory effects of endogenous IL-12 may have been inhibited and the cats which were inoculated with the IL-12 and FeLV DNA vaccine constructs may have been less able to mount an effective immune response to the challenge virus. This may explain why the coinoculation of IL-12 DNA constructs with the FeLV DNA vaccine may actually have reduced vaccine efficacy.

A recent paper has described the construction of vectors expressing bioactive heterodimeric and single-chain murine interleukin-12 for gene therapy (Lee et al. 1998). A vector was constructed that contained two CMV promoters, to drive the expression of the p35 and the p40 subunits, respectively, ensuring that a single target cell would co-express both subunit proteins. Additionally, a vector was designed with a linker which fused the p35 cDNA with the p40 cDNA to produce a bioactive single-chain IL-12 protein. This ensured that the p35 and p40 subunits were equally expressed, and also that no free p40 subunits interfered with the biological activity of IL-12. The use of such vectors should be considered if the adjuvant potential of IL-12 is to be evaluated in future FeLV DNA vaccination trials.

#### 4.4.2.2.4 Co-administration of IL-12 and IL-18 as vaccine adjuvants

##### 4.4.2.2.4.1 Proposed mechanisms of action

The cats which received vaccine, IL-12 and IL-18 DNA constructs, in group D, were protected against the development of transient and persistent viraemia and latent FeLV infection. No cat in group D ever tested VI positive (tables 4.11., 4.12. and 4.13.) and only one cat, L30, tested weakly p27 ELISA positive at the first timepoint (tables 4.11. and 4.14.) At the termination of the trial only one cat, L28, harboured latent FeLV infection in her bone marrow and then at a very low level. Moreover, when a bone marrow biopsy from this cat was collected and cultured seven weeks later, virus isolation results then indicated that the marrow was completely free from latent FeLV infection. Late recrudescence of viral infection was not observed in any of the cats in group D, indicating that the vaccine, IL-12 and IL-18 combination established solid immunity. Statistical analysis of virus isolation results (table 4.13.), revealed that group D possessed significantly fewer positives than groups C (vaccine and IL-12 group), and E (control group), three weeks post-challenge, at week 11;  $p < 0.1$  and significantly fewer positives than group E, at week 11;  $p < 0.05$ . Therefore the IL-12 and IL-18 combination acted as a potent vaccine adjuvant. The reason that there are only statistically significant differences between the groups D and E at the first post-challenge bleed is not because the vaccine, IL-12 and IL-18 combination lost its ability to protect cats against FeLV infection as the trial progressed, but that some of the infected cats in the control group, E, cleared the virus and became VI negative, presumably due to the innate, partly age-related resistance to FeLV infection (Hoover and Mullins, 1991).

It is interesting to consider how the IL-12 and IL-18 DNA construct combination mediated its potent adjuvant effect *in vivo*, enhancing protection from FeLV infection. IL-12 and IL-18 activate both innate and acquired immunity (Okamura et al. 1998). *In vitro* studies have illustrated that T cells exposed to the cytokines IL-18 and IL-12, exhibit a synergistic increase in IFN- $\gamma$  production (Micallef et al. 1996) and, as explained above, IFN- $\gamma$  is integral to the development of a functional cell-mediated immune response. Therefore, IL-12 and IL-18 may have mediated their

immunostimulatory, adjuvant effect *in vivo* by means of a synergistic increase in IFN- $\gamma$  production. However, the IFN- $\gamma$  expressing DNA plasmid used in this trial was not an effective vaccine adjuvant (although IFN- $\gamma$  expressing DNA plasmids have been well documented as effective adjuvants in several DNA vaccination studies (Pasquini et al. 1997), (Hosie et al. 1998)), which could lead to the assumption that IFN- $\gamma$  production was not necessarily the mechanism by which IL-12 and IL-18 mediated their adjuvant effects.

However, the IFN- $\gamma$  construct may have proved to be an ineffective adjuvant because the expression levels of IFN- $\gamma$  protein it produced were too low or too localised to stimulate the immune system. In fact, the synergistic effect of IL-12 and IL-18 in increasing IFN- $\gamma$  production may have resulted in dramatically enhanced levels of IFN- $\gamma$ . Also, as IL-12 and IL-18 are higher up in the cytokine cascade (figure 1.8.), their increased expression may have led to an avalanche effect in the amplification of IFN- $\gamma$  production. The augmented levels of IFN- $\gamma$  would then be able to stimulate the immune system and thus enhance vaccine efficacy. Alternatively, a recent interesting report has documented that IFN- $\gamma$  may directly inhibit the expression of genes driven by the CMV or SV40 promoters (Harms and Splitter, 1995). As the FeLV antigens *gag/pol* and *env A* were under the control of the CMV promoter, perhaps the simultaneous administration of a DNA plasmid expressing IFN- $\gamma$  decreased the expression of the FeLV antigens to such a level that an effective, protective immune response was not elicited. In contrast, administration of the IL-12 and IL-18 adjuvant combination may have resulted in significantly increased endogenous levels of IFN- $\gamma$ , but the delay between the administration of the FeLV antigen constructs and the increase in IFN- $\gamma$  production may have been sufficiently long to ensure that the developing immune response was not effected. Therefore, increased endogenous IFN- $\gamma$  production may indeed have been the mechanism by which the IL-12 and IL-18 DNA constructs mediated their adjuvant effects.

Alternatively, the IL-12 and IL-18 expressing plasmids may have functioned directly, through a mechanism other than increased IFN- $\gamma$  production. Natural killer cells and activated CD8<sup>+</sup> T lymphocytes are integral to the cell-mediated immune response.

They target and lyse cells which express foreign antigens in the context of their MHC class I molecules, such as tumour cells and cells infected with microbial agents. Both IL-18 and IL-12 augment natural killer cell and activated T lymphocyte proliferation and cytolytic activity (Dao et al. 1998), and IL-18 is able to activate NK cells independently of IL-12 (Okamura et al. 1998). Therefore, in this system, the IL-12 and IL-18 expressing plasmids may mediate their adjuvant effect by directly stimulating and expanding the populations of both these cell types, primed by the antigenic component of the FeLV DNA vaccine. Consequently, after viral challenge, enhanced targeting and lysis of FeLV infected cells should result.

Another option to consider is that the IL-18 expressing DNA plasmid alone was responsible for the observed adjuvant effect. Unfortunately, a group of cats co-inoculated with vaccine and IL-18 alone could not be included for logistical reasons, to act as a comparison and to evaluate this hypothesis. IL-12 was considered to possess a broader range of actions, and be capable of mediating these actions alone, while IL-18 often appeared to require the presence of IL-12 to mediate its effects. For example, although IL-18 was found to be essential for the effective induction and activation of Th1 cells by IL-12, the former cytokine was found to be unable to induce the development of Th1 cells, by itself (Okamura et al. 1998). For these reasons, a vaccine and IL-12 group and vaccine and IL-12 and IL-18 group were included in the trial, in preference to a vaccine and IL-18 alone group.

Since the IL-12 expressing DNA plasmid by itself was not an effective vaccine adjuvant, and may actually have reduced vaccine efficacy, it is feasible that the IL-18 component of the IL-12 and IL-18 combination mediated the adjuvant effects. Alternatively, although IL-12 by itself was an ineffective adjuvant, perhaps working in synergism with IL-18, it became an essential part of the adjuvant combination. Future FeLV DNA vaccination trials should include a vaccine and IL-18 alone group, to further investigate and dissect the adjuvant effects of IL-12 and IL-18, alone and in concert.

An interesting recent report on the efficacy of IL-18 as an adjuvant in DNA vaccination studies, has questioned its prototypical Th1 type cytokine role (Kim et al.

1998). When co-inoculated with different plasmids encoding HIV-1 proteins, an IL-12 expressing DNA plasmid polarised the resulting immune response strongly towards cellular immunity, as expected. In contrast, an IL-18 expressing DNA plasmid induced dramatic increases in antibody production, with only modest increases in CTL activity. Therefore, in that study, it appeared that the immunomodulatory characteristics of IL-18 were similar to a Th2 type cytokine, rather than a Th1 type. The results of this study illustrate an important point; that a particular cytokine may mediate different immunomodulatory effects in different systems and species of animal. However, if IL-12 and IL-18 mediated similar effects to the above in the FeLV DNA vaccination trial, with IL-12 strongly enhancing cellular immunity and IL-18 enhancing humoral immunity, this dual enhancement may represent another theory to explain how the cytokine combination mediated its protective adjuvant effect. Again, future FeLV DNA vaccination trials, including the groups detailed previously and fully investigating the cellular and humoral immune responses elicited by vaccination, should dissect the mechanisms of protection more fully.

Another interesting hypothesis to consider is that the cytokines IL-12 and IL-18 were acting as immunotherapeutic agents, rather than vaccine adjuvants, and were, by themselves, responsible for the protection against FeLV infection, observed in the cats in group D. The antigenic component of the vaccine, therefore, would be irrelevant and redundant. Evidence to support this hypothesis is provided by the fact that the antigenic component of the FeLV DNA vaccine did not elicit any FeLV specific antibodies, before viral challenge. Reasons for this apparent lack of humoral immune response are considered in section 4.4.3. Expression of a reporter gene in murine skeletal muscle and biological activity of the encoded enzymes for up to 60 days after inoculation has been reported (Wolff et al. 1990). Consequently, it is feasible that the IL-12 and IL-18 expressing DNA plasmids may have been expressing the respective cytokine proteins *in vivo* at a continual low level, over a prolonged period of time so that when viral challenge was performed, three weeks after the last immunisation, levels of the immunostimulatory IL-12 and IL-18 proteins may still have been elevated in the animals inoculated with these DNA constructs, in group D. The elevated levels of endogenous IL-12 and IL-18 proteins

may then have acted as immunotherapeutic agents, stimulating the development of Th1 cell populations and non-specifically yet dramatically enhancing the immune response raised against the challenge virus, so that all the cats in group D were protected against the development of persistent viraemia. However, although no FeLV specific antibody response was elicited in any of the cats, the cellular immune response to the FeLV vaccine antigens was not investigated. Therefore, the vaccine antigens may have instead generated a protective, FeLV specific cellular immune response, to which the cytokines IL-12 and IL-18 added an adjuvant effect. To evaluate and investigate this hypothesis further, future FeLV DNA vaccination trials should investigate the FeLV specific cellular immune response and include a control group of cats, inoculated with only the DNA plasmids expressing IL-12 and IL-18, to ascertain whether this combination of cytokines is sufficient to protect cats from FeLV challenge. However, the above is an unlikely hypothesis, as the FeLV DNA vaccine alone did initially provide some degree of protection against viral challenge.

In conclusion, IL-12 and IL-18 most likely acted as vaccine adjuvants, enhancing the efficacy of the novel FeLV DNA vaccine. Considering the well documented synergistic effect of IL-12 and IL-18 in increasing IFN- $\gamma$  production (Micallef et al. 1996), elevated endogenous IFN- $\gamma$  levels may indeed have been the mechanism by which the IL-12 and IL-18 DNA constructs enhanced vaccine efficacy. Future FeLV DNA vaccination trials, including the groups and additional tests suggested above, should elucidate the mechanisms of protection more fully.

#### 4.4.2.2.4.2 *The role of viral challenge in establishing protection*

The first post-challenge blood sample was collected from all the cats three weeks after viral challenge. Although for the remainder of the trial all the cats in group D consistently tested negative for p27 antigen and virus, at three weeks after challenge one cat in group D tested weakly p27 antigen positive. For an effective cellular immune response to be produced *in vivo*, cells have to be infected with virus. Therefore, the vaccine and adjuvants may mediate their protective effects by initially allowing the development of low levels of infection after viral challenge, in order to stimulate the CTL response.

Viral challenge may, in fact, act as a booster to the effect of the immunisation course. As the CTL response matured, virus infected cells would be targeted and lysed and complete immunity would subsequently develop. Therefore, the FeLV DNA vaccine may not produce sterilising immunity, but if vaccinated cats are ultimately protected from the development of persistent and latent FeLV infection, this may not necessarily be important. Perhaps if blood samples had been collected earlier, one or two weeks after viral challenge, p27 ELISA and virus isolation results would have been positive for more, or even all, of the cats in group D, indicative of low levels of FeLV infection. Future trials should thoroughly investigate the role of the cellular immune response in establishing immunity to FeLV and include a blood sample collected one or two weeks post viral challenge, to investigate the above hypothesis.

#### *4.4.2.2.4.3 Protection against latent infection*

Latent infection of bone marrow occurs in a significant proportion (between 30 and 60%) of ostensibly immune cats, following recent recovery from FeLV infection and viraemia (Pedersen et al. 1984). It is usually a temporary state (Pacitti and Jarrett, 1985), and by six to eight months post-viraemia, most naturally infected cats have completely eliminated the virus (Pedersen et al. 1984). Although most latently infected animals do not actively excrete virus (Madewell and Jarrett, 1983), transmission of FeLV via the milk of a latently infected nonviraemic queen, to her kittens, has been demonstrated (Pacitti et al. 1986). This illustrates that in a breeding colony, at least, latent FeLV infection may represent a potential source of infective virus. Therefore, the production of a vaccine which could completely protect against the development of persistent and latent FeLV infection would be extremely desirable.

At present, very few commercial or experimental vaccines are able to protect against the development of latent FeLV infection. Two inactivated whole viral vaccines are available which have been demonstrated to be highly efficacious in protecting cats against persistent viraemia and latent FeLV infection: Fel-O-Vax, (Fort Dodge), (Hoover et al. 1996), (Legendre et al. 1991) and Fevaxyn FeLV , (Solvay-

Dulphar/Fort Dodge), (Pedersen, 1993), (Hines et al. 1991). Although Fel-O-Vax is extremely efficient in protecting against the development of persistent viraemia, one study demonstrated that almost one half of the non-viraemic, protected cats were transiently viraemic during the trial (Legendre et al. 1991) and almost half were latently infected, 23 weeks after viral exposure. Similarly, a study involving Fevaxyn FeLV, found that although this vaccine completely protected all vaccinates (10/10), from latent infection (Pedersen, 1993), the strain of FeLV used for challenge did not produce a high proportion of latent infections, when compared with other strains (Pedersen et al. 1984). Therefore, although Fevaxyn FeLV and Fel-O-Vax demonstrate the most consistent protection against FeLV challenge, compared with other commercially available vaccines, neither are able to completely prevent the development of transient or latent FeLV infection.

In contrast, the FeLV DNA vaccine and IL-12 and IL-18 adjuvant combination completely protected all six cats in group D from persistent and transient viraemia; that is, the vaccine achieved 100% protection. Section 1.2. reviews the efficacy of most of the experimental and commercially available FeLV vaccines and, although the size of the protected group D is small (six cats), 100% protection compares extremely favourably with the protection achieved by these other vaccines. Additionally, only one out of six of the cats in group D was latently infected with FeLV, 15 weeks after viral exposure. Moreover, this one cat was retested six weeks later, 21 weeks after viral exposure, and was found to be completely clear of virus, when virus isolation was performed on cultured bone marrow. Again, this compares very favourably with the degree of protection against latency achieved by Fel-O-Vax (Legendre et al. 1991); five out of twelve vaccinated cats were found to possess latent FeLV infection in their bone marrow, 23 weeks after viral exposure. Therefore, the FeLV DNA vaccine and IL-12 and IL-18 adjuvant combination appear to be superior to many of the commercially available and experimental FeLV vaccines, in terms of protecting against the development of transient and persistent viraemia and latent FeLV infection.

### **4.4.3 DNA VACCINATION DOES NOT ELICIT VIRUS NEUTRALISING OR NON-VIRUS NEUTRALISING ANTIBODIES**

Neither virus neutralising nor non-neutralising antibodies were elicited in any of the cats, after immunisation with the FeLV DNA vaccine and adjuvants. However, a significant number of cats were protected against viral challenge, so development of pre-challenge antibodies did not correlate with protection against persistent FeLV infection. The absence of an FeLV specific humoral immune response, pre-viral challenge, may be due to one or more of the following reasons.

#### **4.4.3.1 Potentiation of the cellular immune response**

Firstly, the nature of the DNA used for immunisation and the route of immunisation employed may have influenced the type of immune response elicited. Unmethylated CpG motifs, present in plasmid DNA, have been shown to possess T-helper type 1 immunostimulatory activity (Pisetsky, 1996) and stimulate the development of cellular immunity, while saline DNA inoculations, intradermal or intramuscular, stimulate predominantly Th1 type immune responses (Feltquate et al. 1997). Therefore, the intramuscular inoculations of naked plasmid DNA, administered to the cats in this trial, may have skewed the immune response towards the cellular arm. Similarly, all the adjuvants co-inoculated with the vaccine were plasmids expressing Th1 type cytokine genes, IL-12, IL-18 and IFN- $\gamma$ , potentially able to steer the immune response further towards the cellular arm. Moreover, *in vitro* studies have documented the importance of the cell mediated response in host defence against FeLV infection (Charreyre and Pedersen, 1991). Perhaps all the above factors contributed to the potentiation of the cellular immune response, at the expense of the humoral immune response, although the complete absence of FeLV specific humoral immunity was an unexpected outcome. Alternatively, immunisation with the FeLV DNA vaccine may prime the immune system to produce FeLV specific antibodies, subsequent to challenge with live virus. Future FeLV DNA vaccine trials should fully investigate the cellular immune response elicited by immunisation, to evaluate the above hypothesis.

#### **4.4.3.2 Cytokine expressing plasmids; vaccine adjuvants or immunotherapeutic agents?**

Another hypothesis to consider, as discussed in section 4.4.2.2.4., is that the cytokine expressing plasmids were acting as immunotherapeutic agents and not as vaccine adjuvants, non-specifically enhancing the immune response elicited to the challenge virus. If the cytokine adjuvants alone were protecting against FeLV infection, and the antigenic component of the vaccine was largely irrelevant, an FeLV specific immune response, humoral or cellular, would not be elicited in any of the animals. However, this is an unlikely hypothesis, as immunisation with the FeLV DNA vaccine alone did appear to protect cats from the development of persistent viraemia, at least initially. At the first post-challenge bleed only two out of six cats in group A (FeLV DNA vaccine alone), tested VI positive, while five out of six of the control cats, in group E, tested VI positive. To fully elucidate the mechanisms of protection conferred by the FeLV antigen and cytokine DNA constructs, the cellular immune response must be investigated in future FeLV vaccination trials.

#### **4.4.3.3 Antigenic competition**

Recent studies investigating the humoral immune response elicited in cats following DNA vaccination have provided conflicting results. Intramuscular DNA vaccination with a defective mutant provirus of feline immunodeficiency virus, FIV, (FIVDeltaRT), provided significant protection against FIV infection without inducing a detectable pre-challenge antiviral antibody titre. However, pre-challenge cytotoxic T-cell (CTL) responses to FIV *Gag* and *Env* were elicited (Hosie et al. 1998). Meanwhile, immunisation of cats with a DNA vaccine encoding the rabies virus glycoprotein G, elicited significant titres of rabies virus neutralising antibody, although the intradermal route of inoculation was found to be superior to the intramuscular (Osorio et al. 1999). A recent FIV DNA vaccination trial has also demonstrated that intramuscular immunisation with DNA plasmids encoding the FIV gp120 protein, induced a humoral immune response (Cuisinier et al. 1997). The above may reflect differences in immunogenicity between different antigens and how

they interact with the feline immune system. Alternatively, the absence of an antigen specific humoral immune response in the cats involved in the aforementioned FIV trial and this FeLV DNA vaccination trial may be due to antigenic competition. Anti-DNA or anti-cytokine antibodies may be elicited as a result of immunisation with plasmid DNA or *in vivo* production of cytokine proteins, respectively, which may then overwhelm the FeLV and FIV antigen specific humoral immune response.

#### 4.4.3.3.1 *Anti-DNA antibodies*

The rapid adoption and implementation of the novel DNA vaccination strategy carries with it a number of important safety and efficacy concerns. One of these is that DNA vaccination may induce responses against self-antigens, such as the induction of anti-DNA antibody production. These anti-DNA antibodies may subsequently interfere with the humoral immune response raised against the antigenic components of DNA vaccines and trigger the development of autoimmune disease (Klinman et al. 1997). In fact, a recent study has documented a three-fold increase in the number of B cells secreting immunoglobulin G (IgG) anti-DNA autoantibodies in BALB/c mice immunised and boosted with any of three DNA plasmids (Mor et al. 1997). This correlated with a transient increase in serum anti-DNA autoantibody titers but was not associated with the development of glomerulonephritis or autoimmune disease. Therefore, the absence of an FeLV specific humoral immune response, in the cats involved in this trial, may have been due to the generation of such anti-DNA antibodies.

#### 4.4.3.3.2 *Anti-cytokine antibodies*

Low amounts of high-affinity autoantibodies to various cytokines have been detected in serum samples from healthy human donors (van der Meide and Schellekens, 1997). Their levels, although highly variable, were increased in the circulation of patients subjected to cytokine therapy or suffering from a variety of immunoinflammatory diseases. It has been suggested that these autoantibodies could play various roles, including a regulatory role in the intensity and duration of an immune response, and as carrier proteins, preventing the rapid elimination of cytokines from

the circulation, thus increasing their bioactivity. However, they may also neutralise endogenously produced cytokines with possible pathological consequences. It has been hypothesised that immunisation with DNA plasmids encoding cytokine genes, as vaccine adjuvants, may elicit such autoantibodies. These, in turn, may interfere with the humoral immune response raised against the antigenic components of DNA vaccines. Therefore, in theory, the absence of an FeLV specific humoral immune response, in the cats involved in the FeLV DNA vaccination trial, may be due to the induction of such auto-antibodies.

In conclusion, the inability of the FeLV DNA vaccine to elicit an FeLV specific humoral immune response, most likely reflects differences in immunogenicity between different antigens and how they interact with the feline immune system, and the very nature of the immunising DNA itself. Relatively few DNA vaccination trials have been conducted in cats and those that have investigated the humoral immune response have provided conflicting results. As more DNA vaccination trials involving cats are performed, researchers may develop a greater understanding of the humoral and cellular immune responses elicited in the feline immune system, by this novel method of vaccination. Of interest and concern is the theory that auto-antibodies raised against the immunising DNA and expressed cytokine proteins, may have overwhelmed the FeLV antigen specific humoral immune response. Serum samples obtained from the cats involved in this trial, 48 hours after each immunisation, may be examined, retrospectively, for the presence of such anti-DNA and anti-cytokine antibodies.

#### **4.4.4 PCI-NEO; AN EFFECTIVE PLASMID FOR DNA-BASED VACCINATION**

The pCI-neo Mammalian Expression Vector (Promega) was the plasmid into which all the cytokine adjuvant genes were cloned and from which the pUSE vector series was derived. This plasmid contains the four features considered necessary for an effective DNA vaccine plasmid to possess (Davis, 1997). These are, firstly, a bacterial origin of replication that allows amplification of large quantities of plasmid

DNA for purification; the pCI-neo plasmid contains the f1 origin of replication. Secondly, a prokaryotic selectable marker gene, such as an antibiotic resistance gene; the pCI-neo plasmid contains the neomycin phosphotransferase selectable marker gene, expression of which confers resistance to the antibiotic G418 (Promega, 1998).

Thirdly, eukaryotic transcription regulatory elements, that are usually strong viral promoter/enhancer elements, allowing high levels of gene expression in a wide range of different cell types, are desirable; the pCI-neo plasmid contains the CMV (cytomegalovirus) immediate-early enhancer/promoter region. Studies have demonstrated the promiscuous nature of the CMV enhancer/promoter in transgenic mice, where expression of the chloramphenicol acetyltransferase (CAT) gene, under the control of the CMV enhancer/promoter, was observed in 24 out of the 48 tissues examined (Schmidt et al. 1990). Moreover, several researchers have demonstrated that the CMV promoter is significantly stronger in driving gene expression in muscle tissue than the RSV (respiratory syncytial virus) promoter, another strong viral promoter (Norman et al. 1997), (Davis et al. 1993). Finally, the ideal DNA vaccine plasmid should also contain a polyadenylation sequence to ensure appropriate termination of the expressed mRNA and enhance RNA stability and translation. The pCI-neo plasmid contains the efficient SV40 late polyadenylation signal which has been shown to increase the steady-state level of RNA approximately five-fold more than the SV40 early polyadenylation signal (Carswell and Alwine, 1989).

Additionally, the pCI-neo plasmid contains a chimaeric intron, downstream of the CMV promoter. This is composed of the 5' donor site of the first intron of the human beta-globin gene and the branch and 3' acceptor site from the intron of an immunoglobulin gene heavy chain variable region. The chimaeric intron is situated upstream of the inserted gene to prevent the utilisation of possible cryptic 5' donor splice sites within the inserted cDNA sequence. Transfection studies have reported that an intron flanking the inserted cDNA frequently increases the level of gene expression (Huang and Gorman, 1990). The increase in expression levels due to the presence of the chimaeric intron varies according to the particular DNA insert; increases in expression levels of between three and twenty fold have been reported, with the luciferase gene and the CAT gene, respectively (Brondyk, 1994). Obviously,

the presence of the chimaeric intron and the CMV promoter/enhancer in the plasmid DNA constructs employed in this trial was desirable, to allow maximal enhancement of the expression levels of both the FeLV antigen and adjuvant genes.

#### **4.4.5 DEVELOPMENT OF POST-CHALLENGE VN ANTIBODIES APPEARS TO CORRELATE WITH PROTECTION AGAINST FeLV INFECTION**

The generation of virus neutralising antibodies is generally thought to correlate with protection against FeLV infection (Hoover et al. 1976), although very occasionally some cats resistant to infection do not develop VN antibodies, and occasionally detectable VN antibody titres are found in persistently viraemic cats (Charreyre and Pedersen, 1991). In this vaccine trial, the development of post-challenge virus-neutralising antibodies correlated with protection against persistent viraemia, as demonstrated in tables 4.11. and 4.12. None of the persistently viraemic cats demonstrated a detectable VN antibody titre. Moreover, all cats which tested virus isolation negative thirteen weeks post-challenge possessed a significant VN antibody titre, with the exception of four cats. However, one of these cats, L17, in group C (vaccine and IL-12), had tested p27 ELISA and VI positive at the first and second post-challenge bleeds, had tested p27 ELISA positive and VI negative at the third and fourth post-challenge bleeds and then tested virus isolation positive, again, at the final pre-euthanasia bleed, fifteen weeks post-viral challenge. Therefore, the absence of virus neutralising antibodies at thirteen weeks post-challenge is not surprising, as this cat had obviously developed an incomplete immune response, which was incapable of completely clearing the virus.

The other three cats that consistently tested virus isolation negative throughout the trial, in the absence of a significant VN antibody titre, all belonged to group B (vaccine and IFN- $\gamma$ ), L8 (VN=16), L11 (VN=0) and L12 (VN=0). It is interesting to consider why none of these solidly protected cats developed a significant VN antibody response. Firstly, the possibility that these three cats were ineffectively challenged, rather than protected from infection by the vaccine and adjuvant

constructs, should be considered. Intraperitoneal inoculation, although not a difficult technique, is prone to operator error; vaccines may be inoculated into the bladder or gastro-intestinal tract, rather than the peritoneal cavity. However, failure of the challenge technique is not a likely possibility, for several reasons. Firstly, it would be an unusual coincidence if the three cats which were challenged incorrectly all belonged to the same group. Secondly, it does appear that at least one of the cats, L11, was exposed to the challenge virus; this cat tested weakly p27 ELISA positive, three weeks after viral challenge. Finally, even if intraperitoneal challenge was ineffective in these three cats, they would still be exposed to a second FeLV challenge later in the trial, when the persistently infected cats they were housed with began to excrete productive virus.

The reason why none of these three solidly protected cats, L8, L11 and L12, receiving the FeLV DNA vaccine and the IFN- $\gamma$  adjuvant constructs, developed a significant VN antibody response, remains unknown. One hypothesis is that the IFN- $\gamma$  adjuvant potentiated the development of the cellular immune response, at the expense of the humoral immune response. IFN- $\gamma$  is known to be central to the development of functional cell-mediated immunity. Perhaps this Th1 type cytokine skewed the immune response towards the cellular arm to such an extent that significant post-challenge virus neutralising antibody titres were not elicited in any of the animals. Future FeLV DNA vaccination trials should investigate the cellular immune response, by examining the CTL (cytotoxic T lymphocyte) response, to FeLV infected cells *in vitro*.

#### **4.4.6 HAEMATOLOGICAL CHANGES ASSOCIATED WITH EARLY FeLV INFECTION**

Clinically, during the initial stages of persistent FeLV infection, fever, depression and generalised lymphadenopathy may be observed. Six to eight weeks after exposure, when the virus has invaded the bone marrow and infected precursor cells, laboratory evaluation may reveal anaemia, leukopenia, thrombocytopenia, or pancytopenia, and death during this stage may be directly related to haematological

abnormalities (Baldwin and Ledet, 1994). Moreover, during acute viral infection neutrophils may migrate into tissues at a rate which exceeds their release from bone marrow, also resulting in a clinical neutropenia. However, persistently infected cats normally recover from this stage and experience a period of apparent good health, before succumbing to FeLV-related conditions associated with stage 2 of disease (Baldwin and Ledet, 1994). These conditions are invariably fatal, and are described in section 1.1.6.3.

The main change observed in the haematology results collected during the course of this trial was a profound neutropenia in four persistently infected cats, L2 (group A, vaccine alone), L9 (group B, vaccine and IFN- $\gamma$ ), L10 (group B, vaccine and IFN- $\gamma$ ) and L18 (group C, vaccine and IL-12), six weeks after viral challenge, at the second post-challenge bleed. This neutropenia was, however, transient, as neutrophil numbers returned to the low end of the normal range, over the next six weeks. This presumably reflected the balance between the rate of marrow precursor cell development and differentiation and the rate of viral replication and infection of these precursor cells. No significant differences were detected between the four neutropenic persistently infected cats and the other persistently infected cats in the cohort, either in terms of haematology results, FeLV status (VI, p27 ELISA result and VN antibody titres) or which combination of DNA constructs the cats had received. The cats which became profoundly neutropenic may simply have elicited a less effective initial immune response against the invading virus, thus temporarily allowing neutrophil precursor cell development to be almost completely arrested.

Interestingly, although concurrent neutropenia and lymphopenia are well documented in FeLV infection (Bush, 1991), the persistently infected cats involved in this trial possessed lymphocyte counts within normal ranges, at all times. Aside from one low total white cell count in cat L9, at the first post-challenge bleed, the only cell type to be affected by FeLV infection was found to be neutrophils. This may reflect the fact that the cats were blood sampled at an extremely early stage of FeLV infection. Perhaps if the trial had continued over a longer time period haematological changes may have been observed in other cell types, such as lymphocytes. Natural field cases of FeLV infection are usually only identified when clinical signs become evident and

therefore disease progression is already fairly advanced. In conclusion, the haematological data collected during the trial may be an important source of information, documenting the haematological changes in the very earliest stages of experimental FeLV infection.

#### **4.4.7 IMPORTANCE OF REMOVAL OF CONTAMINATING ENDOTOXIN FROM DNA TO BE USED FOR VACCINATION**

Endotoxins are derived from components of the cell wall of gram-negative bacteria and consist of lipopolysaccharide and variable amounts of protein and lipid. The biological effects of endotoxin are numerous and include activation of complement and coagulation pathways and modulation of the activity of platelets, neutrophils, monocytes/macrophages and endothelial cells (Morrison and Ulevitch, 1978). Bacterial endotoxin may induce pathogenic states in mammals, such as septic shock (Lamping et al. 1998). A recent DNA vaccination study demonstrated that the IgG response to DNA-encoded antigens, contaminated with high levels of bacterial endotoxin, was inhibited in a dose-dependent manner, when the intra-dermal route of DNA immunisation was utilised (Boyle et al. 1998). Thus, the presence of contaminating endotoxin may actually interfere with the immune response generated by DNA vaccine antigens. As the plasmid DNA for this trial was grown in *Escherichia coli* bacteria, and endotoxins are released during the lysis step of plasmid purification, it was absolutely imperative that all contaminating bacterial endotoxin was removed, prior to the injection of DNA into kittens.

The Qiagen EndoFree plasmid Giga kit used to prepare the DNA for the trial included an efficient endotoxin removal step before plasmid purification on a Qiagen-tip. This preparation method effectively reduced endotoxin levels, in all seven DNA samples, to almost undetectable levels; less than 0.5 EU per millilitre of plasmid DNA, as determined by the LAL endotoxin assay, (performed by Q1 Biotech). As the DNA was supplied to Q1 Biotech at a concentration of one milligram per millilitre, 1mg/ml, this meant that endotoxin levels were less than 0.5EU per milligram of plasmid DNA. The minimum pyrogenic dose of endotoxin

for man, administered intravenously, is 4 - 8 EU per kilogram bodyweight and for rabbits the figure is 10 - 15 EU/kg (Hochstein, 1987). As the one to two kilogram cats were immunised with 100µg of each construct, receiving a minimum of one and a maximum of five constructs and, therefore, only 100µg - 500µg of DNA at any timepoint, this meant the endotoxin levels these animals received were almost negligible and well below the accepted limits. As expected, no adverse reactions after vaccination with this ultrapure, endotoxin free DNA were observed in any of the kittens.

#### **4.4.8 PRODUCTIVE VIRUS IS NOT PRODUCED AS A RESULT OF HOMOLOGOUS RECOMBINATION BETWEEN VACCINE ANTIGENS AND ENDOGENOUS RETROVIRAL SEQUENCES**

The FeLV DNA vaccine, consisted of two separate pUSE1<sup>-</sup>CMVT series plasmids, one expressing FeLV *gag/pol* genes, and the other expressing FeLV subgroup A *env* gene. Although the expressed structural and regulatory FeLV proteins were assembled into infective FeLV virions, these “empty” virions lacked a functional viral genome, and were therefore not capable of replication, proviral integration or establishing infection. However, there was a theoretical concern that the FeLV antigen constructs would be able to recombine with exogenous non-pathogenic or endogenous feline retroviral sequences, enFeLV, resulting in the production of replication competent virions, capable of establishing productive FeLV infection in cats. In fact, FeLV B subgroup variants are thought to arise through recombination events between exogenous FeLV A subgroup isolates and endogenous feline retroviral sequences (Neil et al. 1987), (Stewart et al. 1986).

The consistently negative virus isolation and p27 ELISA results (illustrated in table 5.9.), obtained after screening the pre-viral challenge blood samples, determined that all the cats were free from FeLV infection and productive virus was not produced by homologous recombination between FeLV vaccine antigens and exogenous non-pathogenic or endogenous feline retroviral sequences. It was obviously very important to establish the above, in order to determine that the vaccine was safe to

administer and would not, by itself, cause disease or any adverse side-effects, such as pyrexia, inappetance, or local reactions at the vaccination site. To this end, the kittens were carefully observed by cattery staff for the twenty-four hour period following each immunisation. Twenty-four hours after each immunisation the temperature, pulse, respiration rate and general demeanour of every kitten was recorded and the vaccination site was examined. No local or systemic reactions were observed in any of the kittens at any time after immunisation with the FeLV antigen or cytokine adjuvant DNA constructs. Therefore, it was concluded that the FeLV DNA vaccine and cytokine constructs were safe to administer to cats.

#### **4.4.9 SUMMARY**

In conclusion, the FeLV DNA vaccine and cytokine constructs were determined to be safe to administer to cats. Although the FeLV DNA vaccine alone did not protect cats from the development of FeLV infection, the addition of IL-12 and IL-18 expressing DNA constructs markedly enhanced vaccine efficacy. IL-12 and IL-18, administered together, appeared to act as potent vaccine adjuvants, when co-inoculated with the novel FeLV DNA vaccine, providing protection against the development of transient and persistent viraemia and latent bone marrow infection. In contrast, IL-12 alone and IFN- $\gamma$  were not effective vaccine adjuvants in this system. Interestingly, FeLV DNA vaccination did not elicit any FeLV specific antibodies.

**5. CHAPTER FIVE; DETERMINATION OF  
DISTRIBUTION OF PLASMID DNA CONSTRUCTS IN  
PERIPHERAL BLOOD USING PCR**

## 5.1 INTRODUCTION

The advent of DNA vaccination technology has generated much excitement regarding the potential of these new vaccines to be employed in preventative and therapeutic vaccine strategies. As discussed in chapter one, Wolff et al demonstrated that the injection of pure DNA or RNA directly into mammalian skeletal muscle resulted in significant expression of the gene encoded protein within the muscle cells (Wolff et al. 1990). Moreover, the expression of the reporter gene in skeletal muscle and biological activity of the encoded enzymes were detected for up to 60 days after inoculation. Since this initial finding many research groups have continued to explore the therapeutic potential of DNA vaccination, achieving effective immunisation against viruses by intramuscular injection of DNA plasmids encoding viral proteins (Ulmer et al. 1993), (Donnelly et al. 1997a).

However, before DNA vaccines can be considered completely safe for use in human clinical trials, preclinical toxicology and pharmacokinetics studies must be conducted. There are widely held concerns regarding the uptake and long-term persistence of plasmid DNA in human tissues, such as the testes and ovaries, with the obvious implication that plasmid DNA may integrate into the germline. Integration of plasmid DNA into the host genome may lead to insertional mutation, activation of oncogenes and, therefore, possible tumourigenesis (Ramsay et al. 1997). Furthermore, aside from the safety aspect, information regarding the tissue distribution of the DNA vaccine plasmids at specific time points after inoculation may further elucidate the mode of action of DNA vaccination.

Performing pharmacokinetic studies with nucleic acids, however, presents a challenge. The therapeutic potential of a DNA vaccine construct is based on its specific nucleotide sequence. Therefore, when determining the distribution of a DNA vaccine construct post-inoculation, ascertaining that its specific sequence is present is obviously important. The application of traditional radiolabelling techniques in these

circumstances is extremely limited. Nucleic acids are degraded very rapidly in the body, to individual nucleotides, and these nucleotides may then be reincorporated into endogenous DNA (Winegar et al. 1996). Hence, the detection of radiolabel in tissues or blood may not indicate that an intact DNA sequence is present. Molecular biology techniques may overcome these difficulties and allow sequence-specific quantification.

The polymerase chain reaction (PCR) allows exponential amplification of specific DNA sequences (Mullis and Faloona, 1987). DNA isolated from tissue and body fluid samples may be added to a reaction mix containing a pair of sequence specific primers, *Taq* polymerase and deoxyribonucleotides. Following repeated cycles of denaturation, annealing and extension, up to  $10^{12}$  copies of the DNA target sequence may be obtained (Winegar et al. 1996). A recent study has described a method designed to examine the tissue distribution and level of a DNA plasmid encoding the HIV *gag/pol* genes, following DNA inoculation in rabbits (Winegar et al. 1996). A quantitative, competitive PCR technique, which was designed to minimise the potential risk of contamination, was employed by this research group. By varying the amounts of an internal standard sequence that was competitively co-amplified with the target sequence, the copy number of the target sequence could be determined with a reasonable degree of accuracy.

Using the above technique, this study examined a variety of tissues and body fluids at specific time points after exposure to plasmid DNA. Plasmid DNA was found predominantly in the skin and muscle at the injection site and in blood plasma. Initially, four hours after dosing with 400µg of DNA, the plasmid was detected in the muscle at higher copy numbers than in the skin. However, plasmid copy number declined rapidly in muscle in the first 24 hours after inoculation and was undetectable at 7 and 28 days post-inoculation. In contrast, the decline in plasmid copy number was slower in skin and, in fact, plasmid was still detectable in this tissue at 28 days post-inoculation (Winegar et al. 1996).

In an attempt to establish the presence or absence of DNA constructs in plasma and PBMC samples collected at specific time points after immunisation with the FeLV

DNA vaccine, a PCR technique was developed. The PCR employed primers which specifically amplified a 2206bp fragment of the plasmid backbone, present in all the cytokine and FeLV antigen vaccine constructs. In order to determine the sensitivity of the PCR a number of positive and negative control reactions were set up each time PCR amplification was performed. Distilled water and total DNA isolated from serum samples were spiked with various copy numbers of the pCI-neo plasmid. Additionally, serum samples were spiked with various copy numbers of the pCI-neo plasmid before total DNA was isolated from these samples. Therefore, although this PCR technique was not quantitative, the inclusion of the three sets of positive controls allowed the determination of the lowest copy number of plasmid able to be amplified in each separate batch of reactions. Blood samples were collected from the kittens 48 hours after each consecutive immunisation and at three week intervals post-viral challenge, as described in section 4.2.1. It was hoped that this technique would determine whether DNA constructs were detectable in the plasma or cellular compartments of the peripheral blood at these times.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 SAMPLE COLLECTION**

Section 4.2.1.4. describes the blood sampling schedule employed in the vaccine trial and details when blood samples were collected and subjected to PCR analysis to determine the presence or absence of the DNA constructs. To summarise, pre-immunisation blood samples were collected from all 29 kittens, to confirm the absence of the DNA constructs, pre-trial, in the plasma and PBMC fractions. Blood samples were then collected forty-eight hours after each immunisation and at three, six and nine weeks after the last immunisation, (weeks 8, 11 and 14, respectively, of the trial.)

## **5.2.2 DNA PURIFICATION FROM PLASMA AND PBMCs**

DNA was extracted from plasma and PBMC samples in a tissue culture laminar flow hood, in a separate site from where the PCR reactions were set up and from where the PCR reaction products were visualised by polyacrylamide gel electrophoresis, in order to avoid contamination. Anti-aerosol tips and dedicated micropipettes were employed and positive control DNA samples were synthesised at different times from the test samples.

### **5.2.2.1 Test samples**

Plasma and PBMC samples had been previously stored at -70°C. DNA was extracted from plasma and PBMC samples, using the QIAamp Blood Kit (Qiagen), according to the manufacturer's protocol, with the following modifications. Due to the size of the kittens, small volumes of blood were collected. Therefore, DNA was purified from 100µl of plasma diluted with 100µl of PBS, rather than purifying from a 200µl volume of plasma, as recommended by the protocol. Similarly, DNA was purified from 100µl of the PBMC fraction diluted with 100µl of PBS. The yield of DNA for the plasma and PBMC samples was predicted to be less than 5µg. Therefore, elution of the DNA was performed using 100µl of buffer AE, as the protocol recommended that for samples containing less than 5µg of DNA elution in 100µl rather than 200µl increased the final DNA concentration in the eluate. Finally, the eluted DNA samples were stored at -20°C prior to the performance of PCR amplification.

### **5.2.2.2 Positive and negative controls**

To ensure that DNA extraction was being performed correctly, PCR amplification was working efficiently and to allow the determination of the lowest copy number of plasmid able to be amplified each time the PCR procedure was carried out, two sets of positive control DNA samples were synthesised. Firstly, 100µl of serum from a SPF cat was aliquoted into six 1.5ml tubes. Using the formula;

1 mole of plasmid = length in bp x 660

1 mole of pCI-neo plasmid was determined to weigh  $3.6 \times 10^6$ g.

Dividing the above by Avagadro's number, one molecule, or one copy number of pCI-neo was calculated to weigh  $6 \times 10^{-12}$ μg. Using this information, the following series of dilutions of pCI-neo was then made;  $10^{11}$  copy numbers/μl,  $10^9$  copy numbers/μl,  $10^6$  copy numbers/μl,  $10^3$  copy numbers/μl,  $10^2$  copy numbers/μl and 10 copy numbers/ μl. 1μl of these six dilutions of pCI-neo were then added to the six tubes containing 100μl of SPF cat serum and DNA was extracted from the spiked serum samples using the QIAamp Blood Kit (Qiagen), according to the manufacturer's protocol. This set of positive control samples was termed set 1.

A second set of positive control samples, set 2, was created by extracting DNA from SPF cat serum using the QIAamp Blood Kit, and then spiking the newly extracted DNA with the dilutions of pCI-neo described above. Finally, to ensure that contamination was not occurring during the DNA extraction process, DNA was extracted from 100μl of SPF cat serum, (with no pCI-neo DNA added), each time DNA was isolated from test plasma or PBMC samples. This negative control DNA sample was subsequently subjected to PCR amplification, to ensure that contamination was not occurring during the PCR procedure.

### **5.2.3 PCR AMPLIFICATION TO DETECT DNA CONSTRUCTS**

DNA extracted from plasma and PBMC samples, collected from all 29 kittens forty-eight hours after each of three immunisations and at three, six and nine weeks after the last immunisation, was subjected to PCR amplification as described below. All PCR reactions were performed in duplicate. PCR reactions were set up in a tissue culture laminar flow hood, in a different site from where the DNA extraction was performed and from where the PCR reaction products were visualised by polyacrylamide gel electrophoresis. Anti-aerosol tips and dedicated micropipettes were also employed, in order to avoid contamination.

### 5.2.3.1 Design of primers

Primers were designed to amplify a 2206 bp fragment from nucleotide 3268 to 5474 in the backbone of the pCI-neo vector, the plasmid into which all the cytokine adjuvant genes were cloned. The pUSE1<sup>-</sup> vector, into which the FeLV *gag/pol* and *env A* antigens genes were cloned, also contained this 2206 bp fragment, as this vector series was derived from the pCI-neo plasmid. The following primers were employed in the PCR amplification;

5' primer: 5'-CTGGGTTTAAACATGACCGACCAAGCGAC-3'

3' primer: 5'-ATCTGGCGCGCCATGTGAGCAAAAAG-3'

### 5.2.3.2 Reaction conditions

Reactions were performed in 50 µl volumes in 0.5 ml tubes. A master reaction mix was prepared by combining 5µl of 10 x PCR buffer, 4µl of 25mM MgCl<sub>2</sub> solution, 5µl of deoxynucleotide (dNTP) mix (1.25 mM each dNTP), 20µl of dH<sub>2</sub>O, 20pmoles of each primer and 1.25U (0.25µl) of *Taq* polymerase in 4.75µl dH<sub>2</sub>O for each sample. The use of such a master mix minimises losses and inaccuracies associated with pipetting and ensures consistency from tube to tube. 40µl of this mix was then pipetted into each reaction tube, which contained 10µl of DNA template (control or test template). The reaction mix was then overlaid with mineral oil and the tubes were transferred to the thermal cycler. After a 5 minute denaturation incubation at 94°C, the thermal cycler was programmed to give 30 cycles of denaturation (94°C for 1 minute), annealing (59°C for 1 minute) and extension (72°C for 2 minutes), followed by a 10 minute extension step at 72°C and finally by a 4°C 'soak'. PCR reaction products were visualised by polyacrylamide gel electrophoresis as detailed in 2.2.2.5.2, using 10µl of reaction product per well.

### 5.2.3.3 Positive and negative controls

In order to assess the efficiency and sensitivity of the PCR process and to ensure that contamination had not occurred, a number of positive and negative controls were set up each time PCR amplification was performed.

#### 5.2.3.3.1 Positive controls

Three sets of positive controls were set up each time PCR amplification was performed. The synthesis of two of the sets of positive control DNA, sets 1 and 2, is described in section 5.2.2.2. As 10 $\mu$ l of DNA template (one tenth of the total elutate volume) was added into each PCR reaction, 10<sup>10</sup>, 10<sup>8</sup>, 10<sup>5</sup>, 10<sup>2</sup>, 10 and 1 copy number of the pCI-neo plasmid were amplified. The third positive control set (set 3) consisted of 10<sup>9</sup>, 10<sup>6</sup>, 10<sup>3</sup>, 10<sup>2</sup>, 10 and 1 copy number of the pCI-neo plasmid simply diluted with the appropriate volume of dH<sub>2</sub>O and added into the PCR reaction mix.

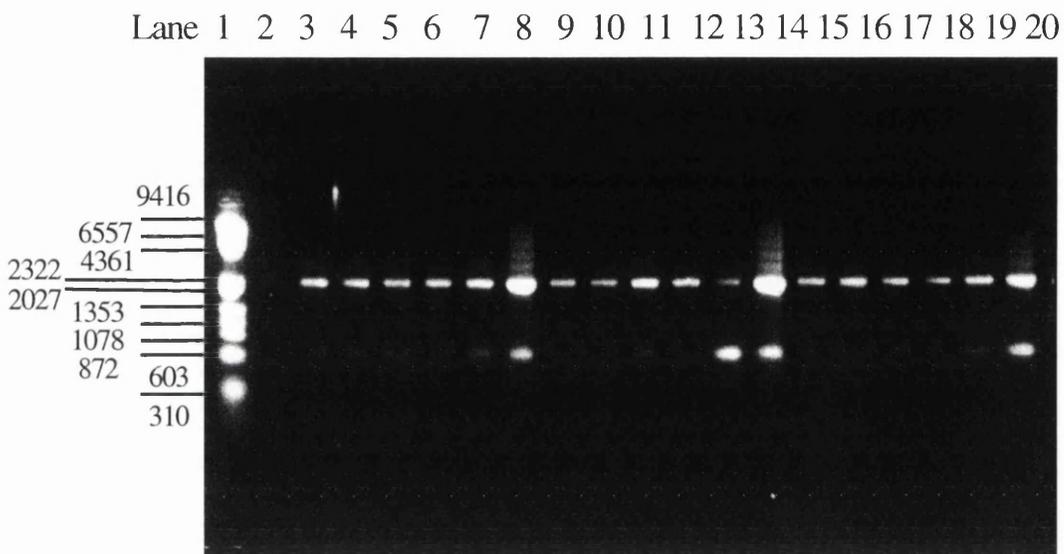
#### 5.2.3.3.2 Negative controls

Each time a series of PCR reactions was set up two negative controls were also included. A reaction mix was prepared containing all PCR components, (primers, dNTPs, MgCl<sub>2</sub> solution, PCR buffer and *Taq* polymerase), except template. This control was performed to check that there was no contamination of the PCR reactions with extraneous template that might serve as a template for PCR amplification. Secondly, 10 $\mu$ l of the negative control DNA sample, the synthesis of which was described in section 5.2.2.2., was also subject to PCR amplification. This control was performed to determine if contamination had occurred in either the DNA extraction process, or in the PCR amplification. The inclusion of both these negative controls allowed the source of any potential contamination and the stage at which it occurred to be determined more accurately.

## 5.3 RESULTS

### 5.3.1 PCR AMPLIFICATION OF POSITIVE CONTROL DNA SAMPLES

Figure 5.1. shows a polyacrylamide gel loaded with the PCR products obtained after amplification with the primers and PCR conditions described in section 5.2.3., and using template from all three sets of positive control DNA, described in section 5.2.3.3.1. Plasmid DNA was able to be amplified from all three sets of positive control DNA with a similar degree of sensitivity; from  $10^{10}$ - $10^9$  copy numbers of plasmid, down to a single copy. In lanes 3, 9 and 15 the presence of a single PCR product of the correct size (approximately 2200bp) indicated that a single copy number of plasmid was able to be amplified by this PCR technique. However, this amplification of a single copy of plasmid was not reliable; perhaps only 1 in 2 or 1 in 3 PCR amplifications achieved this degree of sensitivity. In contrast, 10 copy numbers of plasmid were consistently amplified by this technique, from all three sets of positive control DNA, as demonstrated in lanes 4, 10 and 16. Therefore, the limit of the sensitivity of this PCR technique was determined to be 10 copy numbers of plasmid.



**Figure 5.1. 5% polyacrylamide gel showing the reaction products of a typical PCR reaction used to amplify the positive and negative control DNA samples.**

Lane 1: molecular size markers ( $\phi$ X174 RF DNA/Hae III fragments and  $\lambda$  DNA/*Hind* III fragment markers); Lane 2: negative control (DNA extracted from SPF cat serum); Lanes 3, 4, 5, 6, 7 and 8: 1, 10,  $10^2$ ,  $10^5$ ,  $10^8$  and  $10^{10}$  copy numbers of the pCI-neo plasmid added into the PCR reaction mix, set 1 positive control DNA (pCI-neo DNA added to SPF cat serum before QIAamp DNA extraction); Lanes 9, 10, 11, 12, 13 and 14: 1, 10,  $10^2$ ,  $10^5$ ,  $10^8$  and  $10^{10}$  copy numbers of the pCI-neo plasmid added into the PCR reaction mix, set 2 positive control DNA (pCI-neo DNA added to SPF cat serum after QIAamp DNA extraction); Lanes 15, 16, 17, 18, 19 and 20: 1, 10,  $10^2$ ,  $10^3$ ,  $10^6$  and  $10^9$  copy numbers of the pCI-neo plasmid diluted with the appropriate volume of dH<sub>2</sub>O and added into the PCR reaction mix, set 3 positive control DNA.

### **5.3.2 PCR AMPLIFICATION OF NEGATIVE CONTROL DNA SAMPLES**

No PCR products were ever detected after PCR amplification of either of the negative control reactions, no DNA template and DNA extracted from SPF cat serum; the latter is illustrated in figure 5.1., lane 2. Each time DNA was extracted from test plasma or PBMC samples an internal negative control DNA extraction from SPF cat serum was also performed and each time a batch of PCR reactions was set up both negative PCR controls were included. Therefore, it appeared that contamination of extracted DNA or PCR reactions with extraneous template was not occurring and that positive PCR amplification results obtained were not merely “false positives”.

### **5.3.3 PLASMID DNA CONSTRUCTS WERE NOT DETECTED IN PLASMA SAMPLES BY PCR AMPLIFICATION**

No PCR products were detected after PCR amplification of DNA extracted from any plasma samples, collected either 48 hours after each consecutive immunisation or three, six and nine weeks after the last immunisation. In light of the fact that the PCR amplification process was determined to be extremely sensitive, consistently amplifying as little as 10 copy numbers of plasmid, it appeared that the plasmid DNA constructs were not present in the plasma fraction of the peripheral blood at the times following immunisation, indicated above.

### **5.3.4 PLASMID DNA CONSTRUCTS WERE DETECTED IN PBMC SAMPLES BY PCR AMPLIFICATION**

#### **5.3.4.1 PBMC samples collected 48 hours after each immunisation**

Single PCR products of the correct size were detected after PCR amplification of DNA extracted from some of the PBMC samples collected 48 hours after each intramuscular plasmid DNA immunisation. Table 5.1. indicates after which immunisation and in which kittens the plasmid DNA constructs were identified. The kittens were inoculated with 100µg of each DNA construct, so, in theory the kittens in group D, inoculated with 100µg of five DNA constructs, received five times more DNA than the kittens in group E, inoculated with only 100µg of one DNA construct. Therefore, it was predicted that the kittens inoculated with the greatest amount of DNA would be more likely to be PCR positive. However, this was not found to be the case; the distribution of PCR positive kittens was not related to the group the kitten belonged to or the amount of DNA which the animal had received. Indeed, the distribution of PCR positive cats was fairly random over the three consecutive post-immunisation time points. Interestingly, none of the cats in group A and only one cat in group B ever tested PCR positive and only two cats, L23 and L28, were consistently positive over all three PCR screens, performed on DNA extracted from PBMCs collected 48 hours after each of the three consecutive DNA inoculations.

#### **5.3.4.2 PBMC samples collected 3, 6 and 9 weeks after last immunisation**

No PCR products were detected after PCR amplification of DNA extracted from the PBMC samples collected three, six and nine weeks after the last immunisation. These results indicated that although DNA constructs were detected in some of the PBMC samples obtained 48 hours after intramuscular immunisation, when longer time periods elapsed between immunisation and blood sampling, the PCR technique was unable to detect the DNA constructs in PBMCs. The PCR technique described was extremely sensitive, amplifying as little as 10 copies of pCI-neo plasmid in positive control DNA samples, so it was likely that the DNA constructs had been cleared

from the cellular fraction of the blood by this time rather than the PCR technique failing to amplify the product due to lack of sensitivity.

Cat No	Group	48 hours post	48 hours post	48 hours post
		1 <sup>st</sup> immunisation	2 <sup>nd</sup> immunisation	3 <sup>rd</sup> immunisation
		Week 1	Week 3	Week 5
L1	A			
L2	A			
L3	A			
L4	A			
L5	A			
L6	A			
L8	B		+	
L9	B			
L10	B			
L11	B			
L12	B			
L13	C			
L14	C	+		
L15	C	+	+	
L16	C	+	+	
L17	C	+	+	
L18	C			
L19	E		+	
L20	E		+	
L21	E	+		
L22	E		+	+
L23	E	+	+	+
L24	E	+		
L25	D	+		+
L26	D	+		
L27	D	+		
L28	D	+	+	+
L29	D	+		+
L30	D			+

**Table 5.1. Results of PCR to detect DNA constructs in PBMCs.** The results indicate in which kittens and after which immunisation the plasmid DNA constructs were identified by PCR amplification in DNA extracted from PBMC samples.

**Key: A = vaccine alone, B = vaccine + IFN- $\gamma$ , C = vaccine + IL-12,**

**D = vaccine + IL-12 + IL-18 and E = empty pCI-neo plasmid.**

## 5.4 DISCUSSION

The PCR technique described in this section is indeed sensitive, amplifying as little as 10 copies of pCI-neo plasmid in positive control DNA samples. The PCR technique to assess the tissue distribution of a DNA vaccine, described by Winegar *et al*, was also extremely sensitive; quantitative, competitive PCR detected the presence of as little as 10 copy numbers of plasmid in rabbit skin, four hours after DNA inoculation (Winegar et al. 1996). Using the DNA extraction and PCR techniques described in this chapter, plasmid DNA constructs were not detected in any plasma samples. Given the extreme sensitivity of the PCR technique, it seemed most likely that the plasmid DNA constructs were, in fact, rapidly cleared from the plasma fraction of the peripheral blood soon after immunisation. Significantly, Winegar *et al* were only able to detect plasmid DNA in plasma samples collected from two out of six rabbits, four hours after they were immunised with 400µg of plasmid DNA. This indicated that plasmid DNA entered the bloodstream soon after inoculation. However, after this time DNA constructs were not detected in the plasma fraction (Winegar et al. 1996) and were assumed to have been cleared from this compartment of the peripheral circulation by the next sampling time, 24 hours after DNA inoculation. Therefore, in this trial, plasmid DNA constructs may have been detected in plasma if blood samples had been collected from the kittens in the first few hours following DNA inoculation.

Using the DNA extraction and PCR techniques described in this chapter, DNA constructs were detected in some of the PBMC samples collected 48 hours after each intramuscular plasmid DNA immunisation, in a random assortment of cats among the groups. However, DNA constructs were not detected in PBMC samples collected three, six and nine weeks after the last immunisation, indicating that the DNA constructs had been cleared from the cellular fraction of the blood by this time. Significantly, Winegar *et al* were only able to detect plasmid DNA in one out of six PBMC samples collected four hours after immunisation with 400µg of plasmid DNA. After this time DNA constructs were not detected in the PBMC fraction (Winegar et al. 1996) and were assumed to have been cleared from this compartment

of the peripheral circulation by the next sampling time, 24 hours after DNA inoculation. Therefore, the results obtained from this trial indicate that plasmid DNA is detectable in PBMCs for a longer time after DNA inoculation, 48 hours at least, than had been previously determined (Winegar et al. 1996). Future trials should consider collecting PBMCs for PCR amplification at time points between two days and three weeks post-immunisation, to determine exactly how long the DNA constructs were able to persist in the cellular fraction of the blood.

It is interesting to consider why DNA constructs were detected in PBMC samples 48 hours after each immunisation and yet were not detected in plasma samples. Recent studies have indicated that following intramuscular DNA vaccination, a significant proportion of DNA appears to leave the muscle, in blood or lymphatic fluid, and travel to the spleen or lymph nodes (Torres et al. 1997). Once there, the DNA may transfect resident APCs (Winegar et al. 1996), which then present antigen and initiate an immune response. Perhaps plasmid DNA free in plasma is more able to enter lymph nodes or the spleen and transfect APCs than plasmid DNA bound to the cellular fraction of the blood. Therefore, plasmid DNA bound to PBMCs would be more likely to persist in the peripheral circulation. Another important point to consider is the random nature of the distribution of PCR positive cats among the different groups and over the three consecutive post-immunisation time points, as described in section 5.3.4.1. This was perhaps due, at least in part, to the exact manner in which inoculations were performed and the site of plasmid DNA inoculation. Whether DNA was delivered carefully or with a more destructive, aggressive technique into the body of the quadriceps femoris muscle, between layers of muscle, or even into a small blood vessel, may have influenced exactly how much DNA reached the peripheral circulation and was able to be amplified by the PCR technique.

As DNA vaccination is such a recently developed technology, it is too early to unequivocally define it as a completely safe method of immunisation. However, to date, no experimental evidence for chromosomal integration of injected DNA has been produced (Nichols et al. 1995), (Wolff et al. 1992) and several researchers, investigating the tissue distribution of DNA plasmids following immunisation, have

found no evidence to suggest that uptake of recombinant DNA by the testes or ovaries occurs (Winegar et al. 1996), (Nabel et al. 1992). At the end of the FeLV DNA vaccination trial tissues such as the liver, lungs, spleen, lymph nodes, (mesenteric, popliteal, prescapular and iliac), bone marrow, brain, thymus, kidney; heart, muscle, (two samples; one from the quadriceps injection site and one from the triceps), and ovaries or testes were collected from each cat and stored at  $-70^{\circ}\text{C}$  for future analysis. Future work will involve the extraction of DNA from these tissues, in particular from mesenteric lymph nodes, bone marrow, thymus, spleen and, importantly, brain and gonads, and the performance of PCR amplification, in an attempt to identify the presence of plasmid DNA constructs. The results obtained will determine the tissue distribution of the DNA vaccine constructs; whether plasmid DNA is taken up and is able to persist in these important target tissues. Obviously this information is crucial when assessing whether this novel mode of vaccination will be suitable and safe for widespread use in humans.

## **6. CHAPTER SIX; GENERAL DISCUSSION**

## **6.1 INTRODUCTION**

The primary aims of this project were to clone and sequence the feline homologues of the cytokines IL-12 and IL-18, express the IL-18 protein in a mammalian expression system and investigate the potential of both IL-12 and IL-18 to act as genetic adjuvants in FeLV DNA vaccination studies. These objectives were achieved and provide the background for future work investigating the role of these cytokines as vaccine adjuvants against feline disease. Furthermore, the production of IL-12 and IL-18 recombinant proteins will facilitate the generation of specific polyclonal antibodies, through the immunisation of rabbits with the respective cytokine proteins. This, in turn, will help to elucidate the role of these cytokines in feline health and disease. This chapter briefly considers the advances that have been made in the introduction of cytokines to feline medicine and then considers the potential clinical applications of IL-12 and IL-18 in cats; as vaccine adjuvants, anti-microbial agents and anti-cancer agents.

## **6.2 THE THERAPEUTIC USE OF CYTOKINES IN FELINE MEDICINE**

Much interest has been shown in the potential applications of cytokines in domestic animals (Dunham, 1999), where the animals are used as models for human disease, such as FIV as a model for HIV and AIDS (Willett et al. 1997) and in the therapy and prophylaxis of animal diseases, such as the use of IL-12 as an anti-tumour agent against an established canine mammary tumour cell line (Okano et al. 1997). Initial studies employed human or murine homologues as species specific cytokines were either not available, or their supply was extremely limited. This approach, however, was restricted by two main factors. In the case of several cytokines there is little or no activity in the non-native species. This is especially the case for cytokines which are not well conserved between species, such as IL-3. In fact the human and murine IL-3 proteins share only 29% sequence identity (Yang et al. 1986) and show no cross

species activity (Gearing et al. 1999). Secondly, even when significant cross-species activity does exist, the administration of a heterologous cytokine may result in allergic reactions and prolonged administration may lead to the generation of neutralising antibodies to the cytokine, in a proportion of animals (Gieger, 1992).

To date, several recombinant human cytokines have been employed in the treatment of feline disease, with limited success. Recombinant human erythropoietin, granulocyte colony-stimulating factor (G-CSF), interferon alpha and IL-2 have all been evaluated in experimental trials. Recombinant human erythropoietin has been used in cats to treat the non-regenerative anaemia that accompanies chronic renal failure. Administration resulted in increased red blood cell (RBC) counts and clinical improvement (Cowgill et al. 1998). However, in a significant number of patients a non-regenerative anaemia was observed, more severe than the pre-treatment anaemia, attributable to the generation of antibodies to the heterologous recombinant cytokine which also cross-react with endogenous feline erythropoietin. Recombinant human GCS-F, a lineage specific haemopoietic growth factor, acting principally to increase circulating neutrophil counts and upregulate effector functions such as phagocytosis and chemotaxis, has been administered to normal cats. Administration has been shown to produce a rapid increase in circulating neutrophil numbers (Fulton et al. 1991). However, prolonged use led to a decrease in neutrophil counts to the normal range, again attributable to antibody formation to the heterologous cytokine.

Small scale trials have demonstrated that recombinant human interferon alpha may be a useful adjunct to the adoptive transfer of activated lymphocytes to FeLV infected cats. With the administration of zidovudine, the above therapy was found to reverse viraemia (Zeidner et al. 1995). Interleukin 2 (IL-2), a key cytokine in the regulation of T-lymphocyte immune responses, has been widely studied as a potential anti-tumour agent, though to invoke anti-tumour immunity, leading to tumour regression. The systemic administration of recombinant human IL-2 has been successful in the treatment of renal cell carcinomas in human patients (Bukowski et al. 1993). However, the systemic administration of IL-2 commonly results in side-effects, such as pyrexia, shock and respiratory distress and in more extreme cases, comas and death. Local administration of IL-2 is thought to be safer and some

success has been achieved with this approach in cats. Cats with fibrosarcoma have shown less frequent relapses and prolonged survival time when treated with the combination of surgery, radiotherapy and the local injection of a human cell line expressing the human IL-2 protein, compared to cats receiving surgery and/or radiotherapy (Quintin-Colonna et al. 1996).

Although heterologous cytokines may be suitable for short-term use, the cloning of species specific cytokines and the subsequent production of recombinant proteins is required if the above problems and limitations are to be circumvented. Unfortunately, the production of recombinant cytokine proteins in large quantities is extremely expensive and, therefore, it is likely that these therapeutic proteins will remain as research tools, in the immediate future. However, the use of alternative methods of cytokine delivery, such as naked DNA vaccination as described in this thesis, will significantly decrease production costs; the cytokine proteins are synthesised endogenously using the host cells' transcriptional machinery. This new technology may enable a new array of cytokines to become available for clinical use.

### **6.3 POTENTIAL CLINICAL USES OF FELINE IL-12 AND IL-18**

The clinical applications of IL-12 and IL-18 as anti-microbial and anti-tumour agents in human and mouse models have been discussed in sections 3.1.2.6. and 3.1.3.6., respectively and their use as vaccine adjuvants has been reviewed in section 1.4.3.2. Although almost all of these studies have been conducted in the murine model, the encouraging results clearly indicate that both IL-12 and IL-18 have many potential uses in the treatment and prevention of feline and ultimately human disease, used as single agents or in concert. Indeed, the results obtained from this research and the work of other investigators (Osaki et al. 1999), (Zhang et al. 1997), (Qureshi et al. 1999) indicate that the administration of both cytokines may be the most potent combination for use in therapeutic and prophylactic strategies.

Although IL-12 and IL-18 (recombinant proteins or adenoviral vectors expressing cytokine proteins) have been administered together, in anti-tumour (Osaki et al.

1999) and anti-microbial (Qureshi et al. 1999) treatment regimes and both IL-12 (Sin et al. 1999) and IL-18 expressing plasmid constructs (Sin et al. 1999) have been evaluated separately as DNA vaccine adjuvants, to the author's knowledge, this is the first study to evaluate the combined adjuvant potential of plasmids expressing IL-12 and IL-18. This is also the first study to attempt to produce a DNA vaccine against FeLV. The results obtained clearly demonstrate that the IL-12 and IL-18 combination mediated potent adjuvant activity, when coinoculated with the novel FeLV DNA vaccine, protecting all immunised cats from FeLV infection. This success leads to the idea of investigating the potential of IL-12 and IL-18 to act as adjuvants in FIV DNA vaccination, another significant feline retroviral pathogen and an animal model for HIV/AIDS (Willett et al. 1997).

To date, limited success has been achieved in the production of an efficacious FIV vaccine, with the exception of a whole inactivated virus vaccine (WIV) able to protect against homologous challenge with a weakly pathogenic isolate (Petaluma) (Hosie et al. 1995) but not against a heterologous FIV isolate (GL-8) which was distinct from the Petaluma strain in virus neutralisation. Recently, DNA vaccination has been investigated as an alternative approach to FIV vaccine development. In a recent DNA vaccination trial immunisation with a defective mutant provirus of FIV, FIVDeltaRT, in conjunction with a plasmid expressing feline IFN- $\gamma$ , gave the highest proportion of protected cats, with only two of five vaccinates showing evidence of infection following challenge (Hosie et al. 1998). As IL-12 and IL-18 are known to synergistically increase the production of IFN- $\gamma$ , perhaps the coadministration of both these cytokines with the novel FIV DNA vaccine may prove to be a more effective adjuvant combination. At present, this work is ongoing in the FIV research group, Department of Veterinary Pathology, University of Glasgow. In cats already infected with FeLV and FIV IL-12 and IL-18 may also be effective as immunotherapeutic agents, acting to non-specifically enhance the cell-mediated immune response against these retroviral pathogens.

As discussed in section 3.1. both IL-12 and IL-18 have been shown to be effective anti-microbial agents in many murine models of disease (McDyer et al. 1998), especially against intracellular bacteria such as *Mycobacterium tuberculosis* (Cooper

et al. 1995) and *Yersinia enterocolitica* (Bohn et al. 1998) and chronic viral infections, such as type 2 herpes simplex virus (HSV-2) and murine cytomegalovirus (Gately and Mulqueen, 1996). Therefore, these cytokines, administered as DNA constructs or recombinant proteins, may be effective therapeutic agents in the treatment of chronic infections, such as chlamydia, in cats. Chlamydia, an intracellular bacteria, causes reproductive problems and chronic ocular infections, which can be extremely problematic to treat. IL-12 and IL-18 may also be useful in the treatment of chronic feline herpesvirus type 1 (FHV-1) infections, manifest clinically as upper respiratory tract disease. Due to the nature of feline herpesvirus, latent infections, able to be reactivated by stress and immunosuppression, are common. Interestingly, IL-12 has also been considered as an immunotherapeutic agent in the treatment and/or prevention of atopic and allergic disorders (McDyer et al. 1998). Therefore, this cytokine may be useful in the therapy of the allergic condition, feline asthma.

Due to their multiple immunoregulatory functions, including activation of NK cells, CTLs and Th1 cells, IL-12 and IL-18, alone or in concert, have been utilised as antitumour agents, as discussed in sections 3.1.2.6. and 3.1.3.6. The antimetastatic and antitumour activities of IL-12 have been demonstrated in a number of murine models, and the encouraging results have prompted the use of IL-12 as an anti-tumour agent in the human oncology field. Phase I and II clinical trials in patients suffering from renal cell carcinomas and breast carcinomas are ongoing (Lamont and Adorini, 1996). To date, IL-18 has been evaluated in murine models as an effective anti-tumour agent against malignancies such as fibrosarcoma, melanoma and sarcoma (Osaki et al. 1998), (Tan et al. 1998), (Micallef et al. 1997), (Fukumoto et al. 1997), (Coughlin et al. 1998), (Osaki et al. 1999), although many of these therapeutic strategies have investigated the effects of IL-18 in combination with IL-12. The combined administration of feline IL-12 and IL-18 may therefore prove to be a useful adjunct to chemotherapy or radiotherapy treatment regimes, either by the direct inoculation of recombinant cytokine proteins or DNA plasmids expressing IL-12 and IL-18 into tumours, by the systemic administration of recombinant IL-12 and IL-18 proteins or by the subcutaneous injection of tumour cells transfected with feline IL-12 and/or IL-18 complementary DNA (cDNA). Feline tumours suitable for

cytokine therapy are melanomas, sarcomas and carcinomas, such as squamous cell carcinomas. These cytokines may also be suitable for the treatment of multicentric feline lymphosarcoma as murine IL-12 has been demonstrated to inhibit established experimental pulmonary or hepatic metastases and reduce spontaneous metastases in mice (Brunda et al. 1996).

## 6.4 FUTURE DIRECTIONS

Clearly, feline IL-12 and IL-18 have a wide therapeutic potential in feline medicine and their specific uses and applications have been discussed. Due to the complexity of the cytokine network, the administration of one cytokine *in vivo* may have wide ranging and often deleterious effects, altering the levels of other endogenous cytokines and disturbing the fine balance that exists between the members of this family of glycoproteins. Therefore, the use of cytokines in combination, such as IL-12 and IL-18, in experimental animal models may result in the maximisation of their potential therapeutic value, while minimising the risk of unwanted side effects.

However, the development of cytokines as therapeutic agents in feline medicine depends on a number of other factors, including commercial viability, range of potential clinical applications, efficacy, and cost of production. To date the commercial application of recombinant DNA technology to clinical veterinary medicine has been limited to the development of a recombinant subunit FeLV vaccine consisting of the viral glycoprotein gp70, expressed in *Escherichia coli* (Clark et al. 1991). This vaccine, Leucogen (Virbac), is now in widespread use. Vaccines obviously have a wide application, as all pet cats may be possible recipients, which may compensate for the potentially high research, development and production costs. Therefore, the manufacture of the FeLV DNA vaccine and the plasmids expressing the cytokines IL-12 and IL-18, to be used as vaccine adjuvants, may be a commercially viable venture. Moreover, the wide range of potential clinical applications of IL-12 and IL-18, such as anti-tumour and anti-microbial agents, may result in the widespread use of these cytokines in clinical veterinary practice.

Finally, this research may also have wider implications for the field of human medicine. As discussed previously, FIV has been described as an animal model for HIV and AIDS (Willett et al. 1997). The success of cytokine adjuvant constructs in enhancing the efficacy of DNA vaccines against the feline retroviral pathogens FIV and FeLV, indicate that this approach should be considered in the development of future candidate vaccines against HIV. Moreover, DNA vaccination studies using animal models may provide valuable information regarding the safety and efficacy of this unique method of immunisation, before large-scale human DNA vaccination trials are conducted.

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## **GLOSSARY**

## GENERAL ABBREVIATIONS

°C	degrees Celsius
μl	microlitre(s)
μM	micromolar
2-ME	β-mercaptoethanol
A	adenine or adenosine; one letter code for alanine
A <sub>260/280</sub>	absorbance at 260, 280
Ab	antibody
Ad5	human adenovirus type 5
Ag	antigen
AIDS	acquired immunodeficiency syndrome
AP	alkaline phosphatase
APC	antigen presenting cell
APS	ammonium persulphate
Asn	asparagine
Asp	aspartic acid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCG	<i>Mycobacterium bovis</i> bacillus Calmette-Guerin
BM	bone marrow
BMSN	bone marrow supernatants
bp	base pair
Bq	Becquerel
BSA	bovine serum albumin
C	cytosine or cytidine; one letter code for cysteine
CAT	chloramphenicol acetyltransferase
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
CFA	complete Freund's adjuvant
Ci	curie
CLMF	cytotoxic lymphocyte maturation factor

CMV	cytomegalovirus
CO <sub>2</sub>	carbon dioxide
cpm	counts per minute
CSF	colony stimulating factor
CTL	cytotoxic T lymphocyte
Da	dalton
dATP	deoxyadenosine triphosphate
DC	dendritic cells
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EAE	experimental allergic encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
enFeLV	endogenous FeLV-related sequences
ER	endoplasmic reticulum
EtBr	ethidium bromide
EU	endotoxin units

FBS/FCS	foetal bovine serum/foetal calf serum
FeLV	feline leukaemia virus
FeSV	feline sarcoma virus
FFU	focus forming units
FHV	feline herpes virus
FIV	feline immunodeficiency virus
FOCMA	feline oncornavirus-associated cell membrane antigen
g	gravity; gram(s)
G	gauge; guanine or guanosine; one letter code for glycine
GCG	Genetics Computer Group, Wisconsin Package Version 9.1
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
gp	glycoprotein
GTP	guanosine 5'- triphosphate
HA	haemagglutinin
HAART	highly active antiretroviral therapy
HBS	HEPES-buffered saline
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethanesulphonic acid
HIV	human immunodeficiency virus
HRP	horse radish peroxidase
HSV-2	type 2 herpes simplex virus
HTLV	human T-cell leukaemia virus
ICE	interleukin 1 $\beta$ converting enzyme
Id	idiotype
i.d.	intra dermal
IDDM	insulin-dependent diabetes mellitus
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin
IGIF	interferon gamma inducing factor

IL	interleukin
IL-1Rrp	IL-1 receptor-related protein
IL-12R	IL-12 receptor
i.m.	intramuscular
i.p.	intraperitoneal
IRAK	IL-1 receptor-activating kinase
ISCOM	immunostimulating complex
IU	international unit
JAK	Janus-family kinase
kb	kilobase
kDa	kilodalton
KoAc	potassium acetate
L	litre
LAL	Limulus amoebocyte lysate
LB	Luria Bertani medium
LPS	lipopolysaccharide
LTR	long terminal repeat
M	molar
MAIDS	murine acquired immunodeficiency syndrome
mcg	microgram
ME	mercaptoethanol
mg	milligram(s)
MHC	major histocompatibility complex
mM	micromolar
MoMLV	Moloney murine leukaemia virus
mRNA	messenger ribonucleic acid
NEAA	non essential amino acids
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NK	natural killer cell
NKSF	natural killer cell stimulatory factor
nm	nanometre
NP	nucleoprotein

nt	nucleotide
NTP	nucleoside triphosphate
OD	optical density
oligo(dT)	oligodeoxythymidylic acid
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF	preventable fraction
PHA	phytohaemagglutinin
pNA	para-nitroanilide
poly(A) <sup>+</sup>	polyadenylated (mRNA)
PV	persistent viraemia
RBC	red blood cell
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RSV	respiratory syncytial virus
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
s.c.	subcutaneous
SCF	stem cell factor
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIV	simian immunodeficiency virus
S/P	sample to positive
SPF	specific pathogen free
STAT	signal transducers and activators of transcription

T	thymine or thymidine; one letter code for threonine
TBE	Tris/borate/EDTA
TBS	tris buffered saline
TBS-T	tris buffered saline-tween solution
TBT	tris buffered saline-tween solution
TCF	tissue culture fluid (supernatant) of cultured bone marrow cells.
TE	Tris-EDTA buffer
TEA	Tris/EDTA/acetate
TEMED	<i>N</i> '- tetramethyl-ethylenediamine
Th1	T helper cell type 1
Th2	T helper cell type 2
TK	thymidine kinase
$T_m$	melting (or midpoint) temperature
TMP	tetramethylbenzidine hydrochloride peroxidase
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)aminomethane
Tris-HCl	Tris hydrochloride
tRNA	transfer ribonucleic acid
UV	ultraviolet
UWGCG	University of Wisconsin Genetics Computer Group
VI	virus isolation
VN	virus-neutralising
WBC	white blood cell
WIV	whole inactivated virus vaccine
Xgal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside

## **APPENDIX**

## APPENDIX

### Haematology results from the FeLV DNA vaccination trial

DATE	ANIMAL	WBC	RBC	HB	HCT	MCV	MCH	MCHC
09/06/98	L1	10.5	5.60	8.7	26.4	47	15.5	32.9
09/06/98	L2	11.2	6.33	9.1	28.6	45	14.3	31.8
09/06/98	L3	9.8	6.24	9.7	29.6	47	15.5	32.7
09/06/98	L4	16.2	7.14	9.1	28.6	40	12.7	31.8
09/06/98	L5	15.8	7.86	11.8	35.9	46	15.0	32.8
09/06/98	L6	8.1	6.48	9.0	28	43	13.8	32.1
09/06/98	L8	17.3	6.48	9.7	31.1	48	14.9	31.1
09/06/98	L9	12.2	6.95	11.1	33.3	48	15.9	33.3
09/06/98	L10	14.1	6.70	10.3	31.8	47	15.3	32.3
09/06/98	L11	14.6	6.42	8.7	27.4	43	13.5	31.7
09/06/98	L12	9.2	5.78	9.2	28.9	50	15.9	31.8
09/06/98	L13	18.9	5.87	8.3	26.4	45	14.1	31.4
09/06/98	L14	13.9	6.36	9.7	29.2	46	15.2	33.2
09/06/98	L15	12.8	6.83	9.4	29.2	43	13.7	32.1
09/06/98	L16	15.6	6.39	9.3	29.2	46	14.5	31.8
09/06/98	L17	7.9	6.46	8.8	27.4	42	13.6	32.1
09/06/98	L18	13.0	6.39	9.0	28	44	14.0	32.1
09/06/98	L19	20.9	6.21	9.7	30.2	49	15.6	32.1
09/06/98	L20	12.0	7.04	10.7	31.5	45	15.1	33.9
09/06/98	L21	24.2	5.75	8.9	27.4	48	15.4	32.4
09/06/98	L22	8.8	5.47	8.0	25.2	46	14.6	31.7
09/06/98	L23	12.3	6.30	9.4	29.6	47	14.9	31.7
09/06/98	L24	9.4	5.84	8.3	26.1	45	14.2	31.8
09/06/98	L25	10.4	6.58	9.7	29.2	44	14.7	33.2
09/06/98	L26	12.4	6.48	9.3	28	43	14.3	33.2
09/06/98	L27	15.6	6.15	9.9	29.2	47	16.0	33.9
09/06/98	L28	12.0	6.58	8.8	27.4	42	13.3	32.1
09/06/98	L29	10.3	6.52	9.3	28.3	43	14.2	32.8
09/06/98	L30	6.8	5.44	7.6	23.9	44	13.9	31.7

### Pre-trial haematology results

**Abbreviations;** **WBC**, total white blood cell count x 10<sup>9</sup>/L; **RBC**, total red blood cell count x 10<sup>12</sup>/L; **HB**, haemoglobin, g/dl; **HCT**, haematocrit (synonymous with PCV, packed cell volume); **MCV**, mean red cell volume; **MCH**, mean red cell haemoglobin; **MCHC**, mean red cell haemoglobin concentration; **BNEU**, band (immature) neutrophil count x 10<sup>9</sup>/L; **NEU**, neutrophil count x 10<sup>9</sup>/L; **LYMPH**, lymphocyte count x 10<sup>9</sup>/L; **MONO**, monocyte count x 10<sup>9</sup>/L; **EOS**, eosinophil count x 10<sup>9</sup>/L; **BASO**, basophil count x 10<sup>9</sup>/L

DATE	ANIMAL	BNEU	NEU	LYMPH	MONO	EOS	BASO
09/06/98	L1	0.105	5.775	3.570	0.525	0.525	0.000
09/06/98	L2	0.112	6.496	3.584	0.224	0.560	0.112
09/06/98	L3	0.000	6.370	2.058	0.490	0.784	0.098
09/06/98	L4	0.000	8.424	6.480	0.486	0.810	0.000
09/06/98	L5	0.000	6.478	7.584	0.316	1.422	0.000
09/06/98	L6	0.162	4.455	2.754	0.324	0.405	0.000
09/06/98	L8	0.000	11.072	4.498	1.038	0.692	0.000
09/06/98	L9	0.000	5.856	5.246	0.488	0.488	0.122
09/06/98	L10	0.000	7.614	5.358	0.282	0.846	0.000
09/06/98	L11	0.292	10.804	2.920	0.292	0.292	0.000
09/06/98	L12	0.000	4.416	4.140	0.184	0.368	0.000
09/06/98	L13	0.000	11.151	5.103	0.567	2.079	0.000
09/06/98	L14	0.000	9.313	3.336	0.556	0.417	0.000
09/06/98	L15	0.128	6.400	4.992	0.768	0.512	0.000
09/06/98	L16	0.156	7.488	6.708	0.312	0.780	0.088
09/06/98	L17	0.079	4.424	3.239	0.000	0.158	0.000
09/06/98	L18	0.130	6.890	4.550	0.390	1.040	0.000
09/06/98	L19	0.418	11.913	7.315	0.418	0.836	0.000
09/06/98	L20	0.120	7.560	3.360	0.240	0.360	0.240
09/06/98	L21	0.968	18.876	2.662	0.726	0.726	0.242
09/06/98	L22	0.000	5.016	3.080	0.264	0.352	0.000
09/06/98	L23	0.000	5.289	3.567	0.123	3.198	0.000
09/06/98	L24	0.000	6.204	2.350	0.094	0.752	0.000
09/06/98	L25	0.000	5.408	3.848	0.312	0.624	0.104
09/06/98	L26	0.124	7.564	3.348	0.248	0.992	0.124
09/06/98	L27	0.156	7.644	7.332	0.156	0.312	0.000
09/06/98	L28	0.120	7.440	3.000	0.600	0.720	0.000
09/06/98	L29	0.000	4.223	5.253	0.412	0.412	0.278
09/06/98	L30	0.000	3.400	2.788	0.204	0.340	0.000

## Pre-trial haematology results

**Normal values; WBC, 5.5-15.5 x 10<sup>9</sup>/L; RBC, 5.0-10.0 x 10<sup>12</sup>/L; HB, 10.0-15.0g/dl; HCT, 0.30-0.45L/L; MCV, 39.0-55.0fl; MCH, 12.5-17.5 pg; MCHC, 30.0-36.0g/dl; NEU, 2.5-12.5 x 10<sup>9</sup>/L; LYMPH, 1.5-7.0 x 10<sup>9</sup>/L; MONO, 0.00-0.85 x 10<sup>9</sup>/L; EOS, 0-1.5 x 10<sup>9</sup>/L; BASO, Rare**

DATE	ANIMAL	WBC	RBC	HB	HCT	MCV	MCH	MCHC
28/07/98	L1	13.3	7.94	13.3	35.3	44	16.7	37.6
28/07/98	L2	12.6	8.00	11.6	32.2	40	14.5	36.0
28/07/98	L3	15.2	7.88	11.9	32.8	42	15.1	36.2
28/07/98	L4	13.2	7.88	9.8	28.4	36	12.4	34.5
28/07/98	L5	17.2	9.39	14.6	38.8	41	15.5	37.6
28/07/98	L6	12.6	7.97	11.5	31.9	40	14.4	36.0
28/07/98	L8	24.2	8.00	11.2	32.5	41	14.0	34.4
28/07/98	L9	11.4	9.46	15.2	41	43	16.0	37.0
28/07/98	L10	15.8	8.28	13.1	34.1	41	15.8	38.4
28/07/98	L11	14.0	7.33	9.9	28.4	39	13.5	34.8
28/07/98	L12	12.6	6.99	11.4	31.2	45	16.3	36.5
28/07/98	L13	17.0	8.37	11.0	31.6	38	13.1	34.8
28/07/98	L14	14.1	7.60	12.1	32.2	42	15.9	37.5
28/07/98	L15	20.1	8.43	11.5	31.2	37	13.6	36.8
28/07/98	L16	19.2	8.28	12.4	33.1	40	14.9	37.4
28/07/98	L17	6.2	8.09	11.4	31.6	39	14.0	36.0
28/07/98	L18	12.2	7.57	11.5	31.6	42	15.1	36.3
28/07/98	L19	23.9	8.09	12.0	34.1	42	14.8	35.1
28/07/98	L20	15.8	7.97	10.9	31.6	40	13.6	34.4
28/07/98	L21	30.7	7.36	11.3	31.2	42	15.3	36.2
28/07/98	L22	12.3	8.00	12.6	33.8	42	15.7	37.2
28/07/98	L23	14.2	7.54	11.9	33.4	44	15.7	35.6
28/07/98	L24	18.0	7.17	10.0	28.7	40	13.9	34.8
28/07/98	L25	13.9	7.30	10.3	28.7	39	14.1	35.8
28/07/98	L26	9.8	7.57	12.2	31.9	42	16.1	38.2
28/07/98	L27	19.1	7.94	13.0	34.7	44	16.3	37.4
28/07/98	L28	14.1	7.24	9.8	28.1	39	13.5	34.8
28/07/98	L29	10.6	8.09	11.6	32.5	40	14.3	35.6
28/07/98	L30	12.3	7.54	11.0	30.6	41	14.5	35.9

Haematology results from day of viral challenge

DATE	ANIMAL	BNEU	NEU	LYMPH	MONO	EOS	BASO
28/07/98	L1	0.133	5.719	5.320	0.931	0.931	0.266
28/07/98	L2	0.000	6.174	5.292	0.126	0.756	0.252
28/07/98	L3	0.000	10.944	2.432	0.456	1.064	0.304
28/07/98	L4	0.000	6.072	5.940	0.264	0.924	0.000
28/07/98	L5	0.000	6.364	8.600	0.688	1.204	0.344
28/07/98	L6	0.252	7.056	3.780	0.630	0.756	0.126
28/07/98	L8	0.000	15.004	5.566	1.210	2.420	0.000
28/07/98	L9	0.000	3.762	6.840	0.114	0.570	0.114
28/07/98	L10	0.000	6.952	6.794	0.474	1.422	0.158
28/07/98	L11	0.000	7.700	5.180	0.280	0.840	0.000
28/07/98	L12	0.000	6.048	5.670	0.126	0.252	0.378
28/07/98	L13	0.170	7.820	6.460	0.510	2.040	0.000
28/07/98	L14	0.000	5.640	7.050	0.141	0.987	0.282
28/07/98	L15	0.000	9.447	9.648	0.402	0.603	0.000
28/07/98	L16	0.000	8.448	7.296	0.960	2.304	0.192
28/07/98	L17	0.062	2.480	3.100	0.248	0.310	0.000
28/07/98	L18	0.000	6.344	4.880	0.244	0.732	0.000
28/07/98	L19	0.239	10.994	10.516	0.239	1.673	0.239
28/07/98	L20	0.000	7.900	6.004	0.316	1.422	0.158
28/07/98	L21	0.614	20.876	6.754	0.921	1.228	0.307
28/07/98	L22	0.000	5.043	6.027	0.246	0.984	0.000
28/07/98	L23	0.000	6.958	4.260	0.568	2.272	0.142
28/07/98	L24	0.000	9.720	5.220	0.720	2.160	0.180
28/07/98	L25	0.139	8.340	4.031	0.278	0.973	0.000
28/07/98	L26	0.000	2.548	5.978	0.196	0.882	0.196
28/07/98	L27	0.000	9.741	8.404	0.191	0.382	0.382
28/07/98	L28	0.141	7.050	4.794	0.141	1.692	0.141
28/07/98	L29	0.000	4.452	5.194	0.318	0.530	0.106
28/07/98	L30	0.123	5.781	4.674	0.246	1.476	0.000

## Haematology results from day of viral challenge

DATE	ANIMAL	WBC	RBC	HB	HCT	MCV	MCH	MCHC
18/08/98	L1	12.6	8.19	12.9	34	42	15.7	37.9
18/08/98	L2	6.9	7.81	10.8	29.9	38	13.8	36.1
18/08/98	L3	10.8	8.26	11.6	31.5	38	14.0	36.8
18/08/98	L4	17.4	9.41	11.6	31.8	34	12.3	36.4
18/08/98	L5	11.9	9.12	13.6	35.2	39	14.9	38.6
18/08/98	L6	10.4	9.22	12.2	34.3	37	13.2	35.5
18/08/98	L8	17.6	8.07	10.3	30.5	38	12.7	33.7
18/08/98	L9	3.9	8.99	14.5	35.5	39	16.1	40.8
18/08/98	L10	12.4	8.16	12.3	33.3	41	15.0	36.9
18/08/98	L11	14.5	7.56	9.7	27.4	36	12.8	35.4
18/08/98	L12	12.5	7.12	11.2	31.1	44	15.7	36.0
18/08/98	L13	11.2	9.02	11.3	32.1	36	12.5	35.2
18/08/98	L14	8.0	8.16	12.1	31.8	39	14.8	38.0
18/08/98	L15	10.4	7.94	10.0	27.7	35	12.5	36.1
18/08/98	L16	15.5	8.86	12.2	34.6	39	13.7	35.2
18/08/98	L17	6.0	8.51	11.5	31.5	37	13.5	36.5
18/08/98	L18	9.2	8.13	11.6	32.1	39	14.2	36.1
18/08/98	L19	16.4	7.28	10.0	29.2	40	13.7	34.2
18/08/98	L20	9.5	7.66	9.7	28.6	37	12.6	33.9
18/08/98	L21	11.2	6.55	9.0	25.8	39	13.7	34.8
18/08/98	L22	6.2	6.81	9.7	28.3	42	14.2	34.2
18/08/98	L23	11.4	8.22	12.4	34.6	42	15.0	35.8
18/08/98	L24	11.5	6.81	8.6	26.7	39	12.6	32.2
18/08/98	L25	10.7	7.97	10.3	30.2	38	12.9	34.1
18/08/98	L26	11.1	7.09	10.6	28.6	40	14.9	37.0
18/08/98	L27	19.2	7.62	11.6	31.5	41	15.2	36.8
18/08/98	L28	21.0	7.28	9.3	27.7	38	12.7	33.5
18/08/98	L29	13.1	7.81	10.7	30.8	39	13.7	34.7
18/08/98	L30	15.2	7.24	10.0	28.9	40	13.8	34.6

Haematology results from three weeks post-challenge

DATE	ANIMAL	BNEU	NEU	LYMPH	MONO	EOS	BASO
18/08/98	L1	0.000	5.670	5.670	0.252	0.882	0.126
18/08/98	L2	0.000	3.588	2.484	0.207	0.621	0.000
18/08/98	L3	0.000	6.480	3.456	0.216	0.540	0.108
18/08/98	L4	0.174	4.872	11.136	0.696	0.348	0.174
18/08/98	L5	0.000	4.403	6.902	0.119	0.357	0.119
18/08/98	L6	0.104	6.448	2.704	0.208	0.832	0.104
18/08/98	L8	0.352	8.272	5.104	0.880	2.816	0.176
18/08/98	L9	0.000	0.897	2.769	0.000	0.195	0.039
18/08/98	L10	0.000	7.068	4.216	0.248	0.868	0.000
18/08/98	L11	0.145	8.410	5.075	0.290	0.580	0.000
18/08/98	L12	0.000	6.375	5.375	0.250	0.500	0.000
18/08/98	L13	0.000	5.264	4.368	0.448	1.120	0.000
18/08/98	L14	0.000	3.440	3.680	0.080	0.640	0.080
18/08/98	L15	0.000	4.472	5.200	0.104	0.624	0.000
18/08/98	L16	0.000	5.580	8.215	0.465	1.085	0.155
18/08/98	L17	0.000	3.180	2.520	0.120	0.120	0.060
18/08/98	L18	0.000	4.324	4.232	0.184	0.460	0.000
18/08/98	L19	0.164	7.872	7.380	0.328	0.492	0.164
18/08/98	L20	0.000	4.370	4.180	0.095	0.665	0.190
18/08/98	L21	0.000	4.928	5.152	0.336	0.672	0.112
18/08/98	L22	0.000	2.542	2.108	0.310	0.992	0.186
18/08/98	L23	0.000	4.560	4.788	0.228	1.710	0.114
18/08/98	L24	0.000	5.750	4.025	0.690	1.035	0.000
18/08/98	L25	0.000	6.206	2.996	0.214	1.070	0.214
18/08/98	L26	0.000	5.106	5.217	0.222	0.555	0.000
18/08/98	L27	0.000	8.832	9.408	0.000	0.960	0.000
18/08/98	L28	0.210	9.450	7.980	1.050	1.890	0.420
18/08/98	L29	0.000	5.502	6.812	0.000	0.786	0.000
18/08/98	L30	0.000	9.424	4.712	0.456	0.608	0.000

Haematology results from three weeks post-challenge

DATE	ANIMAL	WBC	RBC	HB	HCT	MCV	MCH	MCHC
08/09/98	L1	11.9	8.77	14.0	36.5	42	15.9	38.3
08/09/98	L2	8.3	8.80	12.3	33.3	38	13.9	36.9
08/09/98	L3	11.9	8.58	12.4	32.4	38	14.4	38.2
08/09/98	L4	16.6	9.41	11.2	31.5	33	11.9	35.5
08/09/98	L5	13.6	9.22	13.5	36.2	39	14.6	37.2
08/09/98	L6	9.9	9.51	12.8	36.5	38	13.4	35.0
08/09/98	L8	16.8	8.61	11.2	31.5	37	13.0	35.5
08/09/98	L9	6.3	9.89	15.4	38.7	39	15.5	39.7
08/09/98	L10	7.4	9.15	13.8	36.2	40	15.0	38.1
08/09/98	L11	16.0	8.03	10.3	28.3	35	12.8	36.3
08/09/98	L12	12.0	8.32	13.0	33	40	15.6	39.3
08/09/98	L13	15.3	9.83	12.4	34.9	36	12.6	35.5
08/09/98	L14	11.4	8.22	12.7	33.3	41	15.4	38.1
08/09/98	L15	10.8	8.99	11.6	31.5	35	12.9	36.8
08/09/98	L16	15.1	9.35	13.1	36.8	39	14.0	35.5
08/09/98	L17	5.1	7.53	11.0	30.2	40	14.6	36.4
08/09/98	L18	6.6	7.84	10.9	29.9	38	13.9	36.4
08/09/98	L19	22.2	8.77	12.6	34.9	40	14.3	36.1
08/09/98	L20	15.9	8.45	11.0	31.5	37	13.0	34.9
08/09/98	L21	8.2	7.02	9.9	27.7	39	14.1	35.7
08/09/98	L22	10.2	5.65	9.6	27.7	49	16.9	34.6
08/09/98	L23	8.2	8.35	12.4	32.7	39	14.8	37.9
08/09/98	L24	17.8	8.38	10.6	28.9	34	12.6	36.6
08/09/98	L25	10.2	8.10	10.8	30.5	38	13.3	35.4
08/09/98	L26	9.3	8.00	12.3	32.4	40	15.3	37.9
08/09/98	L27	20.1	8.67	13.9	36.5	42	16.0	38.0
08/09/98	L28	15.7	7.37	9.7	27.4	37	13.1	35.4
08/09/98	L29	12.9	8.16	10.9	29.9	37	13.3	36.4
08/09/98	L30	16.1	8.00	10.5	28.9	36	13.1	36.3

Haematology results from six weeks post-challenge

DATE	ANIMAL	BNEU	NEU	LYMPH	MONO	EOS	BASO
08/09/98	L1	0.000	3.570	6.664	0.595	0.714	0.357
08/09/98	L2	0.000	2.241	5.727	0.166	0.083	0.083
08/09/98	L3	0.000	5.950	3.927	0.238	1.785	0.000
08/09/98	L4	0.000	6.142	9.130	0.332	0.830	0.166
08/09/98	L5	0.136	5.440	6.936	0.272	0.816	0.000
08/09/98	L6	0.099	4.851	3.960	0.792	0.198	0.000
08/09/98	L8	0.000	8.568	5.544	0.504	2.016	0.168
08/09/98	L9	0.063	0.819	5.355	0.000	0.000	0.063
08/09/98	L10	0.000	0.444	6.734	0.222	0.000	0.000
08/09/98	L11	0.000	6.400	8.960	0.320	0.320	0.000
08/09/98	L12	0.000	4.440	6.600	0.120	0.840	0.000
08/09/98	L13	0.000	7.497	5.967	0.153	1.530	0.153
08/09/98	L14	0.114	5.016	5.700	0.456	0.114	0.000
08/09/98	L15	0.000	3.240	7.236	0.324	0.000	0.000
08/09/98	L16	0.000	4.983	8.758	0.453	0.906	0.000
08/09/98	L17	0.000	2.652	1.734	0.510	0.102	0.102
08/09/98	L18	0.066	1.518	4.950	0.066	0.000	0.000
08/09/98	L19	0.000	7.992	12.654	0.666	0.666	0.222
08/09/98	L20	0.000	7.791	6.678	0.318	1.113	0.000
08/09/98	L21	0.082	3.198	4.674	0.082	0.082	0.000
08/09/98	L22	0.000	2.652	6.630	0.204	0.408	0.204
08/09/98	L23	0.082	3.772	3.362	0.492	0.410	0.000
08/09/98	L24	0.178	11.036	6.052	0.178	0.356	0.000
08/09/98	L25	0.000	4.182	4.692	0.408	0.918	0.000
08/09/98	L26	0.000	2.139	5.766	0.372	0.930	0.093
08/09/98	L27	0.000	6.030	13.266	0.000	0.603	0.201
08/09/98	L28	0.157	6.908	5.966	0.471	2.198	0.000
08/09/98	L29	0.000	3.870	8.127	0.129	0.774	0.000
08/09/98	L30	0.000	6.118	8.855	0.161	0.966	0.000

Haematology results from six weeks post-challenge

DATE	ANIMAL	WBC	RBC	HB	HCT	MCV	MCH	MCHC
02/10/98	L1	18.0	8.45	14.0	38.1	45	16.5	36.7
02/10/98	L2	7.6	8.85	12.3	34.8	39	13.8	35.3
02/10/98	L3	10.1	9.31	13.7	39.1	42	14.7	35.0
02/10/98	L4	16.1	9.90	12.9	35.5	36	13.0	36.3
02/10/98	L5	20.0	9.80	14.7	39.5	40	15.0	37.2
02/10/98	L6	11.4	10.31	13.7	39.8	39	13.2	34.4
02/10/98	L8	21.2	8.72	11.8	34.8	40	13.5	33.9
02/10/98	L9	11.3	9.77	14.9	40.2	41	15.2	37.0
02/10/98	L10	11.0	9.44	14.7	40.5	43	15.5	36.2
02/10/98	L11	18.1	8.29	11.1	30.8	37	13.3	36.0
02/10/98	L12	14.0	7.51	11.8	33.1	44	15.7	35.6
02/10/98	L13	11.0	7.66	11.1	35.8	43	15.5	36.7
02/10/98	L14	13.0	8.06	12.5	33.5	42	15.5	37.3
02/10/98	L15	14.5	9.24	12.6	35.1	38	13.6	35.8
02/10/98	L16	19.5	10.31	14.9	40.2	39	14.4	37.0
02/10/98	L17	7.6	8.75	13.2	35.1	40	15.0	37.6
02/10/98	L18	9.9	8.42	12.4	33.5	40	14.7	37.0
02/10/98	L19	24.0	10.24	14.0	41.2	40	13.6	33.9
02/10/98	L20	19.3	9.90	13.5	38.5	39	13.6	35.0
02/10/98	L21	13.1	7.38	10.7	31.1	42	14.4	34.4
02/10/98	L22	17.8	8.75	14.2	39.8	45	16.2	35.6
02/10/98	L23	14.3	9.28	14.4	40.5	44	15.5	35.5
02/10/98	L24	11.9	7.80	10.1	29.1	37	12.9	34.7
02/10/98	L25	12.2	8.75	11.5	34.1	39	13.1	33.7
02/10/98	L26	11.8	7.97	12.6	34.8	44	15.8	36.2
02/10/98	L27	22.0	9.08	15.2	40.2	44	16.7	37.8
02/10/98	L28	15.2	8.00	11.0	31.8	40	13.7	34.5
02/10/98	L29	10.9	8.39	12.0	34.1	41	14.3	35.1
02/10/98	L30	11.6	8.62	11.7	33.5	39	13.5	34.9

Haematology results from nine weeks post-challenge

DATE	ANIMAL	BNEU	NEU	LYMPH	MONO	EOS	BASO
02/10/98	L1	0.000	8.820	8.100	0.180	0.720	0.180
02/10/98	L2	0.000	1.596	5.548	0.228	0.228	0.000
02/10/98	L3	0.000	5.050	3.737	0.303	0.909	0.101
02/10/98	L4	0.000	5.635	9.660	0.161	0.644	0.000
02/10/98	L5	0.000	5.800	13.000	0.200	1.000	0.000
02/10/98	L6	0.114	5.472	4.560	0.456	0.684	0.114
02/10/98	L8	0.000	10.176	8.268	0.848	1.908	0.000
02/10/98	L9	0.000	2.373	7.797	0.339	0.791	0.000
02/10/98	L10	0.000	2.860	7.810	0.220	0.110	0.000
02/10/98	L11	0.000	6.516	9.955	0.724	0.905	0.000
02/10/98	L12	0.000	6.440	6.720	0.140	0.560	0.140
02/10/98	L13	0.000	6.712	6.360	0.175	0.567	0.000
02/10/98	L14	0.000	6.890	5.200	0.130	0.780	0.000
02/10/98	L15	0.000	5.510	7.975	0.145	0.725	0.145
02/10/98	L16	0.000	7.215	9.360	0.585	2.145	0.195
02/10/98	L17	0.000	2.508	4.560	0.304	0.228	0.000
02/10/98	L18	0.000	2.673	6.831	0.297	0.099	0.000
02/10/98	L19	0.000	12.480	10.320	0.720	0.240	0.240
02/10/98	L20	0.193	10.615	6.755	0.193	1.351	0.193
02/10/98	L21	0.524	6.681	5.502	0.393	0.000	0.000
02/10/98	L22	0.000	5.518	10.502	0.356	1.246	0.178
02/10/98	L23	0.143	5.577	7.007	0.286	1.287	0.000
02/10/98	L24	0.119	5.236	5.474	0.357	0.714	0.000
02/10/98	L25	0.000	4.758	6.100	0.244	1.098	0.000
02/10/98	L26	0.118	2.478	8.260	0.236	0.472	0.236
02/10/98	L27	0.000	8.140	12.320	0.440	0.660	0.440
02/10/98	L28	0.304	7.600	5.168	0.304	1.824	0.000
02/10/98	L29	0.000	4.251	5.886	0.000	0.763	0.000
02/10/98	L30	0.000	6.032	4.408	0.116	0.928	0.116

Haematology results from nine weeks post-challenge

DATE	ANIMAL	WBC	RBC	HB	HCT	MCV	MCH	MCHC
27/10/98	L1	12.6	8.57	15.0	38.5	45	17.5	38.9
27/10/98	L2	9.8	8.93	12.8	34.5	39	14.3	37.1
27/10/98	L3	10.2	9.12	14.7	39.1	43	16.1	37.5
27/10/98	L4	14.9	9.64	13.1	35.5	37	13.5	36.9
27/10/98	L5	15.0	10.45	15.9	41.8	40	15.2	38.0
27/10/98	L6	12.8	9.70	13.4	36.8	38	13.8	36.4
27/10/98	L8	21.3	9.44	13.7	38.8	41	14.5	35.3
27/10/98	L9	11.6	10.42	16.9	43.8	42	16.2	38.5
27/10/98	L10	10.2	9.96	15.6	42.2	42	15.6	36.9
27/10/98	L11	16.1	8.22	11.8	31.1	38	14.3	37.9
27/10/98	L12	11.2	8.12	13.2	35.5	44	16.2	37.1
27/10/98	L13	17.7	10.06	13.5	38.1	38	13.4	35.4
27/10/98	L14	12.0	8.44	13.7	35.8	42	16.2	38.2
27/10/98	L15	13.4	9.09	13.0	34.5	38	14.3	37.6
27/10/98	L16	15.4	10.52	15.9	43.8	42	15.1	36.3
27/10/98	L17	7.8	9.06	13.4	35.5	39	14.7	37.7
27/10/98	L18	9.2	8.83	13.3	36.1	41	15.0	36.8
27/10/98	L19	19.9	9.47	14.2	38.8	41	14.9	36.5
27/10/98	L20	18.7	8.80	12.8	35.8	41	14.5	35.7
27/10/98	L21	16.5	8.28	12.9	35.1	42	15.5	36.7
27/10/98	L22	12.4	8.83	14.2	39.1	44	16.0	36.3
27/10/98	L23	10.1	9.19	15.5	42.8	47	16.8	36.2
27/10/98	L24	13.0	7.68	10.6	30.1	39	13.8	35.2
27/10/98	L25	10.8	8.93	12.3	34.5	39	13.7	35.6
27/10/98	L26	10.0	8.25	14.0	36.5	44	16.9	38.3
27/10/98	L27	16.0	8.32	14.4	37.8	45	17.3	38.0
27/10/98	L28	17.8	8.15	11.9	33.5	41	14.6	35.5
27/10/98	L29	17.1	9.77	14.4	39.8	41	14.7	36.1
27/10/98	L30	12.2	8.57	12.5	34.8	41	14.5	35.9

Haematology results from thirteen weeks post-challenge

DATE	ANIMAL	BNEU	NEU	LYMPH	MONO	EOS	BASO
27/10/98	L1	0.000	3.402	7.812	0.378	0.882	0.126
27/10/98	L2	0.000	4.410	4.998	0.098	0.294	0.000
27/10/98	L3	0.000	4.794	4.386	0.510	0.408	0.102
27/10/98	L4	0.000	5.960	7.897	0.298	0.745	0.000
27/10/98	L5	0.000	3.450	10.500	0.150	0.600	0.300
27/10/98	L6	0.000	6.400	5.248	0.384	0.384	0.384
27/10/98	L8	0.000	9.585	7.029	0.852	3.621	0.213
27/10/98	L9	0.000	2.668	8.468	0.000	0.348	0.116
27/10/98	L10	0.000	2.754	6.630	0.306	0.408	0.000
27/10/98	L11	0.000	6.279	9.177	0.161	0.483	0.000
27/10/98	L12	0.000	4.032	5.936	0.112	1.008	0.112
27/10/98	L13	0.000	9.381	5.133	0.354	2.478	0.354
27/10/98	L14	0.000	4.920	6.000	0.240	0.600	0.240
27/10/98	L15	0.000	4.422	8.576	0.134	0.134	0.134
27/10/98	L16	0.000	4.928	8.162	0.308	1.848	0.154
27/10/98	L17	0.000	3.276	3.978	0.156	0.312	0.078
27/10/98	L18	0.000	3.036	5.612	0.184	0.368	0.000
27/10/98	L19	0.000	10.149	8.756	0.597	0.398	0.000
27/10/98	L20	0.000	8.789	7.667	0.561	1.309	0.374
27/10/98	L21	0.165	10.065	5.115	0.330	0.825	0.000
27/10/98	L22	0.000	3.472	7.316	0.372	1.116	0.124
27/10/98	L23	0.000	4.545	3.838	0.505	1.111	0.101
27/10/98	L24	0.000	5.460	6.370	0.260	0.910	0.000
27/10/98	L25	0.000	5.292	4.536	0.324	0.648	0.000
27/10/98	L26	0.000	2.400	6.800	0.100	0.600	0.100
27/10/98	L27	0.000	5.920	9.280	0.320	0.480	0.000
27/10/98	L28	0.000	7.654	6.764	0.534	2.670	0.178
27/10/98	L29	0.000	5.814	10.089	0.171	1.026	0.000
27/10/98	L30	0.000	5.856	5.612	0.122	0.610	0.000

Haematology results from thirteen weeks post-challenge

