STUDIES OF ACUTE PHASE PROTEINS AND TUMOUR NECROSIS FACTOR RECEPTORS AS INFLAMMATORY MARKERS IN THE CAT

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ABSTRACT

The measurement of acute phase proteins is used by human clinicians to give valuable information about a patient's inflammatory response, both when monitoring clinical disease and when assessing the effect of therapy. Levels of soluble receptors for the cytokine, tumour necrosis factor, also increase as a result of inflammatory stimuli and are useful prognostic markers over the asymptomatic phase of human immunodeficiency virus infection. The aim of the work presented in this thesis was to determine whether these markers are of value when investigating feline disease.

Reference ranges for two acute phase proteins, α1-acid glycoprotein (AGP) and haptoglobin were determined by measuring their concentrations in serum samples from healthy cats. Analysis of samples from cats with feline infectious peritonitis (FIP) and from cats suffering from conditions with a similar clinical presentation revealed that measurement of AGP can be a useful adjunct to other laboratory tests when reaching a diagnosis. In contrast, measurement of haptoglobin was not found to be of value. Despite increases in the levels of pro-inflammatory cytokines in samples taken from cats during the asymptomatic phase of feline immunodeficiency virus (FIV) infection, no changes were detected in the levels of AGP and haptoglobin. It was concluded that these acute phase proteins are of no benefit as prognostic markers in FIV.

The L929 bioassay was used to investigate anti-TNF-α activity in cell culture fluids from feline splenic cells. Cytotoxic activity was demonstrated in very few of the samples whilst anti-cytotoxic activity was detected in the majority of samples. This anti-cytotoxic activity was attributed to the presence of feline soluble TNF receptor type 1 (sTNFR-1) binding to and inhibiting the effects of TNF-α. This was not confirmed because of the lack of specific neutralising antibody. Subsequent work was therefore directed towards the development of immune-based species-specific assays for feline soluble TNF receptors (sTNFRs).

The polymerase chain reaction was used to amplify the sequences coding for feline sTNFRs. Most of the extracellular domain of feline TNFR-1 and part of the intracellular domain of feline TNFR-2 were cloned and sequenced using this technique. The amplified regions demonstrated 85% and 77% homology at the nucleic acid level and 83% and 67% homology at the amino acid level to the corresponding regions of the human sequences for TNFR-1 and 2 respectively. Feline sTNFR-1 was expressed as a glutathione-S-transferase fusion protein. After purification, concentration and electrophoresis, the appropriate protein band was excised and used to inoculate a sheep. Antiserum taken from the sheep post-inoculation recognised the expressed protein by western blotting, but results were inconsistent and analysis of the antiserum was hampered by the very small amounts of expressed protein available.

Two peptides were synthesised based on regions of antigenicity in feline sTNFR-1 and were used to inoculate sheep. Antiserum to peptide A showed a strong reaction against peptide A in an ELISA and gave a positive result when used as the primary antibody to stain healthy feline liver tissue.

In conclusion, both antiserum to expressed feline sTNFR-1 and anti-peptide antibody based on a region of feline sTNFR-1 have been raised in sheep and are available for the development of an assay for this protein. Further expression of feline TNFR-1 will be required before these antisera can be analysed fully.

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Duthie, S, Nasir, L and Eckersall, PD (1997) Feline tumour necrosis factor receptors: identification, sequencing and potential clinical application *World Small Animal Veterinary Association Congress Proceedings*

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DEDICATION

To mum, dad and Neil.

Chapter 1:
General Introduction

1.1. Summary of Chapter

This thesis presents work investigating serological markers of inflammation in the cat including acute phase proteins and soluble receptors for the cytokine, tumour necrosis factor. The introductory chapter gives a review of the inflammatory response, the markers studied in this work and two feline diseases, feline infectious peritonitis (FIP) and feline immunodeficiency virus (FIV) infection, that were used as models for studying these markers. An outline of the objectives which prompted the work is also given.

1.2. The Acute Phase Response

1.2.1. Definition

The acute phase response is a mechanism which attempts to restore normal bodily function after homeostasis has been interrupted by noxious stimuli and to remove the initiating stimulus (Koj and Gordon, 1985b). It consists of both local and systemic effects and mechanisms exist to limit the response since, if excessive or prolonged, it can prove detrimental to the host.

1.2.2. Induction of the Acute Phase Response

Cells of the mononuclear phagocytic system such as monocytes and macrophages are the principal cells involved in the initiation of the acute phase response (Baumann and Gauldie, 1994). Cytokines, particularly γ-interferon (IFN-γ) (Svedersky et al. 1984; Woessner, 1992), induced by inflammatory stimulants such as lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria, play a definitive role in activating macrophages. These activated cells are then responsible for a number of pathways (figure 1.1.) which assist the elimination of pathogens and the restoration of homeostasis. They display increased expression of Fc receptor proteins and major histocompatibility complex (MHC) class II molecules, enhancing

antigen uptake and presentation (De Maeyer and De Maeyer-Guignard, 1994), and of costimulatory or cell surface molecules (Doherty, 1995), which play a part in macrophage interaction with T-cells. IFN-γ also stimulates the secretion of reactive oxygen intermediates and hydrogen peroxide from macrophages, augmenting the killing of intracellular parasites (Nathan et al. 1983).

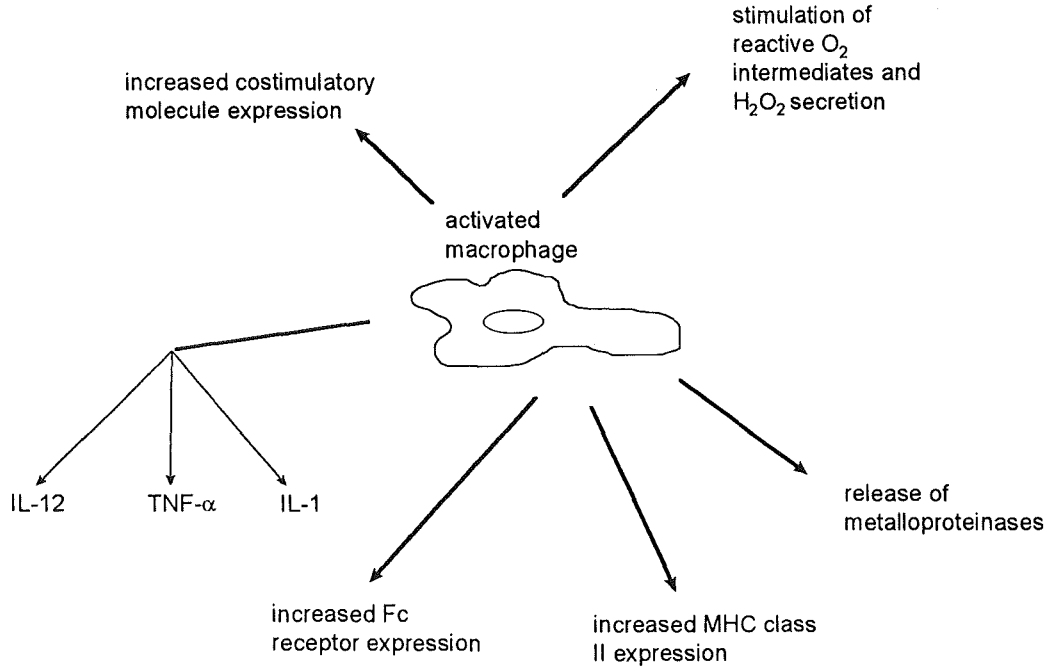
Activated macrophages produce metalloproteinases which assist the digestion of endothelial basement membrane and allow migration of these cells from blood to tissues (Wahl et al. 1974). There, they are responsible for the release of further mediators, in particular the cytokines interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) which initiate the cascade of events constituting the acute phase response. The action of IL-1 and TNF-α results in a secondary wave of cytokines, of which IL-6 is of particular importance in the acute phase response, and chemokines from neighbouring cells which include chemotactic agents to attract cells into the damaged area to assist repair. Interleukin-12 production by macrophages directs the development of a T helper cell type 1 (Th1)-like or cell-mediated immune response (Hsieh et al. 1993).

Several cytokines including interleukins 4, 10 and 13 and transforming growth factor-β have been implicated in causing the deactivation of macrophages (Bogdan and Nathan, 1993) although it is now recognised that macrophages can respond to cytokine stimulation in different ways depending on the local environment (Doherty, 1995).

1.2.3. Mechanisms of the Acute Phase Response

The local and systemic processes of the acute phase response are summarised in figure 1.2. On activation, leukocytes invading infected or inflamed tissue, release substances which leak into the extracellular space (Evans and Whicher, 1992). These low molecular weight mediators such as the arachidonic acid metabolites act locally to alter vascular tone and increase leakage from blood vessels causing tissue oedema and redness (Cotran et al. 1994), and, along with molecules liberated during the clotting cascade, result in the production of pain. One of these mediators, nitrous oxide (NO), is a soluble free radical gas which has been established as a crucial mediator of macrophage function. Treatments blocking the production of NO

Figure 1.1. Activities of the activated macrophage

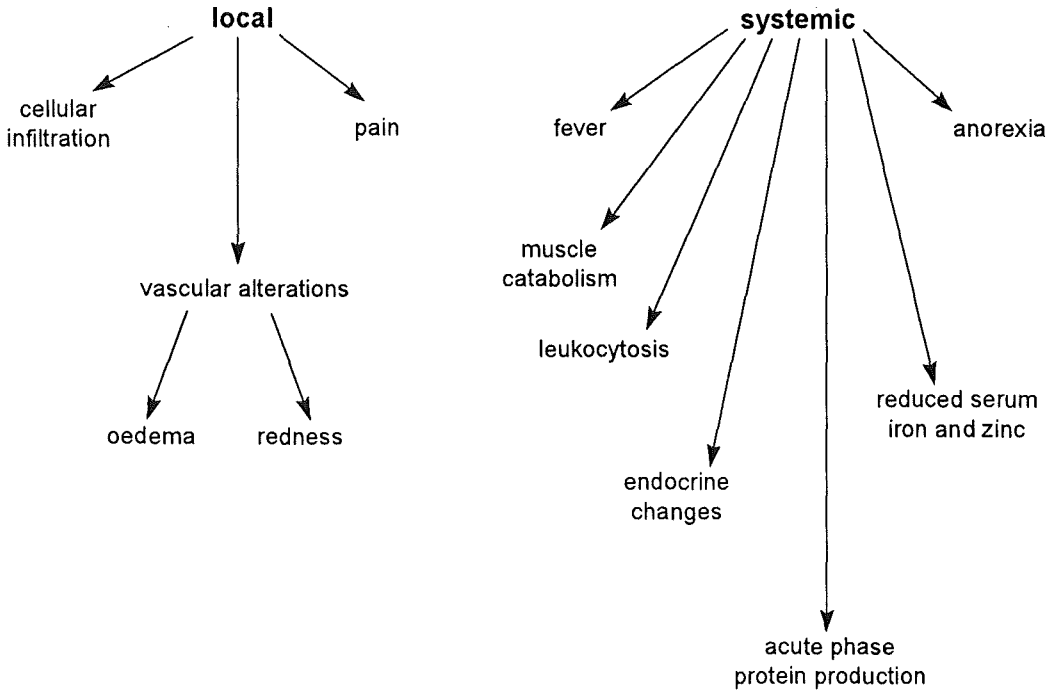


prevent the tumoricidal and bactericidal functions of macrophages (Lowenstein and Snyder, 1992). NO is also produced by endothelial cells, resulting in relaxation of adjacent vascular smooth muscle cells. In a similar manner to other mediators of inflammation, NO can have both helpful and detrimental effects and it is implicated as being a cause of hypotension (Evans and Cohen, 1996) during uncontrolled inflammatory diseases, consequently therapeutic roles for inhibitors of NO and the enzyme NO synthase have been hypothesised (Groeneveld et al. 1997; Nathan, 1992).

Systemically, release of acute phase cytokines can be associated with a multitude of reactions including fever, anorexia, leukocytosis, activation of complement, a rise in serum glucocorticoids, altered lipid metabolism, a reduction in serum iron and zinc levels, an enhanced uptake of amino acids with muscle catabolism and the production of acute phase proteins by the liver (Moshage, 1997; Whicher and Westacott, 1992). The febrile response arises through the central action of the inflammatory cytokines on the hypothalamus (Dinarello, 1994) and induction of prostaglandin E_2 (Dinarello et al. 1991) which alters the balance between peripheral vasoconstriction and sweating and increases muscle metabolism, becoming apparent as shivering (Atkins and Bodel, 1972). An elevated incubation temperature has been shown to enhance lymphocyte function *in vitro* (Roberts and Steigbigel, 1977); fever is considered to be fundamental to the inflammatory response and ultimately host survival (Carmichael et al. 1969). Optimisation of the mechanisms of the inflammatory response in the pyretic state reduce their potential toxic effect on the body during homeostasis. The central action of the pro-inflammatory cytokines on the hypothalamus is also responsible for anorexia (Johnson, 1997).

Corticosteroid synthesis is increased during inflammation as a result of both hypothalamic and adrenal stimulation by the inflammatory cytokines. These glucocorticoids are responsible for alterations in carbohydrate metabolism (Gordon, 1985). Conditions inducing an acute phase response such as severe infection and major surgery can result in lower circulating levels of thyroxine. It is suggested that this alteration in thyroid function is mediated by the direct and indirect effects of TNF-α and other cytokines on pituitary and thyroid cells (Wong and Hershman, 1992).

Figure 1.2. Local and systemic effects of the acute phase response



IL-1 and IL-6 induce the release of cells from the bone marrow and the production of granulocyte and macrophage replication factors. Levels of iron are reduced initially (Myers et al. 1984) due to an increase in vascular permeability and subsequently through sequestration at the site of injury probably by binding to lactoferrin which is released from neutrophils. Zinc is lost from plasma initially (within 2 hours) through extravasation of its carrier protein, albumin, and subsequently by hepatic uptake when the ratio of zinc to albumin becomes reduced. IL-1 and IL-6 also stimulate release of corticotrophin-releasing hormone (Busbridge and Grossman, 1991), and lead to increased cortisol production, which inhibits cytokine expression by a negative feedback loop. In addition, these cytokines have been shown to cause corticosteroid release directly from adrenal gland cells *in vivo*.

An increase in urinary nitrogen excretion during inflammation reflects muscle proteolysis although the precise mechanisms involved are unclear (Webel et al. 1997). Studies *in vitro* have shown that IL-1 (leukocytic pyrogen) induces PGE_2 in isolated muscles which increases protein breakdown, a response that is enhanced at 39°C when compared to 37°C (Baracos et al. 1983). The released amino acids are used for lymphoid proliferation, antibody production and the production of acute phase proteins by the liver. TNF-α stimulates enhanced lipolysis releasing additional substrates for utilisation in the liver. Both of these catabolic processes lead to weight loss. The inflammatory mediators cause alterations in the normal metabolic pathways of the liver and induce the production of acute phase proteins by hepatocytes.

1.2.4. TNF-α and the Acute Phase Response

At the turn of the century, it was observed that haemorrhagic necrosis of tumours occurred in human patients during the course of a concurrent bacterial infection (Coley, 1919) and the same observation was made in cancer patients inoculated with killed bacteria. Further studies of this phenomenon led to the hypothesis that a substance, tumour necrosis factor (TNF), mediated endotoxin-induced tumour necrosis, and that it may be responsible for the suppression of transformed cells that is seen in the presence of activated macrophages (Carswell et al. 1975). Indeed, TNF was later purified and characterised by Aggarwal and others (Aggarwal et al. 1985). The same protein was also purified by a group investigating a toxic mediator responsible for the chronic wasting seen in inflammation and named cachectin

(Beutler et al. 1985b). It became clear that both tumour necrosis and cachexia were due to the same 17kDa protein, now known as TNF-α (Beutler et al. 1985a). However, it was also apparent that any clinical value the protein may have in cancer therapy might be limited by the severe systemic toxicity that it could also initiate (Beutler and Cerami, 1989).

Macrophages and monocytes are believed to be the major source of TNF-α (Mannel et al. 1980) although a variety of other cells have also been identified that are capable of producing the cytokine (Xing et al. 1993). One of the most potent stimuli of TNF-α synthesis is bacterial LPS (endotoxin) (Tracey et al. 1988), which interacts with the CD14 membrane protein on monocytes and macrophages. TNF-α production is also stimulated by other microbial products such as enterotoxins and some antigenic determinants. Non-covalent oligomerisation of three monomers of TNF-α after secretion results in a trimeric structure (Sprang and Eck, 1992) which is essential for biological activity; disruption of the trimer results in loss of activity (Lin, 1992). TNF-α bound to the plasma membrane can also have bioactivity (Perez et al. 1990). It is not known whether all secreted TNF-α arises through cleavage of the membrane bound form or whether it can be released without formation of the intermediate.

As discussed briefly in section 1.2.1., TNF-α in combination with IL-1 is released from activated macrophages at the site of tissue damage. It interacts with endothelial cells to induce the release of further IL-1 (Nawroth et al. 1986). These cytokines then act on neighbouring stromal cells, causing a secondary release of cytokines such as IL-6 and initiating the cascade events of the acute phase response. TNF-α is chemotactic for phagocytic cells and enhances their phagocytic activity and is thus involved in the initial non-specific cellular response to invasion. TNF-α also plays a role in the up-regulation of expression of endothelial surface molecules such as the major histocompatibility complex antigens HLA-A and HLA-B (Collins et al. 1986). The TNF-α-induced increased expression of these molecules on tumour cells might make them more susceptible to cytotoxic cell attack (Vanky et al. 1998). The intercellular adhesion molecule, ICAM-1, is also up regulated by TNF-α, slowing leukocyte flow, and enhancing migration from the circulation into tissue.

TNF-α is involved in the febrile response to inflammation through the induction of prostaglandin E_2 (Dinarello et al. 1991). The hypothalamic centres that regulate body temperature and appetite do not have a blood-brain barrier and systemically administered TNF-α crosses into this area leading to fever and anorexia. Levels of TNF-α in serum become rapidly elevated in response to an inflammatory stimulus (Beutler et al. 1985c) but also decline rapidly, disappearing from serum in less than 6 hours in the case of experimental bacteraemia or endotoxaemia (Redl et al. 1995).

The related but distinct cytokine, TNF-β or lymphotoxin-α, is a member of the same gene family, and shares 35% amino acid homology and many activities with TNF-α including the ability to kill L929 cells. However, TNF-β is produced by activated lymphocytes and has a role in the development of T-cells and in the mediation of graft rejection (Ruddle, 1994).

1.3. Serum Markers of the Acute Phase Response

1.3.1. Acute Phase Proteins

Acute phase proteins alter in concentration during an inflammatory reaction and, in general, are produced to assist the defence of the host organism against tissue damage and infection. They act in a variety of ways such as contributing towards haemostasis (fibrinogen) and opsonisation (C-reactive protein (CRP)), inhibiting proteinases (α1-antichymotrypsin) and acting as immunomodulators (α-1 acid glycoprotein (AGP)). They also aid wound healing (serum amyloid A (SAA)) and act as carriers for certain ions and other proteins (haptoglobin) (Whicher and Westacott, 1992). Most acute phase proteins are produced by the liver although a small fraction are synthesised in extra-hepatic sites (Ramadori et al. 1985).

The rapid rise in the concentration of positive acute phase proteins in serum as a result of tissue damage has led to their measurement being used in man as a marker of inflammation in clinical diagnosis and prognosis (Thompson et al. 1992) and to monitor response to treatment (Casl et al. 1993). For many years, the erythrocyte sedimentation rate (ESR) has been used to quantify the overall acute

phase protein response. The ESR is a measure of the rate at which red blood cells settle in a vertical column of anti-coagulated blood. It gives an indirect measurement of plasma acute phase protein content since the large, highly glycosylated proteins produced during the response influence this rate (Bull, 1981). However, the ESR is greatly affected by fluctuations in the haematocrit, is subject to significant diurnal variation related to ingestion of food (Mallya et al. 1982), and mainly reflects the concentration of the large protein fibrinogen which is a slow acute phase reactant. For these reasons, particularly in acute disease, analysis of individual proteins has been recommended (International Committee for Standardization in Haematology Expert Panel on Blood Rheology, 1988; Sliwinski et al. 1983; Yocum and Doerner, 1957).

The plasma concentration of acute phase proteins may either increase or decrease as a result of inflammation. Those proteins which are reduced in concentration, for example albumin, have been called negative acute phase reactants or concentration depressed acute phase plasma proteins (Koj, 1985a). The positive acute phase proteins rise in concentration following inflammation and have been classified into three groups according to their magnitude of increase (Kushner and Mackiewicz, 1987). Group 1 or minor acute phase proteins increase by around 50%, group 2 or moderate acute phase proteins increase up to two to five-fold and group 3 or major acute phase proteins rise up to a thousand fold from a low or negligible normal value. A table listing some acute phase proteins and the mode in which they respond in man is given (figure 1.3.).

Acute phase proteins can also be classified by the cytokines which are responsible for their induction. In this classification system, type I acute phase proteins are those induced by the IL-1-like cytokines which are IL-1α, IL-1β, TNF-α and lymphotoxin-α (also known as TNF-β). The proteins induced by this group include AGP, SAA, CRP, complement component C3 and haptoglobin (rat). Type II proteins, including fibrinogen, α1-antitrypsin and haptoglobin (human) are induced by the IL-6-like cytokines, which are IL-6, leukaemia inhibitory factor, IL-11, oncostatin M, ciliary neurotrophin factor and cardiotrophin-1 (Moshage, 1997). Stimulation of type-1 acute phase protein genes is enhanced by IL-6-type cytokines whereas the IL-1-type cytokines may inhibit type-2 acute phase protein gene expression (Baumann and Gauldie, 1994).

Figure 1.3. Classification of some acute phase proteins based on changes in their concentration in response to inflammation in man.

Major (group3)	Moderate (group 2)	Minor (group 1)	Negative
CRP	fibrinogen	SAP	albumin
SAA	haptoglobin	ceruloplasmin	transferrin
	AGP		

CRP = C-reactive protein
SAA = serum amyloid A
SAP = serum amyloid P
AGP = α-1 acid glycoprotein

Major acute phase proteins have received most attention as aids to diagnosis in clinical situations. The protein most frequently monitored in man is CRP which was originally identified and named because of its ability to bind to pneumococcal C-polysaccharide (Tillett and Francis, 1930). CRP is a very sensitive marker for the acute phase response in man, rising to peak levels of up to 20 times preoperative concentrations around 48 hours after minor, uncomplicated surgery and falling steadily afterwards (Colley et al. 1983). This protein has been used as the primary biochemical marker of inflammation in man for several years.

There is considerable variation in the acute phase protein response between species (Gruys et al. 1994), a prime example being CRP which is present in significant amounts in serum from the healthy bovine and does not appear to rise in response to inflammation in this species (Maudsley et al. 1987). It is therefore important to determine for each species the proteins which behave as major acute phase reactants, and thus may be of value in the clinical situation (Kent, 1992). A more complete review of the acute phase protein response in the species of veterinary interest is given in Chapter 2.

1.3.2. Acute phase proteins and viral infections

A study of human patients with a variety of infections, monitoring blood levels of two acute phase proteins that act as major responders in man, CRP and serum amyloid A (SAA), found that concentrations were higher in bacterial infections than in viral infections (Nakayama et al. 1993). Although, in the vast majority of patients, both proteins were higher in samples obtained during the acute than the convalescent stages, determination of SAA concentration was found to be more sensitive than CRP for monitoring viral infections. Indeed, in some situations the lower sensitivity of CRP to detect viral infections is considered useful in differentiating symptoms that may be of bacterial or viral origins such as meningitis (Clarke and Cost, 1983).

Patients with acquired immunodeficiency syndrome (AIDS) as a result of human immunodeficiency virus (HIV) infection exhibit higher acute phase protein levels than healthy controls reflecting the additional inflammatory stimulus of secondary infections (Husebekk et al. 1995), although it has also been found that the CRP response in patients with pneumococcal pneumonia is impaired if the patient also has AIDS (Klenerman et al. 1993).

1.3.3. TNF-α Receptors

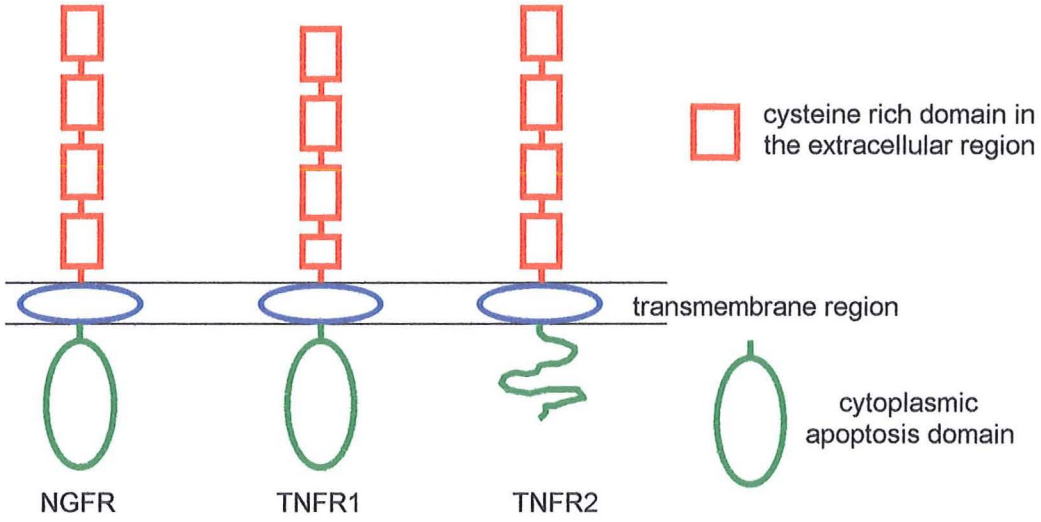
It is known that in man and the mouse, TNF can exert its effect through two separate receptors which bind TNF-α and TNF-β with equal affinity and are widely distributed on virtually all cells except red blood cells (Aggarwal and Reddy, 1994; Beutler et al. 1985). However, the genes encoding the two receptors are differentially expressed in different cell types (Vandenabeele et al. 1995). The smaller, 55-60kDa receptor, known as Type 1 (TNFR-1), is expressed mainly on epithelial cells; the larger, 75-80kDa receptor, known as Type 2 (TNFR-2), is expressed mainly on myeloid cells (Hohmann et al. 1989).

The receptors are members of the nerve growth factor receptor super-family which are characterised by cysteine rich repeat domains in the extracellular region. The extracellular regions of the receptors show 25% homology in amino acid sequence both to each other and to other members of the same family (see figure 1.4.). However the cytoplasmic domains are not homologous. Crystallographic studies indicate that three TNFR-1 molecules bind to one TNF-α trimer (Banner et al. 1993) and a more recent report shows that either TNF-α or TNF-β binding causes activation of TNFR-1 through aggregation of the cytoplasmic domains (Naismith et al. 1995). Receptor-specific antibodies can mimic TNF activity (Engelmann et al. 1990a) suggesting that clustering of the receptors is sufficient to initiate the intracellular signalling mechanisms involved.

TNFR-1 appears to be the main receptor in mediating cell cytotoxicity, LPS toxicity (Espevik et al. 1990) and gene induction in epithelial cells (Mackay et al. 1993). Transgenic mice deficient in TNFR-1 are resistant to endotoxic shock when treated with bacterial LPS or staphylococcal enterotoxin B and D-galactosamine and yet have a reduced ability to fight infection with *Listeria monocytogenes* (Pfeffer et al. 1993) illustrating that TNFR-1 is crucial for both toxic and protective effects of TNF.

The role of TNFR-2 is less well defined although it has been implicated in the cellular proliferation effects of TNF-α (Tartaglia et al. 1993a). In addition, in association with the function of TNFR-1, both 'ligand-passing' and 'decoy' roles have been proposed for TNFR-2 although these mechanisms may not be mutually exclusive (Bigda et al. 1994). The 'ligand-passing' model hypothesises that TNFR-2, as it has a higher affinity and dissociation rate than TNFR-1, preferentially binds TNF

Figure 1.4. Outline of the structure of nerve growth factor receptor (NGFR), TNFR1 and TNFR2. The extracellular domains of each receptor share 25% homology.



at low concentrations and passes it to neighbouring TNFR-1 molecules (Tartaglia et al. 1993b). This 'enhancing' role for the receptor is supported by the finding that mice deficient in TNFR-2 show an increased, although not complete, resistance to TNF-induced death and a reduction in tissue necrosis as a result of subcutaneous TNF injection (Erickson et al. 1994). The 'decoy' model hypothesises that TNFR-2 binds TNF, thus preventing its action through TNFR-1. TNFR-2 may also be important in mediating the effects of membrane-bound TNF (Grell et al. 1995).

Removal of the cytoplasmic domain of TNFR-1 results in a non-functional receptor (Tartaglia and Goeddel, 1992) whereas less complete deletions within the cytoplasmic region demonstrate a region of about eighty amino acids essential for cytotoxicity, antiviral activity and NO synthase signalling (Tartaglia et al. 1993), designated the death domain. A TNFR-1 associated death domain protein has been identified which interacts with this area and leads to NF-κB activation. Phosphoproteins and a serine protein kinase that interact with the more membrane-proximal region of the intracellular domain of TNFR-1 have also been isolated (Van Arsdale and Ware, 1994).

Proteins interacting with the intracellular portion of TNFR-2 have been identified and designated TRAF proteins (TNFR-2 associated factors) although these can also interact with TNFR-1. The precise function of this protein family is not known but they have an area characteristic of DNA-binding proteins thus may represent cytoplasmic transcription precursors (Vandenabeele et al. 1995). Over-expression of TRAF2 is responsible for NF-κB activation (Kaye et al. 1996). Vandenabeele and others propose that TNFR2-mediated intracellular signalling pathways require the co-operation of other cytokine-receptor-stimulated pathways such as the IL-1 pathway (Vandenabeele et al. 1992).

Human TNF-α has been shown to bind to murine TNFR-1 but not murine TNFR-2, indicating that TNFR-2 exhibits more species specificity (Lewis et al. 1991).

1.3.4. Soluble TNFRs

The extracellular portions of both receptors can be cleaved to release soluble proteins (sTNFR-1 and sTNFR-2) (Engelmann et al. 1989; Kohno et al. 1990; Nophar et al. 1990; Seckinger et al. 1989). These soluble receptors were first

identified in urine by their ability to inhibit TNF-α induced cytotoxicity in bioassays (Engelmann et al. 1990b). They bind TNF-α in a reversible manner, inhibiting activity by preventing its interaction with cells, but also forming a reservoir of TNF-α activity (Aderka et al. 1992; Mohler et al. 1993). Soluble TNF receptors have been identified in different body fluids of both healthy and diseased patients including urine (Novick et al. 1989), serum (Godfried et al. 1993) and synovial fluid (Cope et al. 1992).

Viral genes have been isolated from Shope fibroma, myxoma and cowpox viruses that encode proteins which interact with TNF-α and thus interfere with the host's defence mechanisms (Smith et al. 1991; Smith et al. 1996; Upton et al. 1991). These proteins are also members of the nerve growth factor receptor family and show most homology to the soluble portion of TNFR-2.

Diez-Ruiz and others reviewed the use of sTNFR measurement in clinical laboratory diagnosis and suggest that their levels in serum show high accuracy in the follow-up and prognosis of diseases such as HIV infection and sepsis (Diez-Ruiz et al. 1995). Increased levels of endogenous sTNFRs reflect activation of the TNF-α/TNF receptor system and assist quantification of the Th1-type cell-mediated immune response. Redl and others have reported on elevation of plasma levels of sTNFR-1 (from a healthy mean of 1.7ng/ml to a mean in affected animals of 10.4ng/ml) in a baboon model of bacteraemia (Redl et al. 1995). Since levels of sTNFR-1 remained elevated for the eight hours of the experiment but TNF-α returned to basal levels within six hours, sTNFR-1 may represent a more useful marker of inflammation than TNF-α.

In patients with human immunodeficiency virus (HIV) infection, sTNFR-1 and -2 levels have been correlated to disease progression (Aukrust et al. 1994; Barcellini et al. 1996; Godfried et al. 1993; Hober et al. 1996; Kalinkovich et al. 1992; Kalinkovich et al. 1993). In addition, measurement of sTNFR-2 in serum has been of value in monitoring the efficacy of treatment in asymptomatic HIV-1 infection (Godfried et al. 1995). Low levels of sTNFR in cancer patients have been correlated with an increased survival time (Langkopf and Atzpodien, 1994) and successful treatment of hairy cell leukaemia is associated with a decrease in sTNFR (Digel et al. 1992). In a study of patients with congestive heart failure, measurement

of sTNFR, in addition to TNF-α, was found to be essential for evaluation of the role of this cytokine and elevated levels of sTNFR-2 correlated with a poor short-term prognosis (Ferrari et al. 1995).

Inhibition of the bioactivity of TNF-α by sTNFR has also stimulated interest in the soluble receptors as therapeutic agents (Olsson et al. 1993). Administration of sTNFR-1 can prevent the adverse pathologic sequelae caused by the exaggerated TNF-α production observed in lethal sepsis in non-human primates (Van Zee et al. 1992). However, a clinical trial in people has shown an increased mortality in patients given TNFR:Fc fusion protein as a treatment for septic shock (Fisher et al, 1996) and it may be that ameliorating effects in this condition is only possible in experimental infection when the soluble receptor can be given prior to TNF-α stimulation. Implantation of mice with copolymers containing human sTNFR1 has been associated with protection from the development of chronic TNF-associated diseases such as lethal wasting and arthritis (Eliaz et al. 1996).

1.4. Feline Infectious Peritonitis

1.4.1. Background

Feline infectious peritonitis (FIP) is an invariably fatal disease of cats caused by infection with a feline coronavirus (FCoV) (Horzinek and Osterhaus, 1978). The *Coronaviridae* are pleomorphic viruses with a single stranded ribonucleic acid (RNA) genome, surrounded by an envelope with a corona of radiating proteins or peplomers which give the virus its name.

1.4.2. Pathogenesis

Infection of the cat in the field probably occurs by inhalation or ingestion (Hoskins, 1993). Replication of the virus in the alimentary tract and subsequent viraemia may be asymptomatic or result in mild disease such as enteritis and be followed by recovery from infection or, if virus is not cleared by the host, be associated with the clinical signs of FIP (Weiss, 1991). Viraemic cats may shed virus in faeces, saliva and urine.

Whether cats recover from infection with FCoV or develop FIP is thought to be determined by a multitude of factors including strain of virus, whether humoral or

cellular mechanisms predominate in the host immune response and whether the cat is already sensitised from a previous infection (Hoskins, 1993). More recently, it has been suggested that increased viral replication in the gastrointestinal tract, such as that exhibited when the host is immunosuppressed enhances the mutation rate in the viral genome, and makes it more likely for strains of enhanced virulence to arise and cause disease (Poland et al. 1996).

Persistent infection with FCoV causes continued stimulation of the immune system which results in an immune-stimulated vasculitis and the condition known as FIP. Two forms of FIP been described based on the predominant clinicopathological findings (Pedersen, 1976). In the classical, effusive presentation, the vasculitis is responsible for the characteristic exudative inflammation of the pleural and/or peritoneal cavities (figures 1.5. and 1.6.). The other, non-effusive form (figures 1.7. and 1.8.) is caused by pyogranulomatous deposits in a variety of organs including the eye and the central nervous system and clinical signs are dependent upon the organs involved.

1.4.3. Clinical Biochemistry and Diagnosis

Diagnosis of FIP can present a considerable problem for the clinician. Clinical biochemical and haematological findings in FIP are non-specific although the albumin and globulin content (Weiss, 1991) and protein electrophoresis (Shelly et al. 1987) of effusion samples are considered to be parameters that assist diagnosis. Positive antibody titres to FCoV are not diagnostic since cats that have been infected with FCoV can develop antibody and then recover from infection (Scott, 1979). The only definitive diagnostic test for FIP is histopathological examination of a representative biopsy sample, which is an invasive procedure (Addie, 1989).

Reports on inflammatory cytokines and acute phase proteins during the course of FIP are limited. IL-1 (Goitsuka et al. 1991) and IL-6 (Goitsuka et al. 1990) activities have been detected in ascitic fluid samples and serum (IL-6 only) from cats with FIP. Gouffaux and others described elevated acute phase proteins and reduced albumin levels in experimental and field cats with FIP illustrating an inflammatory process (Gouffaux et al. 1975). More recently, Stoddart and others studied the acute phase protein response in experimental cats inoculated with

Figure 1.5. The typical appearance of a cat with feline infectious peritonitis and an associated abdominal effusion.

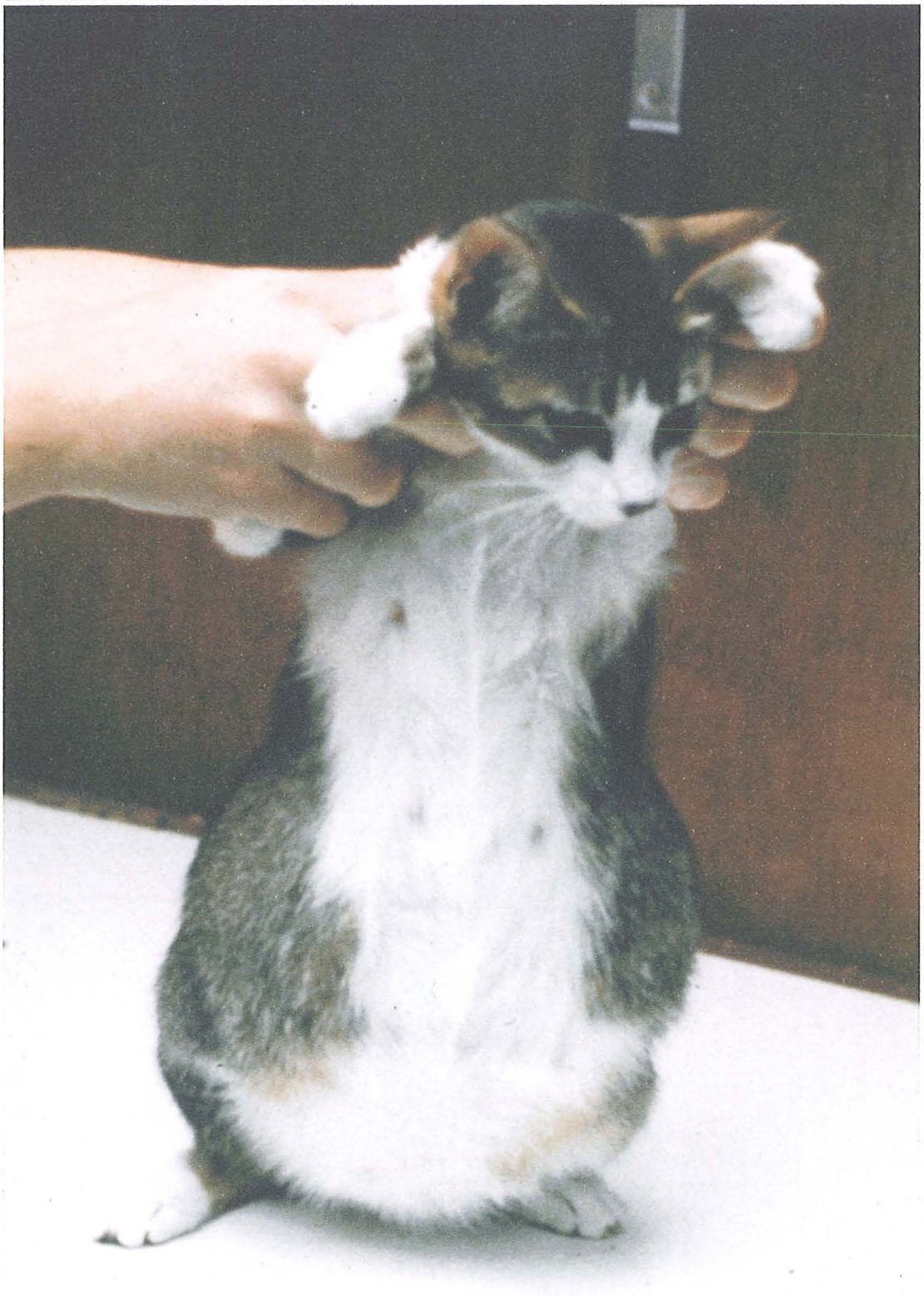


Figure 1.6. An abdominal radiograph of a cat with effusive FIP. Visceral detail within the abdomen is obliterated by the presence of fluid. Only gas within the gastrointestinal tract, bone and organs within the thorax can be discerned.

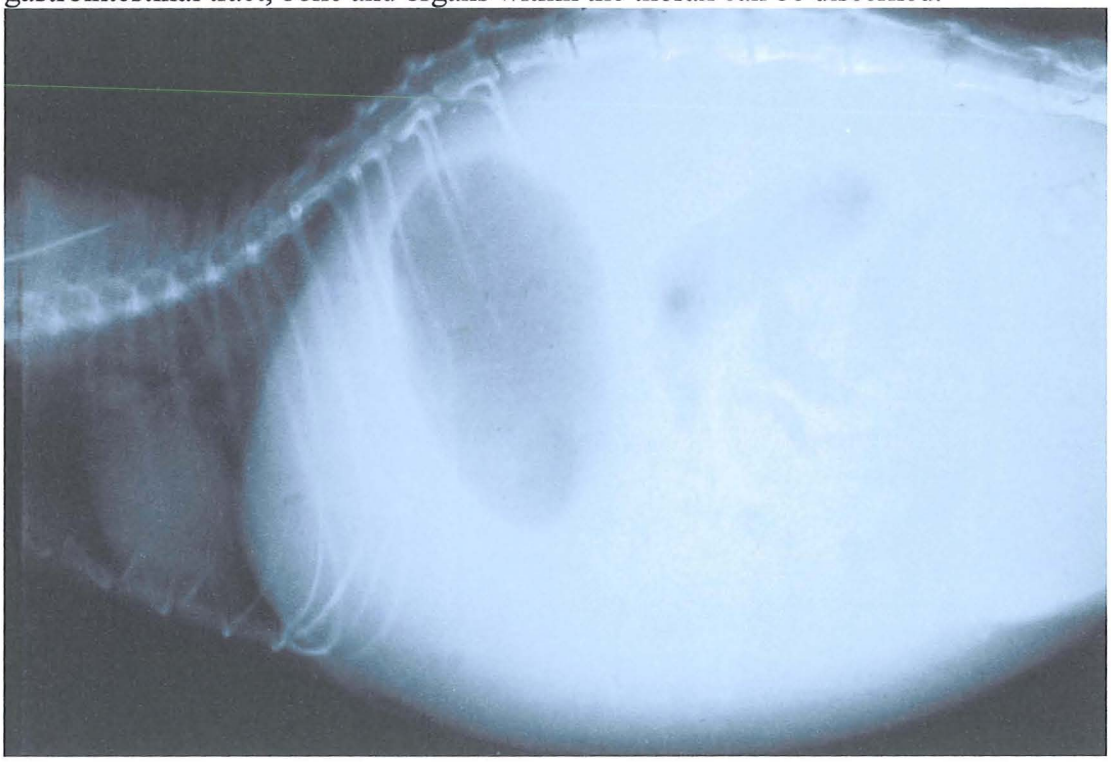


Figure 1.7. An emaciated cat with non-effusive FIP.

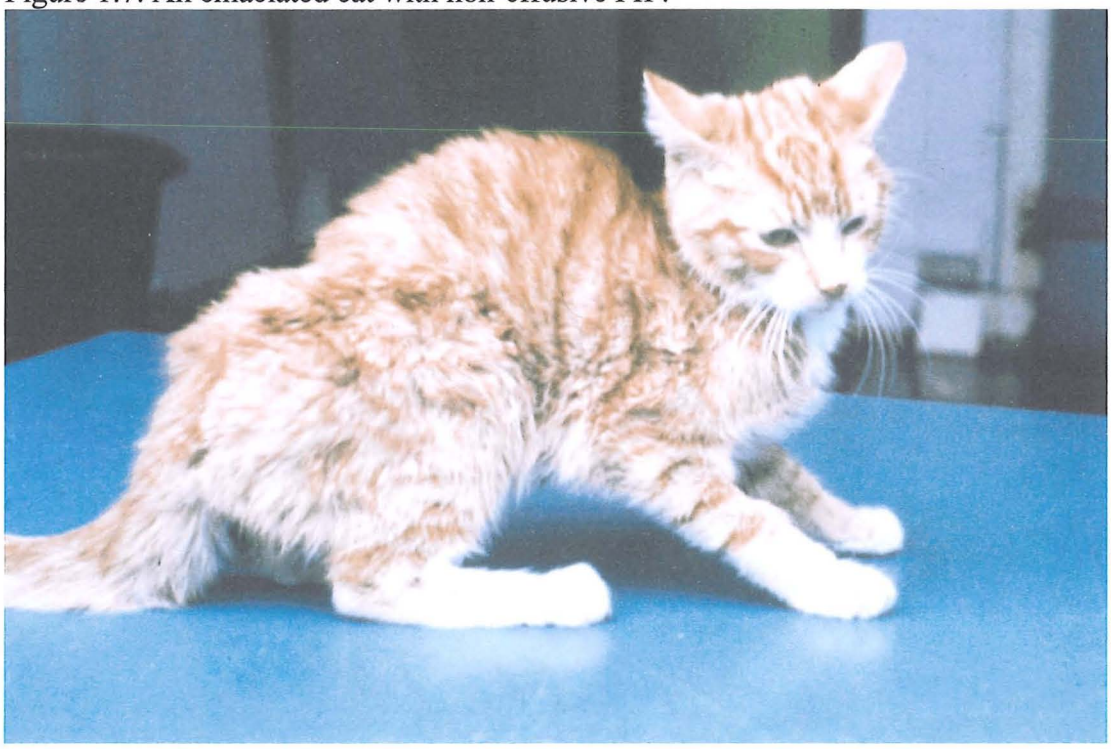
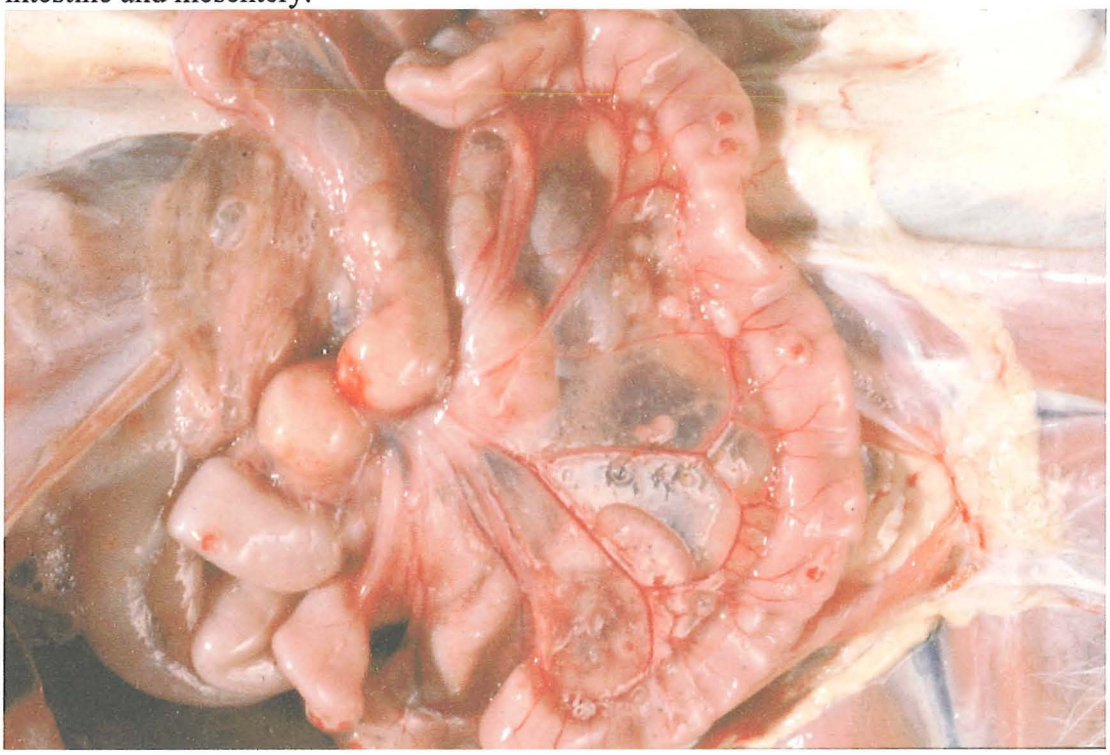


Figure 1.8. The gross abdominal pathology of the cat in figure 1.7. Enlarged mesenteric lymph nodes can be seen, with multiple granulomata on the small intestine and mesentery.



coronavirus to produce FIP in an attempt to identify changes useful in the early diagnosis of the disease (Stoddart et al. 1988). They used electrophoretic methods to examine changes in serum protein levels, found a biphasic response of an initial small peak of proteins shortly after inoculation followed by a large negative drop, and concluded that the technique was of no value in the diagnosis of FIP.

1.5. Feline Immunodeficiency Virus Infection

1.5.1. Background and Epidemiology

Feline immunodeficiency virus (FIV) is a lentivirus that was first isolated in 1986 from a colony of cats in which several suffered from an immunodeficiency-like syndrome (Pedersen et al. 1987). Lentiviruses, which include human immunodeficiency virus (HIV), have an RNA genome and are members of the family *Retroviridae* (Calisher et al. 1992). FIV is genetically distinct from HIV although both cause a similar clinical disease in their respective hosts (Pedersen, 1995). A study of blood samples taken from cats in the UK determined the prevalence of FIV infection as 19% in sick cats and 6% in healthy cats (Hosie et al. 1989), emphasising the major significance of this pathogen. Both this and further epidemiological studies have identified that older, free-roaming male cats are most likely to be infected with FIV (Grindem et al. 1989; Gruffydd-Jones et al. 1988; Hosie et al. 1989; Ishida et al. 1989; Peri et al. 1994; Yamamoto et al. 1989) which reflects the proposed mode of transmission through fighting and biting. Although the virus was only discovered in 1986, retrospective analysis of stored serum samples has detected antibody to FIV prior to this time (Bennett et al. 1989; Gruffydd-Jones et al. 1988).

1.5.2. Pathogenesis

Cats infected with FIV can shed virus in their saliva and this is enhanced by oral inflammation, a frequent symptom of FIV infection (Sparkes et al. 1993). Consequently, the major mode of transmission of natural infection appears to be fighting and biting (Sparger, 1993), although infection from mother to kitten has also been reported (Callanan et al. 1991). Transmission *in utero* and *intrapartum* can occur as well as infection whilst suckling, and vertical transmission is enhanced when

the mother has a low $CD4^+$ count, a prolonged duration of infection or clinical signs of immunodeficiency (O'Neil et al. 1996).

Recently, it has been demonstrated that some FIV strains use the chemokine receptor CXCR4 for entry into the cell (Willett et al. 1997). Once the virus has entered a cell, it uncoats and uses its reverse transcriptase enzyme to make a DNA copy of its RNA genome. This viral DNA then enters the nucleus and integrates into the host DNA, becomes known as the provirus and is translated resulting in the production of further virions which are released from the cell.

Within 6 weeks of experimental infection with FIV, cats enter an acute phase of general malaise (Pedersen et al. 1989) which is associated with a lymphadenopathy lasting up to 1 year (Callanan et al. 1992). During this period, leukopenia, in particular neutropenia, is apparent and cats show an increased susceptibility to infection (Dua et al. 1994; Yamamoto et al. 1988). By semi-quantitative analysis using the polymerase chain reaction (PCR), Dean and others showed higher levels of FIV provirus in $CD4^+$ T cells at this stage of disease than in B cells ($CD21^+$) and $CD8^+$ T cells (Dean et al. 1996). Within 10 days of infection, when compared to negative controls, plasma TNF-α levels are elevated and the response of peripheral blood mononuclear cells (PBMCs) to mitogens is depressed (Lawrence et al. 1992). A strong humoral antibody response becomes apparent from 1 to 2 months after infection (Callanan, 1995; Dua et al. 1994) and in the acute phase of the infection there is a rapid expansion of $CD8^+$ lymphocytes and a more gradual decline in the peripheral $CD4^+$ lymphocyte population (Willett et al. 1993).

After the acute phase, cats enter the asymptomatic phase when, as the name suggests, they are free from clinical signs but remain persistently viraemic although most of the virus is cell-associated (Sparger, 1993). During this period, an inversion in the $CD4^+$:$CD8^+$ ratio has been demonstrated (Ackley et al. 1990; Lawrence et al. 1995; Novotney et al. 1990), and viral DNA is present in greater numbers of B cells than $CD4^+$ and $CD8^+$ T cells (Dean et al. 1996). PBMCs taken from cats during this period exhibit reduced proliferation in response to mitogen stimulation (Ishida et al. 1992). However, the changes in cytokine levels over this period appear less consistent with both an increase in levels of IL-1, IL-6 and TNF-α in supernatants from stimulated PBMCs (IL-1, IL-6) or plasma (TNF-α) (Lawrence et al. 1995a), contrasting with reports of a reduction in TNF-α and no change in IL-6 production

from stimulated cells (Ma et al. 1995). The latter study suggests that the lack of TNF stimulation may be due to the absence of secondary disease as it examined experimental specific pathogen free (SPF) cats. However the population of cats in the first study were also SPF.

The asymptomatic stage may last for years until, as immunosuppression progresses, secondary disease such as opportunistic infection or neoplasia becomes apparent. In HIV, progression from the asymptomatic to clinical stages of disease has been associated with a shift from predominantly cell-mediated immune responses (via Th1-type cytokines) towards a predominance of Th-2 cytokine influences and a humoral immune response (Clerici and Shearer, 1994) but this has not been documented in FIV. Eventually cats succumb to secondary disease, or more commonly, are euthanased when presented to veterinarians as seropositive, sick cats.

1.5.3. Diagnosis

Diagnosis of FIV infection is relatively straightforward as most infected cats have anti-FIV serum antibodies (Macy, 1994). In addition, immunoassays are available to detect the presence of FIV antigen, virus isolation can be performed (Pedersen and Barlough, 1991) and FIV nucleic acid sequences can be demonstrated in infected samples by the polymerase chain reaction (Tomonaga and Mikami, 1996).

1.5.4. Treatment and Prognosis

Treatment is largely palliative and directed towards any secondary infections or other diseases that are present, although some anti-viral drugs have been investigated in the treatment of FIV. It has been suggested that cytokine-based therapy that enhances cell-mediated immunity should be instituted in HIV in combination with anti-retroviral drugs (Clerici and Shearer, 1994) but this avenue remains largely unexplored in the cat. Although treatment strategies are currently fairly restricted, it is essential that markers of disease progression are identified in FIV so that the effect of treatment can be monitored and a prognosis over the prolonged asymptomatic period can be given.

Numerous cellular and serological markers have been monitored in HIV infection and an attempt made to correlate these with disease progression. The parameter said to reflect stage of disease most closely is the CD4+ lymphocyte count

(Stein et al. 1992), which gradually declines over the course of infection. The serological markers studied and correlated with CD4+ lymphocyte counts and to clinical outcome include the cytokine tumour necrosis factor-α (TNF-α) (Aukrust et al. 1999) and its soluble receptors (see section 1.2.7) (Godfried et al. 1993; Hober et al. 1996). Although a reduction in CD4+ lymphocyte numbers have been recorded in both naturally (Hoffman-Fezer et al. 1992; Novotney et al. 1990) and experimentally (Ackley et al. 1990) infected FIV positive cats, with an associated inversion in the CD4+:CD8+ ratio, little has been reported on serological markers of FIV disease progression.

1.6. Objectives

The ultimate aim of the work presented in this thesis was to assist clinicians by identifying markers of inflammation in the cat that it might be useful to monitor in the clinical situation. This was approached, firstly, by studying the feline acute phase protein response in FIP and FIV infection. Thereafter, the possibility of using an *in vitro* bioassay to identify TNF inhibitory activity was investigated. Finally, work was directed towards developing species-specific assays for feline soluble tumour necrosis factor receptors for use in the management of cats with FIV infection.

Chapter 2:
The Acute Phase Protein Response In The Cat

2.1. Summary of Chapter

The aim of the work presented in this chapter was to characterise the feline acute phase response by monitoring the levels of two acute phase proteins, α1-acid glycoprotein (AGP) and haptoglobin (Hp). Reference ranges for these proteins were determined by measuring their concentrations in serum samples from healthy cats. Subsequently, levels of AGP and Hp were measured in serum and effusion samples from sick cats with a clinical presentation suggestive of feline infectious peritonitis (FIP) and in serum samples from cats taken during the initial acute, asymptomatic and terminal stages of feline immunodeficiency virus (FIV) infection. It was found that these acute phase proteins are present in the serum of healthy cats and their concentrations rise during certain periods of these diseases. In particular, AGP analysis was of value in differentiating cases of FIP from conditions with a similar clinical presentation.

2.2. Acute Phase Proteins in the Species of Veterinary Interest

2.2.1. General introduction

A summary of major and moderate acute phase proteins as well as those that are most commonly monitored in clinical diagnosis in a number of species is given in figure 2.1. The acute phase response has certainly not been as well documented in the species of veterinary interest as it has in man. As mentioned briefly in Chapter 1, there are considerable interspecies differences in the production of acute phase proteins thus conclusions on the response in animals cannot be drawn directly from clinical investigations in man. In the veterinary literature, the erythrocyte sedimentation rate (ESR) has been acknowledged as providing useful information for clinical evaluation and in detecting occult disease, particularly in the dog (Jain, 1986) and should be of equal benefit in the cat. However horse erythrocytes naturally settle

very rapidly in anti-coagulated blood and bovine erythrocytes show little sedimentation so the ESR is unreliable in these species (Jain, 1993). Despite the long-recognised assistance with diagnosis that this test may provide in the assessment of small animal cases in the field, it is very seldom used in practice possibly because of the dependency of the test on other parameters such as packed cell volume. Instead, clinicians have increasingly directed their attention towards the measurement of individual acute phase reactants to provide assistance with diagnosis.

In a review of the acute phase proteins that have been studied and utilised in the veterinary species, Gruys and others emphasise the value of testing for these proteins in the investigation of both sub-clinical and clinical disease (Gruys et al. 1994).

The acute phase protein Hp is an α_2-glycoprotein in man which binds haemoglobin with high affinity (Cohen-Dix et al. 1973) and is involved in its transport around the body. It is thought that through binding to Hp, the harmful effects of haemoglobin on the kidneys are avoided, particularly after a haemolytic crisis. The binding is 'suicidal' since the Hp-haemoglobin complexes are destroyed by the reticuloendothelial system although iron from haemoglobin is recycled. In addition to this haemoglobin transport, it is emerging that Hp also plays a role in the host defence against infection. The protein binds to antigens on invading pathogens (Kohler and Prokop, 1978) and haptoglobin receptors have also been identified on leukocytes; Hp binding to these cellular receptors may modify the cell's response (El Ghmati et al. 1996).

The acute phase protein AGP (also known as orosomucoid) is an α_1-glycoprotein, the function of which is largely unknown although it has been reported to play a part in immunomodulation (Cheresh et al. 1984) and the promotion of fibroblast growth (Sliwinski et al. 1983). It is responsible for binding and transporting drugs (McNamara et al. 1986) and endogenous compounds such as steroids.

Figure 2.1. Acute phase protein changes as a result of inflammation in different species, including major and moderate responders and those most commonly monitored in clinical diagnosis.

Species	Major APP	Moderate APP	APP generally monitored
Man	CRP, SAA	AGP, Hp	ESR, CRP, occ SAA
Mouse	SAA	SAP, Hp	SAA, SAP
Cow	Hp, SAA	AGP	Hp, occ SAA
Horse	SAA	Hp, fibrinogen	fibrinogen
Pig	CRP, pig-MAP	Hp, ASG	CRP, Hp
Dog	CRP	Hp	CRP
Cat	?SAA	CRP, AGP, Hp	

APP, acute phase protein; CRP, C-reactive protein; SAA, serum amyloid A; AGP, α1-acid glycoprotein; Hp, haptoglobin; ESR, erythrocyte sedimentation rate; pig-MAP, pig major acute phase protein; ASG, acid soluble glycoprotein; occ, occasionally.

2.2.2. Acute phase proteins in farm animals

A study in 1986 by Conner and others (Conner et al. 1986) examined the bovine acute phase response in cattle with mastitis by comparing the levels of three acute phase proteins; Hp, ceruloplasmin and α-1 antitrypsin, to levels in healthy controls. Although significantly increased concentrations of all 3 proteins were found in cattle with infection, the most dramatic change occurred in Hp levels which were undetectable in healthy cattle but detectable in most of the mastitis cases. C-reactive protein (CRP) does not appear to act as an acute phase reactant in cattle (Maudsley et al. 1987) although serum amyloid A (SAA) is a major reactant in this species (Boosman et al. 1989; Horadagoda et al. 1993; Yamashita et al. 1994). SAA is more sensitive as an indicator of inflammation than Hp in cattle and it is not affected by haemolysis which may, through binding to haemoglobin, result in a reduction in detectable Hp (Eckersall and Conner, 1988). The assay of SAA, however, is more difficult than that of Hp, hence Hp is still the most assayed acute phase protein in bovine medicine.

Pronounced rises in Hp levels have been recorded in sheep with both induced (Pfeffer and Rogers, 1989) and naturally-acquired inflammation (Skinner and Roberts, 1994) and it has been suggested that in this species protein measurement may be of more value than haematological analysis in the diagnosis of tissue injury and infectious disease.

An interesting application of acute phase protein measurement, particularly in the pig, is that this technique may be of value in assisting the meat inspection process to detect sub-clinical disease. A novel porcine major acute phase reactant has been identified, called pig major acute phase protein (pig-MAP) which is homologous to a human serum protein denominated PK-120. Serum levels of pig-MAP rose 10 to 30 times those recorded pre-injection as a result of turpentine injection in pigs (Gonzalez-Ramon et al. 1995). CRP, Hp and the acid soluble glycoprotein fraction of serum are also elevated as a result of inflammation in pigs and may be of value clinically (Eckersall et al. 1996).

2.2.3. Acute phase proteins in the horse

Acute phase proteins have been used in the horse to monitor inflammatory conditions such as laminitis and colic. Historically, fibrinogen and Hp have been used

but more recently SAA has been described as the preferential protein for monitoring disease (Pepys et al. 1989). In a similar fashion to the situation in the ruminant, CRP does not appear to act as an acute phase protein in the horse.

2.2.4. Acute phase proteins in the dog

Canine CRP was first identified using immunological reagents designed to detect human CRP (Dillman and Coles, 1966), and the authors observed that, although there was considerable individual variability in the CRP response, development of an assay for the canine protein might prove useful to the clinician. CRP has since been cited as the protein of choice to monitor canine inflammation (Caspi et al. 1987) and it has been shown to rise to over 100 times levels found in healthy dogs in response to inflammation (Yamamoto et al. 1993) although levels in serum samples from healthy dogs were higher than baseline levels recorded in man. An immunoturbidimetric assay suitable for routine analysis of canine CRP has been developed (Eckersall et al. 1989) and its clinical value has been assessed (Burton et al. 1994).

Hp can be detected in serum from healthy dogs and, on stimulation, responds as a moderate rather than a major acute phase reactant (Conner et al. 1988). In dogs with experimental acute necrotising pancreatitis, detectable haptoglobin levels were reduced at 24 hours after induction of disease, as a result of complex formation with haemoglobin, thereafter levels became elevated in parallel with fibrinogen (Feldman et al. 1980). The measurement of haptoglobin and α-1 antitrypsin was also found to be a useful prognostic marker in dogs with chronic liver disease. In severe cases, with impaired liver function, levels of these proteins were decreased whereas dogs with normal or increased levels had a longer survival time (Sevelius and Andersson, 1995).

SAA has been monitored in dogs and was found to rise to up to 20 times pre-inoculation levels as a result of experimental *Bordetella bronchiseptica* infection (Yamamoto et al. 1994).

2.2.5. Acute phase proteins in the cat

Analyses of the protein fractions seen in normal cat serum were made over thirty years ago using electrophoretic techniques (Groulade et al. 1965; Okoshi et al. 1968). These papers describe changes in serum protein fractions with age rather than

with disease and note that total protein levels are lower in the newborn. Electrophoresis was later employed to look at protein changes induced during the course of FIP when a rise in the α and β fraction of plasma proteins, which contain the acute phase proteins, was recorded (Gouffaux et al. 1975). Further differentiation by comparison of serum and plasma electrophoresis for fibrinogen, by complex formation with haemoglobin for haptoglobin and by immunoelectrophoresis for transferrin identified that each of these proteins was specifically elevated and could also be detected in ascitic fluid samples. In addition, in experimental disease a steady increase in an α-1 glycoprotein, postulated to be AGP (orosomucoid) was also demonstrated. The elevation in transferrin appears to highlight interspecies variation in the acute phase reaction as this protein acts as a negative acute phase reactant in man (Kushner and Mackiewicz, 1993).

Feline Hp was studied by Harvey and Gaskin, who noted that whilst levels of this protein declined dramatically as a result of haemolysis due to *Haemobartonella felis* infection, it became elevated up to seven-fold during other inflammatory conditions such as turpentine injection, surgical intervention, upper respiratory tract disease and in FIP (Harvey and Gaskin, 1978).

In contrast to these two latter reports, a more recent electrophoretic study of serum samples from cats with experimentally-induced FIP, which included laser densitometric analysis of electrophoresis results, showed a rise in protein bands corresponding to AGP (orosomucoid), haptoglobin and possibly transferrin one day after inoculation with feline coronavirus followed by a large negative response, gradually increasing thereafter to pre-infection levels (Stoddart et al. 1988). As a result of this study, the authors concluded that acute phase protein measurement was of limited value in the diagnosis of FIP.

SAA has been monitored in healthy and sick cats by a group investigating familial amyloidosis in the Abyssinian breed (DiBartola et al. 1989). Using a radial immunodiffusion assay, a mean level of SAA was identified in hospitalised cats (non-Abyssinian), not necessarily suffering from an inflammatory condition, of ten times that in healthy non-Abyssinian cats, although no correlation was made between clinical condition and SAA concentration.

Feline CRP has been isolated and characterised and a reference range for this protein in the serum of healthy cats determined (Watanabe et al. 1992). The

reference range was found to be higher than both that in man and that in the dog. None of the above studies have identified a definitive major acute phase reactant in the cat, therefore a need persists to identify assess markers that may be of value in clinical diagnosis and prognosis.

2.3. Aim of Experimental Studies

The aim of the work presented in this chapter was to characterise the acute phase response in the cat by monitoring levels of haptoglobin and AGP and to identify whether these are useful parameters to measure in clinical cases.

To this end, both proteins were monitored in samples from cats with FIV infection. In the early acute and asymptomatic stages of infection, serial samples were obtained which coincided with peaks in serum levels of inflammatory cytokines. As such cytokines are known to stimulate acute phase protein production, it was expected that analysis of these samples would provide a picture of protein changes in cat serum over the course of an inflammatory reaction.

In addition, Hp and AGP were measured in samples from cats with FIP, and from cats with clinically similar disease. Although FIP has a viral aetiology, and thus may not be expected to induce maximum acute phase protein levels, it is a disease associated with much tissue damage as a result of the associated immune-complex based vasculitis, and this tissue damage might be responsible for a strong acute phase reaction. As described in section 2.2.5., previous reports of the levels of acute phase proteins detected during the course of FIP have been contradictory.

2.4. Materials and Methods

2.4.1. Clinical biochemistry

2.4.1.1. Haptoglobin

The ability of Hp to bind haemoglobin was utilised in the method for its quantification. The Hp assay employed the colorimetric method of Makimura and Suzuki (Makimura and Suzuki, 1982) with the modifications of Eckersall and others (Eckersall et al. 1988), which depends on the binding of Hp to haemoglobin, preventing the inactivation of the peroxidase activity of haemoglobin in an acid environment. Free haemoglobin within a sample interferes with this assay, thus

haemolysed samples tend to give elevated readings and were excluded from the study. A canine serum pool with a high Hp content, quantified using a purified bovine haptoglobin preparation, was used as a working standard. The assay results are therefore reported in g/l (bovine equivalent) based on the assumption that the specific activity of Hp in terms of haemoglobin binding capacity per mg of Hp is similar with bovine, canine and feline Hp. No differences were found when comparing feline Hp binding to cyanmethemoglobin from feline, canine, equine or human sources (Harvey, 1976).

A stock solution of methaemoglobin (30g/l) was diluted one hundred fold for the haemoglobin assay. This was prepared previously from bovine erythrocytes which had been washed with saline, haemolysed with a mixture of distilled water and toluene, and centrifuged to remove the cell membranes. Oxyhaemoglobin was then converted to methaemoglobin in the haemolysate by the addition of potassium ferricyanide. The latter reaction was allowed to proceed for ten minutes after which the solution was filtered through a Sephadex G-25 column equilibrated with 0.15M NaCl to remove potassium and ferricyanide ions. Haemoglobin was quantified by a modified Drabkins method using a haemoglobinometer (Coulter Electronics, Luton, UK).

Aliquots of 5µl of standards covering a range from 0 to 7g/l Hp, control and test samples were pipetted into separate tubes. To each was added 100µl of diluted bovine methaemoglobin solution (0.3g/l), and the tubes were mixed. After 10 minutes, 5ml of saline was added, the tubes were mixed and left for 10 minutes at room temperature. At the end of this incubation, 12µl of the diluted samples were added to 200µl of tetra methyl benzidine solution (0.06g/l) in chromogen buffer (1.3mM di-NaEDTA, 0.1M $NaH_2PO_4.2H_2O$, pH3.8) in separate wells of a 96-well microtitre plate. The plate was incubated for 1 hour at 37°C after which 50µl of a 0.04% H_2O_2 solution was added. When a strong blue colour had developed in the high standards, the reaction was terminated by the addition of 50µl of 2M H_2SO_4. Plates were read in an ELISA plate reader (Titertek Multiscan) at 450nm.

The haptoglobin concentrations in the test samples were calculated from the standard curve. For each assay, negative samples were prepared with saline replacing the sample and these were used as a blank in the plate reader.

2.4.1.2. α1-acid glycoprotein

AGP levels were measured by single radial immunodiffusion using a commercially available kit (Saikin Kagaku Institute Co Ltd., Sendai, Japan) which consisted of an agarose gel containing specific rabbit antiserum to feline AGP (illustrated in figure 2.2.). For quantification of results, standards of purified feline AGP are supplied with the kit.

The agarose gel is supplied in plastic plates, each of which has 10 wells. Five μl of sample or standard were dispensed into each well and the plate was incubated in a humid atmosphere at room temperature for 24 to 48 hours. After incubation, the size of the precipitant ring was measured and AGP quantified in the test samples by comparison to the standard graph. A control feline serum sample was run with each batch of samples.

2.4.1.3. Total protein, albumin and globulin

Total protein (TP) and albumin concentrations were measured in serum and effusion samples from cats in group 5 on an Axon discrete biochemical analyzer (Bayer Diagnostics, Basingstoke, UK) using the standard Biuret method for TP and bromocresyl green method for albumin with reagents provided by the same supplier (Cat nos 011301A4 and 011301A4 respectively). Globulin levels were determined by subtracting albumin from the total protein concentration.

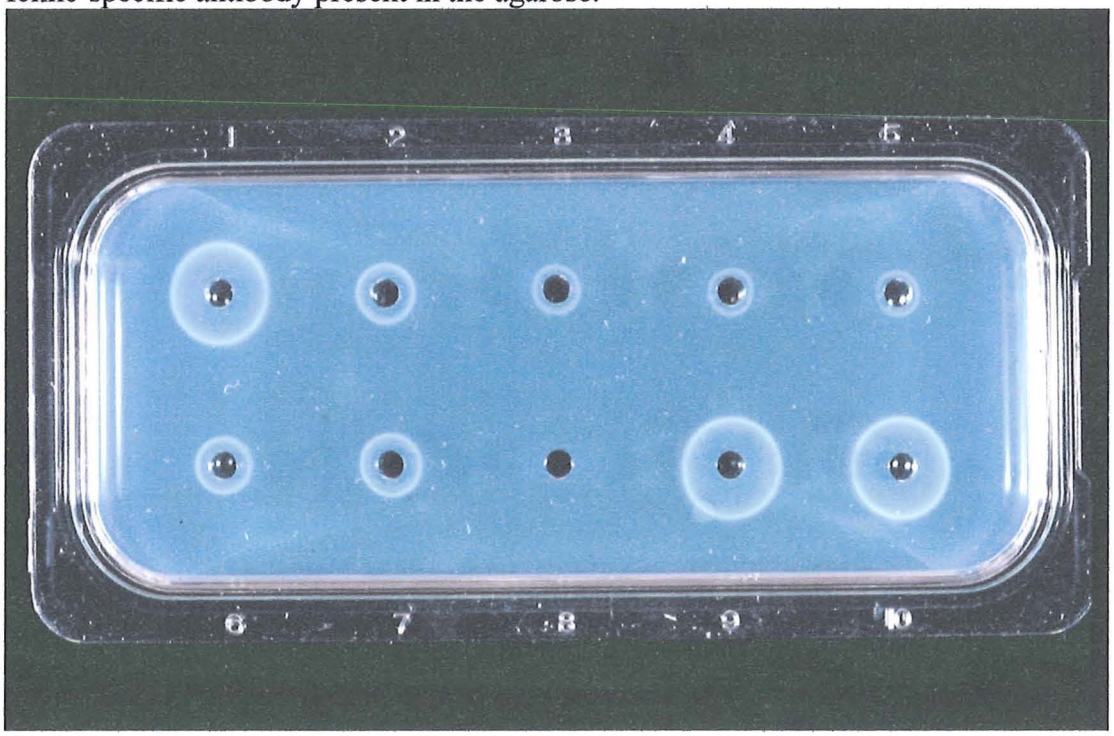
2.4.2. Diagnosis of viral diseases

FIV infection was diagnosed by an enzyme immunoassay for anti-FIV antibody (Reid et al. 1991). FIP and the aetiology of clinically similar conditions were confirmed by post mortem examination and histopathology (Addie et al. 1995). FCoV antibody titres were determined by indirect immunofluorescence (Addie and Jarrett, 1992).

2.4.3. Source of samples

Serum samples from forty adult, specific pathogen free and clinically healthy cats were analysed to determine reference ranges for Hp and AGP. These cats were designated Group 1.

Figure 2.2. An agarose radial immunodiffusion plate used to calculate the AGP concentration of a sample. High and low standards have been applied to wells 1 and 2 respectively and test samples to the remaining wells. AGP was quantified in the samples by comparison of the diameter of their precipitant ring to those of the standards. Well 8 contains a sample from a species that does not cross-react with the feline-specific antibody present in the agarose.



Samples were taken from cats with FIV infection during the early acute, asymptomatic and terminal stages of disease and separated into three groups:

Group 2 consisted of six experimentally-infected specific pathogen free (SPF) 12-week old cats. Serum samples had been obtained from these cats over the initial period immediately following infection with FIV (Glasgow-8 strain) and had been stored for 35 months prior to this investigation. Samples had been harvested at day ten for one pair of cats, at days ten and twenty for the second pair of cats and at days ten, twenty and thirty for the final pair. Three age-matched kittens maintained as controls were sampled simultaneously. Over the course of sampling, these FIV infected kittens exhibited decreased mitogen responsiveness of their peripheral blood mononuclear cells and elevated plasma tumour necrosis factor levels with respect to controls (Lawrence et al. 1992).

Group 3 consisted of five SPF cats 30 months after experimental infection with FIV which were classified as being in the asymptomatic phase of infection. Stored serum samples from these cats obtained at monthly intervals over a six month period coincident with a previously measured peak in IL-6 production were selected for analysis.

Group 4 consisted of six terminally-ill FIV positive cats with either natural or experimental infection which exhibited a variety of clinical signs and from which a single serum or effusion sample was obtained.

Single serum, plasma or effusion samples were obtained from 48 cats suffering from naturally occurring FIP (group 5) and 21 cats with similar symptoms such as peritoneal and pleural effusions but a different underlying aetiology (group 6). All of these samples had been sent to the Feline Virus Unit within the Department of Veterinary Pathology at the University of Glasgow Veterinary School for coronavirus analysis. Forty-five of the cats with FIP had effusive disease, 2 had non-effusive disease and 1 started with effusive signs and then became non-effusive. Samples from cats in groups 5 and 6 were also analysed for total protein, albumin and globulin levels and feline coronavirus antibody titres.

All samples were stored at -20°C until assayed.

2.4.4. Statistical analysis

The inter- and intra-assay coefficients of variation (CV) for both the Hp and the AGP assays were determined by repeated analysis of a control sample. The CV was calculated as (standard deviation x 100)/mean.

Statistical analysis was performed using the 'STATISTIX' Version 4.0 from Analytical Software, Tallahassee, Florida. Reference values were determined on the basis of the 2.5-97.5 percentiles. Inter-group variation was analysed using one-way analysis of variance (Tukey's pairwise comparison), with significance determined as $p<0.05$. The cats sampled shortly after infection with FIV and their controls (group 2) were compared using a two sample t test.

To determine the potential diagnostic value of AGP and Hp measurement and the albumin:globulin ratio as indicators of FIP in the population of cats with FIP-like disease, the optimum cut-off or reference value (defined as the optimum value for separating negative and positive test results) for each parameter in distinguishing groups 5 and 6 was calculated. This was achieved by plotting differential positive rate (DPR) curves of DPR against an arbitrarily selected range of cut-off values for each parameter. The DPR is the difference between true and false positive values and thus is equivalent to (sensitivity-(1-specificity)) where sensitivity is defined as the proportion of positive results in diseased individuals and specificity as the proportion of negative results in individuals without the specified disease. The DPR curve has been used to evaluate and compare diagnostic tests in canine medicine (Jensen and Poulsen, 1992).

2.5. Results

The Hp assay had an intra-assay coefficient of variation (CV) of 7% (n=16) and an inter-assay CV of 29% (n=4) when determined using a sample with a Hp concentration of 2.3g/l. The AGP assay had an intra-assay CV of 3% (n=8) and an inter-assay CV of 13% (n=6) when determined using a sample with an AGP concentration of 0.668g/l.

The haptoglobin and AGP results for all groups are illustrated in figures 2.3. and 2.4. respectively.

Figure 2.3. Concentrations of Hp in serum, plasma or effusion samples from healthy cats, cats with FIV infection and cats with FIP and clinically similar disease. Each column shows the range of Hp results in a clinical group with the red bars representing the median values of each group.
Group 1, healthy cats (n=40); Group 2a, cats in the early stage of FIV infection (n=6); Group 2b, control cats for group 2a (n=3); Group 3, cats with asymptomatic FIV infection (n=5); Group 4, cats terminally ill and FIV positive (n=6); Group 5, cats with FIP (n=48); Group 6, cats with disease clinically similar to FIP (n=21).

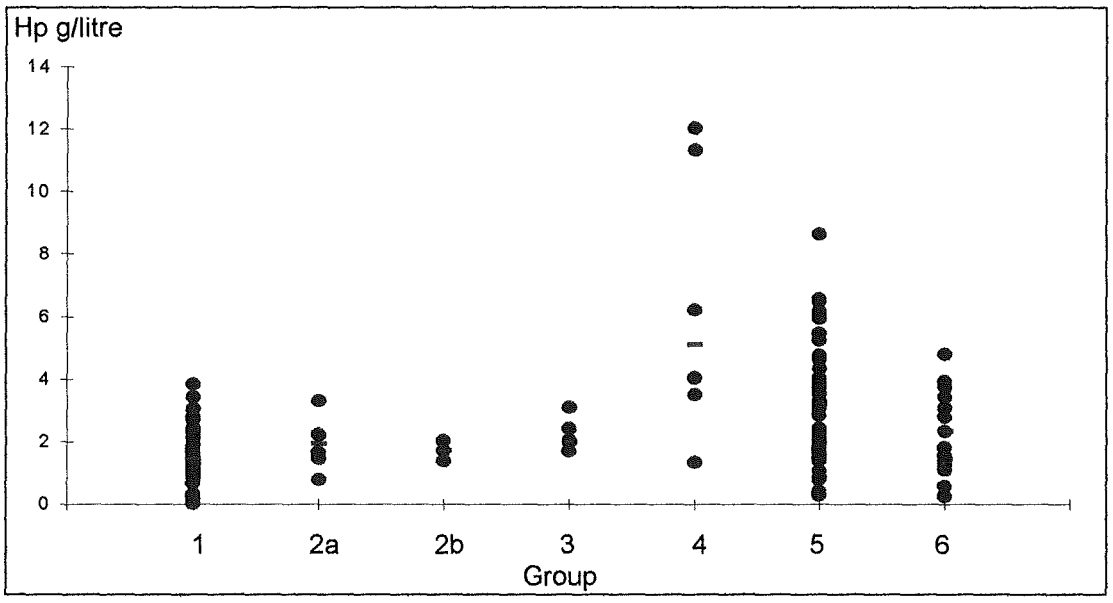
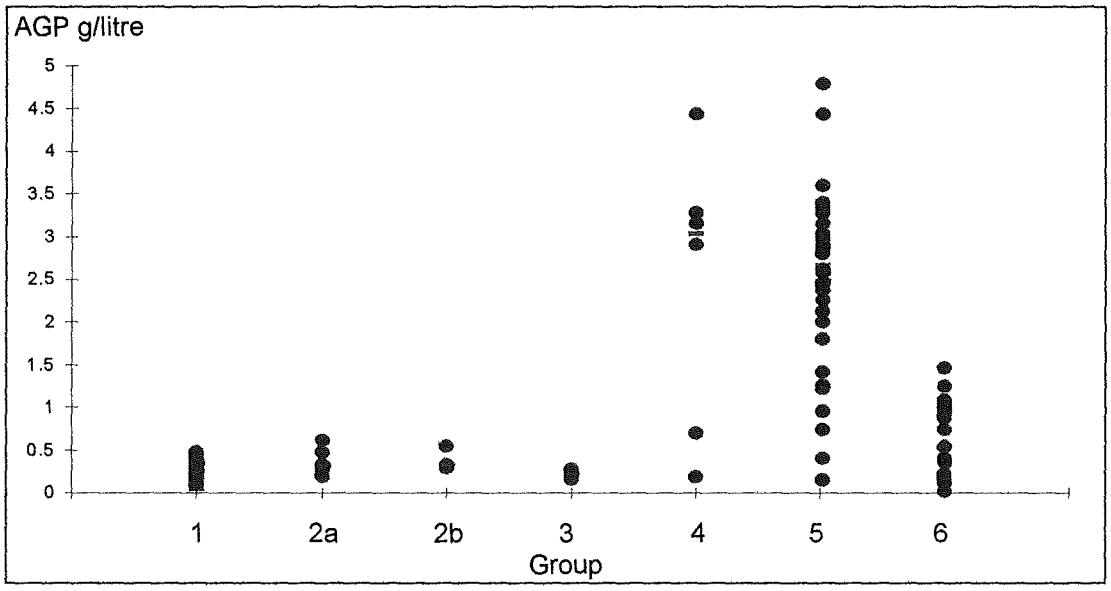


Figure 2.4. Concentrations of AGP in serum, plasma or effusion samples from healthy cats, cats with FIV infection and cats with FIP and clinically similar disease. Each column shows the range of AGP results in a clinical group with the red bars representing the median values of each group. Group 2 is split into infected cats (2a) and controls (2b).
Group 1, healthy cats (n=40); Group 2a, cats in the early stage of FIV infection (n=6); Group 2b, control cats for group 2a (n=3); Group 3, cats with asymptomatic FIV infection (n=5); Group 4, cats terminally ill and FIV positive (n=6); Group 5, cats with FIP (n=48); Group 6, cats with disease clinically similar to FIP (n=21).



2.5.1. Group 1

Analysis of the samples from healthy cats gave a reference range for Hp of 0.04-3.84g/l. and for AGP of 0.1-0.48g/l.

2.5.2. Group 2

The cats sampled shortly after infection with FIV had a range of Hp values from 0.77g/l to 3.3g/l and their uninfected controls had a range from 1.4g/l to 2.04g/l. AGP values in these FIV positive cats ranged from 0.19 to 0.615g/l and those in the controls from 0.29 to 0.547g/l. There was no statistically significant difference between the infected cats and their controls for either AGP or Hp.

2.5.3. Group 3

The samples taken from cats during the asymptomatic stage of FIV infection exhibited a range of Hp values from 1.7 to 3.1g/l and of AGP values from 0.165 to 0.28g/l. There was no significant difference between this group and the reference group (group 1).

2.5.4. Group 4

Cats with FIV-associated terminal disease had Hp values from 1.34 to 12.04g/l, the latter being over three times the maximum reference value, and AGP values ranging from 0.18-4.44g/l, the latter being almost ten times the maximum healthy value. Both AGP and Hp results in this group of cats were significantly different from those of the healthy group.

2.5.5. Groups 5 and 6

In cats with FIP (group 5), Hp concentrations ranged from 0.29 to 8.65g/l, the latter being over two times the maximum value in healthy cats and AGP concentrations ranged from 0.15-4.8g/l the latter being ten times greater than the maximum reference value. Both ranges were significantly different from those of the healthy group. By contrast, cats with FIP-like disease (group 6) exhibited a lower range of Hp and AGP concentrations from 0.26 to 4.81g/l and 0.02-1.46g/l respectively, which did not differ significantly from the healthy group. However, in the majority of cats with FIP-like disease, pleural or ascitic effusions were analysed and these would

not necessarily have the same protein content as serum, so may not be directly comparable to serum samples from healthy cats.

From figure 2.4., it is apparent that the median AGP level in cats with FIP is distinct from that in cats with FIP-like disease, suggesting that this test might be of value in the diagnosis of FIP. The pathological, serological and biochemical findings of the cases with FIP and clinically similar diseases (groups 5 and 6 respectively) are given in figures 2.5. and 2.6. Albumin:globulin ratios are commonly used as an aid to the diagnosis of FIP and the effectiveness of measurements of AGP, Hp and albumin:globulin ratios in differentiating FIP from clinically similar diseases was compared.

The DPR curves used to calculate the optimum cut-off points for these parameters in distinguishing groups 5 and 6 are plotted in figure 2.7. The optimum cut-off point corresponds to the maximum DPR value. For the Hp assay, the maximum DPR value (0.29) was obtained when a cut-off value of >2g/l was used as a positive test for FIP. For the AGP assay, the maximum DPR value (0.85) was obtained when a cut-off value of >1.5g/l was used as a positive test for FIP. A cut-off value of <0.7 was determined to be optimal value for the albumin:globulin ratio as an indicator of FIP in this population of cats with a DPR value of 0.72.

In this sample of the population of cats with FIP and FIP-like disease, the prevalence of FIP was 70%. Using the cut-off points described above, the sensitivity, specificity and overall efficiency for each of the parameters were determined and are given in figure 2.8. AGP had the greatest overall efficiency (90%) in distinguishing cats with FIP from those with clinically similar disease with a sensitivity of 85% and a specificity of 100%. The albumin:globulin ratio had a similar overall efficiency (87%) and sensitivity (87%), but a somewhat lower specificity (85%) and Hp had a much lower overall efficiency (54%) and sensitivity (40%), although a comparable specificity (86%).

Figure 2.5. Concentrations of AGP, Hp, total protein (TP) and albumin:globulin ratio (A:G) and the titre of anti-feline coronavirus antibody (anti FCoV Ab) in serum, plasma and effusion samples from cats with FIP (group 5).

Case no.	sample	AGP g/l	Hp g/l	TP g/l	A:G	anti FCoV Ab	Diagnosis
1	af	4.80	2.43	112	0.47	1280	wet FIP
2	af	4.44	4.77	61	0.74	160	wet FIP
3	af	3.60	haem	69	0.47	1280	wet FIP
4	af	3.60	3.73	102	0.59	40	wet FIP
5	af	3.60	4.64	45	0.61	320	wet FIP
6	af	3.60	3.60	50	0.67	320	wet FIP
7	af	3.60	6.49	70	0.71	1280	wet FIP
8	pl	3.40	5.26	108	0.38	1280	wet FIP
9	af	3.38	5.47	64	0.42	320	wet FIP
10	ser	3.32	6.51	91	0.42	1280	dry FIP
11	af	3.28	6.19	70	0.35	>1280	wet FIP
12	af	3.16	6.58	40	0.67	160	wet FIP
13	af	3.04	1.83	52	0.53	1280	wet FIP
14	af	3.00	3.11	70	0.59	40	wet FIP
15	ser	3.00	haem	78	0.81	n/a	wet FIP
16	pl	2.98	1.06	53	0.56	1280	wet FIP
17	af	2.90	6.06	87	0.36	>1280	wet FIP
18	pl	2.88	haem	91	0.44	1280	wet FIP
19	af	2.86	2.85	73	0.38	1280	wet FIP
20	ser	2.85	5.47	64	0.78	1280	dry FIP
21	af	2.80	2.31	57	0.84	640	wet FIP
22	pf	2.62	3.30	79	0.23	n/a	wet FIP
23	af	2.62	5.96	46	0.53	160	wet FIP
24	ser	2.60	8.65	80	0.36	1280	wet FIP
25	af	2.60	1.75	64	0.39	n/a	wet FIP
26	pf	2.58	3.23	50	0.52	1280	wet FIP
27	pf	2.47	2.17	46	0.28	>1280	wet FIP
28	af	2.47	1.49	60	0.43	1280	wet FIP
29	af	2.45	3.20	70	0.32	160	wet FIP
30	af	2.44	3.09	70	0.30	1280	wet FIP
31	af	2.44	0.29	68	0.31	n/a	wet FIP
32	af	2.44	1.49	33	0.32	640	wet FIP
33	af	2.38	2.84	47	0.62	80	wet FIP
34	af	2.26	1.89	67	0.26	80	wet FIP
35	af	2.26	4.34	51	0.46	640	wet FIP
36	af	2.26	0.85	60	0.36	640	wet FIP
37	pl	2.13	3.89	60	0.33	160	wet FIP
38	af	2.13	3.53	66	0.35	320	wet FIP
39	af	2.00	1.61	42	0.40	1280	wet FIP
40	af	2.00	4.66	57	0.46	>1280	wet>dry FIP
41	af	1.80	2.04	80	0.31	n/a	wet FIP
42	pf	1.42	4.04	106	0.31	>1280	wet FIP
43	af	1.26	1.40	56	0.37	1280	wet FIP
44	pf	1.22	1.65	64	0.23	>1280	wet FIP
45	af	0.96	haem	haem	haem	0	wet FIP
46	af	0.74	0.42	58	0.32	1280	wet FIP
47	af	0.40	3.17	41	0.64	160	wet FIP
48	af	0.15	0.79	30	0.88	0	wet FIP

af, ascitic fluid; pl, plasma; ser, serum; pf, pleural fluid; n/a, not available; haem, haemolysed sample; wet, effusive; dry, non-effusive.

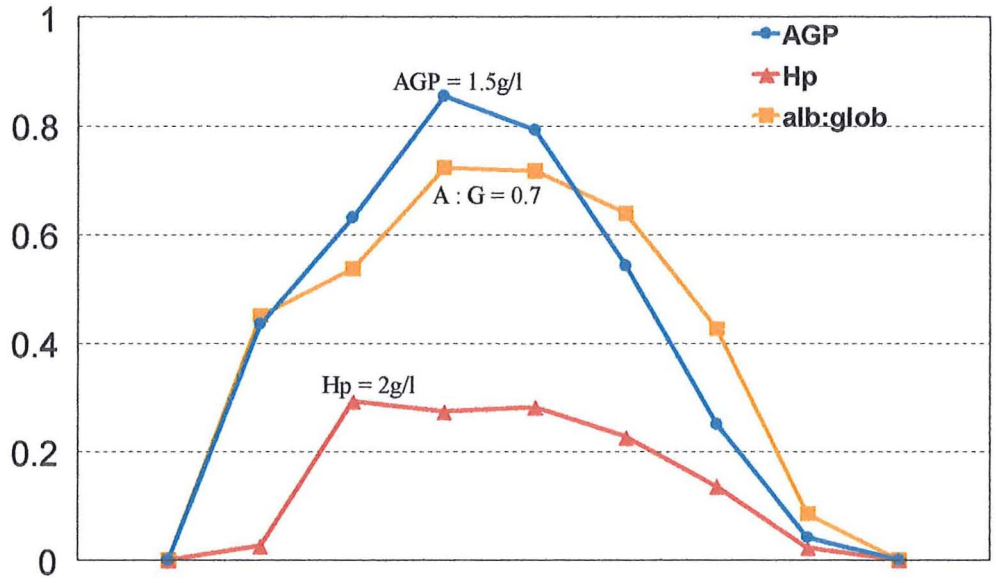
Figure 2.6. Concentrations of AGP, Hp, total protein (TP) and albumin:globulin ratio (A:G) and the titre of anti-feline coronavirus antibody (anti FCoV Ab) in serum, plasma and effusion samples from cats with clinical signs suggestive of FIP (group 6).

Case no.	sample	AGP g/l	Hp g/l	TP g/l	A:G	anti FCoV Ab	Diagnosis
1	af	1.46	3.75	43	1.05	0	biliary adenocarcinoma
2	af	1.25	4.81	56	0.81	0	lymphosarcoma
3	af	1.08	1.83	38	1.00	20	choloangitis and cirrhosis
4	pl	1.04	3.92	25	0.79	n/a	cardiomyopathy
5	af	1.02	haem	51	0.65	0	adenocarcinoma
6	af	1.00	3.09	43	0.72	320	chronic nephritis
7	af	0.96	1.82	65	0.91	n/a	cirrhosis
8	ser	0.92	haem	92	0.67	40	adenocarcinoma
9	af	0.88	1.28	19	0.73	n/a	not FIP
10	af	0.74	1.45	49	0.96	n/a	cardiomyopathy
11	af	0.54	1.56	43	1.10	0	small intestinal adenocarcinoma
12	af	0.40	1.45	47	0.96	n/a	cardiomyopathy
13	af	0.40	haem	haem	haem	n/a	not FIP
14	af	0.36	3.43	40	1.35	n/a	cardiomyopathy
15	af	0.22	2.32	38	0.73	0	chronic cholangitis
16	pf	0.20	0.57	23	0.77	0	cardiomyopathy
17	af	0.16	1.11	6	1.00	0	renal lymphosarcoma
18	af	0.16	2.79	68	1.06	320	pericarditis
19	af	0.16	1.46	47	1.24	0	cardiomyopathy
20	pf	0.12	haem	47	0.81	0	cardiomyopathy
21	pf	0.02	0.26	29	0.53	160	cardiomyopathy

af, ascitic fluid; pl, plasma; ser, serum; pf, pleural fluid; n/a, not available; haem, haemolysed sample; wet, effusive; dry, non-effusive.

Figure 2.7. Calculation of the optimum cut-off values for AGP, Hp and albumin:globulin ratio using a differential positive rate (DPR) curve to differentiate the cats with FIP or clinically similar disease. The value of the optimum cut-off point is marked at the maximum DPR value for each parameter. The table underneath illustrates the individual cut-off values for AGP, albumin:globulin ratio and Hp at which the sensitivity , specificity and, subsequently, DPR were calculated.

DPR = sensitivity - (1 - specificity)



AGP g/l	0	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0
A:G	1.4	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2
Hp g/l	0	1	2	3	4	5	6	8	10

Cut-off value

Figure 2.8. Sensitivity, specificity and efficiency of AGP, Hp and albumin:globulin in differentiating cats with FIP and clinically similar conditions.

Biochemical parameter	Sensitivity %	Specificity %	Overall efficiency %
AGP >1.5g/l	85	100	90
*Hp >2g/l	70	59	67
†albumin:globulin <0.7	87	85	87

*Eight cases missing from Hp analysis owing to haemolysed samples

†Two cases missing from albumin:globulin analysis due to haemolysed samples

Whilst an insufficient number of blood samples was available to perform a detailed analysis on the difference between blood and effusion samples, the mean total protein (TP) concentrations in both types of samples were calculated and compared (TP concentrations are given in figures 2.5. and 2.6.). The mean effusion TP concentration in the FIP group (n=39) was 62g/l and the mean blood TP concentration in the same group was 78g/l (n=8). In the cats with clinical signs of different aetiologies, the mean TP content of effusion samples was 42g/l (n=18). However, only 2 blood samples were analysed in this group, one of which had a very low level (25g/l) so the mean TP level was also low (58g/l).

2.6. Discussion

For any diagnostic test it is important to establish the expected values in a healthy population. Accordingly, the reference ranges for AGP and Hp in the blood of healthy cats were determined. Previous studies where feline Hp was quantified, reported the results in units of cyanmethemoglobin binding capacity which makes the comparison of results difficult (Harvey, 1976; Harvey and Gaskin, 1978). This lack of standardisation between laboratories is a major problem which prevents international harmonisation of acute phase protein assays and has been highlighted by Eckersall and others (Eckersall et al. 1997). Development of international standards for calibration of these assays would be a major achievement.

The inter-assay CV described for the Hp assay in this study is considerably poorer than that described for AGP. This may help to explain why levels of Hp were not as useful as levels of AGP in distinguishing FIP from clinically similar disease. The assay has since been automated to a biochemical analyser (MIRA, Roche Diagnostics) by other workers and has a lower inter-assay CV using this method. It would be of interest to repeat this investigation using the improved technique to determine whether an improvement in precision leads to a greater diagnostic value.

The reference range described here for AGP (0.1-0.48g/l) is similar to that quoted by the manufacturers of the kit (0.27-0.47g/l), which was determined in 23 cats from six months to 11 years of age. The range found in this study has an extended lower limit, possibly because the samples tested came from specific

pathogen free animals which are likely to have less immunological stimulation than cats in the field.

In eight samples the Hp measurement was not recorded as the samples were grossly haemolysed and in two of these, it was impossible to analyse albumin and total protein levels but this had no effect on the AGP immunodiffusion assay.

AGP and Hp levels were highest in cats with FIV associated illness and FIP. The maximum concentrations recorded suggest that in the cat these proteins are only moderate acute phase reactants with an increase of three to tenfold over the maximum values in healthy cats. No acute phase protein response was detected in the kittens shortly after infection with FIV or in the sequential samples taken from cats during the asymptomatic stage of the disease, despite previously recorded alterations in inflammatory cytokines (TNF-α and IL-6 respectively) in both groups of cats. Although sampling was rather infrequent for acute phase proteins detection, it might still be expected that some samples would have exhibited raised acute phase proteins levels had they fluctuated in parallel with the cytokines. Indeed, because they are generally elevated for a longer time period, acute phase proteins are normally regarded as more reliable markers of inflammation than cytokines (Whicher et al. 1993).

It may be that inhibitors such as soluble receptors are also circulating and prevent the interaction of cytokines with the appropriate cells. Further research is required to elucidate the discrepancy between inflammatory cytokine and acute phase protein levels during the early stages of FIV infection described here. Although the terminally-ill FIV-positive cats exhibited elevated acute phase proteins, their measurement in cats with FIV infection is likely to be of limited value as assistance with diagnosis is not required since specific immunoassays for anti-FIV antibody are available. As in HIV infection, acute phase protein measurement may be useful during the clinical stage of FIV infection when monitoring secondary infection (Husebekk et al. 1995).

In previous retrospective studies to identify serological, biochemical and haematological markers of FIP, there were differences in the way that the data were evaluated and in the prevalence of disease in the populations examined (Rohrer et al. 1994; Shelly et al. 1987; Sparkes et al. 1991; Sparkes et al. 1994; Weiss, 1991). In the present study, we selected cases in which FIP was considered a major differential

diagnosis so that the prevalence was high (70%). This does not affect the sensitivity or the specificity calculations, but alters the efficiency of the tests so that only the former two can be compared with previous results. The most frequent diagnoses made in the non-FIP cases were cardiomyopathy (8 out of 21 cases) and neoplasia (6 out of 21 cases) which is similar to that found in previous studies (Shelly et al. 1987; Weiss, 1991). There have also been variations in the 'cut-off' values quoted for A:G in the diagnosis of FIP. Shelly and others (Shelly et al. 1987) selected the optimal value to differentiate FIP and effusions of other origins by visual assessment of all the results on a histogram. They elected to use a cut-off value of 0.81 which is similar to 0.7, the optimum value calculated in our population by plotting a DPR curve.

Both blood and effusion samples were included in the overall comparison between FIP and clinically similar disease. Since the effusion in FIP is exudative in nature, the protein content of the effusion should reflect blood levels. This is supported in this study by the similarity of the mean total protein levels in blood and effusion samples in cats with FIP. In cats with effusions of different aetiologies, protein levels would be expected to be lower if the effusion were a transudates or modified transudate. Indeed, a lower mean total protein concentration in effusion samples is recorded here in cats with FIP-like disease. Consequently, although protein levels in effusions from cats with transudates do not necessarily reflect blood levels, the value of AGP in differentiating these from exudative effusions should be enhanced.

Cats with conditions that might be confused with FIP may be less likely to be have elevated serum AGP levels as the pathology involved is generally less pro-inflammatory e.g. cardiomyopathy. The FIP-like conditions are often chronic and not associated with an infectious agent. In addition, in this study, the cats with neoplastic disease had lower acute phase protein responses than cats with FIP.

Although the samples from cats with FIP that had a normal AGP result were effusions, two of the samples which gave false negative albumin:globulin results were sera and one of the latter was a serum sample from a case of non-effusive FIP. This finding suggests that AGP measurement might be of particular benefit in cases where no effusion sample is available.

A previous study monitoring acute phase protein changes by electrophoresis concluded that this method of analysis was of little value in the diagnosis of FIP

(Stoddart et al. 1988). As the results of electrophoresis were quantified by densitometry, absolute changes in individual proteins would be less easily assessed by this method than by the specific assays used here. Stoddart and others analysed samples taken from cats immediately after inoculation with feline coronavirus until the onset of clinical signs suggestive of FIP, when the cats were euthanased (17 to 28 days after inoculation). The samples in the study presented here were from cats with established clinical signs and this may also help to explain the observed differences. However, even in samples taken at the end of their study, Stoddart and others only detected protein levels returning to their pre-inoculation values and not becoming elevated. An earlier study of cats experimentally infected with feline coronaviruses described elevated Hp levels from the 4th day after inoculation (Gouffaux et al. 1975). As this was also an electrophoretic study, the reasons for these discrepancies remain unclear. A reduction in acute phase protein concentration has been described immediately after the initiation of inflammation due extravasation of protein into tissues but this was only detected in the first few hours (Olsen, 1993).

Our results indicate that AGP is a reliable aid in the diagnosis of FIP when the clinical picture is suggestive of this disease. The assay is readily performed and it is now used routinely in the Feline Virus Unit as part of an 'FIP profile' in conjunction with measurement of albumin and globulin levels and antibody titres to FCoV.

2.7. Further Studies

Although not possible in this study due to the lack of availability of samples, analysis of effusion and blood samples from the same animal would identify the similarity in their protein content in cats with FIP and those with clinically similar disease.

It would be interesting to investigate the glycosylation pattern of the AGP produced in the course of disease using electrophoretic techniques. During acute inflammation, less complex structures are seen, designated Type 1 changes which lead to an increased reactivity with concanavalin A (Con A). However, in the prolonged inflammation seen in cats with FIP, it might be expected that changes in glycosylation would be those of chronic change, or Type 2 when more complex structures are created with reduced Con A reactivity (Fassbender et al. 1991). Although AGP has an effect on immunomodulation, chronically raised levels during

FIP do not appear to limit the immunoglobulin response to any extent as hypergammaglobulinaemia is one of the characteristic clinical biochemistry findings in cats with FIP. Indeed, immune complex formation is the basis of the underlying pathology of the disease. Analysis of circulating cytokine levels might determine the driving influence behind the antibody response.

Analysis of other acute phase proteins such as SAA may also prove to be of benefit in the diagnosis of FIP. The more sensitive major acute phase responders, however, may be less effective at discriminating diseases of such a chronic nature.

For clarification of the discrepancy between cytokine and acute phase protein levels in the early stages of FIV, it would be most interesting to investigate concentrations of cytokine inhibitors such as soluble receptors, if suitable assays for these were available.

2.8. Conclusion

Moderately elevated levels of acute phase proteins were demonstrated in the serum of cats during two infectious diseases, FIV infection and FIP. These results indicate that, whilst not a specific test for FIP, AGP measurement is of considerable value in the diagnosis of this disease, particularly in conjunction with clinical signs, antibody titre and albumin:globulin ratio.

Chapter 3:
Bioassay For Feline Anti-Tumour Necrosis Factor Activity

3.1. Summary of Chapter

This chapter presents work designed to investigate activity against tumour necrosis factor-α (TNF-α) in culture fluids from feline splenic cells. The work in Chapter 2 demonstrated that the measurement of acute phase proteins in cats with asymptomatic FIV infection had no prognostic value. Measurement of levels of soluble tumour necrosis factor receptors (sTNFRs) has given useful prognostic information in human patients with asymptomatic HIV infection, and it is likely that a similar situation exists in cats. The work described in this chapter was directed towards the detection of sTNFRs by their inhibition of the cytotoxic activity of TNF-α in a bioassay. Splenic tissue isolated from healthy cats was incubated for varying lengths of time, either with or without mitogenic stimulation. At the end of the incubation period, culture fluids were harvested, concentrated and analysed by bioassay for their TNF activity and their ability to inhibit the TNF-induced killing of the murine fibroblast cell line, L929. TNF activity was detected in only two of the samples. Inhibition of TNF activity was demonstrated in most samples, and this was not affected by mitogenic stimulation or length of incubation.

3.2. Background

3.2.1. Investigating anti-TNF activity

Anti-TNF activity was initially described in concentrated urine from both healthy (Engelmann et al. 1989) and fevered (Seckinger et al. 1989) human patients. Each of these studies demonstrated inhibition of the cytotoxic effects of TNF-α in bioassays by the addition of concentrated urinary fractions and suggested that this effect was a consequence of a urinary constituent binding TNF thus preventing its interaction with membrane-bound receptor. Further analysis of these urinary fractions revealed two immunologically distinct proteins that were responsible for the inhibitory effect

(Engelmann et al. 1990b). Even at that early stage, the suggestion was raised that there might be a therapeutic potential for these proteins as TNF inhibitors.

Partial amino acid sequencing of the urinary proteins soon led to the development of probes for screening cDNA libraries and the complete nucleotide and amino acid sequences for both inhibitory proteins were elucidated. They were identified as tumour necrosis factor receptor 1 (TNFR-1, p55) (Gray et al. 1990; Loetscher et al. 1990; Nophar et al. 1990) and tumour necrosis factor receptor 2 (TNFR-2, p75) (Kohno et al 1990). The TNFR-2 sequence was derived simultaneously from screening an expression library for TNF-α binding (Smith et al. 1990). Further analysis showed that the urinary proteins consisted of the extracellular portion of the membrane-bound TNF receptors and are most probably derived by proteolytic cleavage of the latter rather than alternative mRNA splicing (Schall et al. 1990; Kohno et al. 1990). These proteins became known as the soluble receptors or sTNFR-1 and sTNFR-2.

A variety of physiological and pharmacological mechanisms have been found to induce the cleavage of soluble receptors from the cell surface (Porteu and Nathan, 1990) including inflammation as a result of endotoxin administration (Van Zee et al. 1992) and HIV infection (Kalinkovich et al 1992). The role of the soluble receptors in binding to circulating TNF appears to be twofold, both protecting against the harmful effects of TNF and creating a reservoir of the cytokine and sustaining the effects of its release (Aderka et al. 1992).

TNF receptors have not been described in the cat although a study on adult feline myocardial cells suggested that TNF-induced release of a heat shock protein was mediated through a TNFR-1 like receptor (Nakano et al. 1996). Identification of sTNFR in the cat will allow development of assays to determine their diagnostic value in different clinical situations and may have therapeutic potential. In the absence of cross-reacting antibody, an assay that is not immunologically based is required to identify sTNFR activity in feline derived fluids. TNF bioassays can be used in the assessment of TNF inhibitory activity as an indirect means of identifying sTNFRs.

3.2.2. TNF bioassay

The use of cell lines to measure TNF-related cytotoxicity was documented by Carswell and others in 1975 (Carswell et al. 1975). The same authors found that three cell lines displayed different susceptibilities to the cytokine. Modifications to an assay employing the tumorigenic murine fibroblast line, L929, including adaptation to a 96-well microtitre plate format (Ruff and Gifford, 1981) and the addition of actinomycin D (Ostrove and Gifford, 1979), resulted in a sensitive method of monitoring serum tumoricidal activity (Flick and Gifford, 1984). The WEHI 164 line has also been used in a similar way to detect TNF activity (Burke and Balkwill, 1991).

A major disadvantage of bioassays is that they do not distinguish between molecules with similar effects on susceptible cells (Thorpe et al. 1992). Although interleukin (IL)-1 shares many biological activities with TNF, L929 cells are not affected by IL-1. A bioassay has the advantages that it only detects biologically active cytokines and tends to be less species-specific than immunoassays. The use of neutralising antibody is recommended in bioassay regimes to ensure that the detected activity is due to the molecule of interest. However, such antibody may not be available against the cytokine and species being investigated.

Molecules, such as soluble receptors, which interfere with the biological availability of cytokines, can result in different values for cytokine levels being obtained with bioassays compared to other techniques (Van Zee et al. 1992), as can the presence of inactive cytokine forms such as monomeric TNF (Corti et al. 1994). The component antibodies of an immunoassay determine its specificity for the form of cytokine that it can detect (Bird et al. 1991). The purification of TNF-α has led to the development of sensitive immunological assays which have largely replaced bioassay techniques in the analysis of human (Michie et al. 1988) and laboratory animal samples. Radiolabeled TNF receptors have also been used to develop a very sensitive assay for TNF (Poltorak et al. 1994).

A further technique which is becoming widely used in experimental disease to quantitate cytokine production is assessment of cellular transcript levels by reverse transcription followed by the polymerase chain reaction (RT-PCR). Transcript levels do not necessarily reflect cellular cytokine release, but they can give an indication of changes in rate of production. Relatively little starting material is required and multiple

cytokines can be investigated in the same sample if a suitable protocol is prepared although the technique is considered, at best, semi-quantitative (Benveniste et al. 1996). Real time PCR is a recent development where the amount of product is monitored continuously during the reaction and allows accurate quantification of the starting levels of mRNA (Orlando et al. 1998).

Serum levels of TNF have been monitored in cattle using species-specific antibody (Horadagoda et al. 1994; Sileghem et al. 1994) and bovine TNF transcripts have been quantified in RNA derived from alveolar macrophages by RT-PCR (Bienhoff and Allen, 1995). The gene sequence of feline TNF-α has been published (McGraw et al. 1990) and the protein has been expressed (Otto et al. 1995). Despite these advances, analysis of feline serum TNF levels has generally been performed using a murine cell line bioassay (Lawrence et al. 1992; Lawrence et al. 1995b; Ma et al. 1995) or immune-based methodology with anti-human TNF antibody (Kraus et al. 1996; Lehmann et al. 1992). In addition, TNF transcript levels in feline peripheral blood mononuclear cells have been analysed using quantitative RT-PCR (Rottman et al. 1995; Dean et al. 1995) and feline cytokine expression has been measured using real time PCR (Leutenegger et al. 1998).

3.2.3. TNF receptor assay

As described in section 3.2.1., the L929-based bioassay was used initially to demonstrate sTNFR in urine (Seckinger et al. 1989; Engelmann et al. 1989). Compared to TNF-α, the effects of TNF-β are only slightly affected by the soluble receptors. A cross-linking assay, where sTNFR in cell culture supernatants are detected by binding to radioactive TNF-α has also been described (Gatanaga et al. 1991). More recently, with the development of appropriate reagents, assays using a combination of antibody capture and radioiodinated TNF binding (Digel et al. 1992) and enzyme linked immunosorbent assays (ELISAs) (Higuchi and Aggarwal, 1992) have been utilised for sTNFR measurement. Indeed ELISAs are now commercially available for the measurement of human and murine sTNFRs (R&D Systems).

3.3. Aim of Experimental Study

The aim of the work presented in this chapter was to determine whether the L929 cell bioassay can be used to detect anti-TNF activity in supernatants from cultured feline splenic cells. This assay has already been developed for analysis of TNF in feline plasma samples and a polyclonal anti-human TNF antibody was used to neutralise feline TNF activity by the same method (Lawrence 1995).

Initial western blots performed as described in chapter 5, using anti-human sTNFR-1 antibody (1mg/ml, R&D Systems) as the primary antibody at a concentration of 1:1000 did not show any reaction with the feline splenic cell supernatant samples described in sections 3.4.1. and 3.4.2. Therefore an immunoassay was not feasible and a bioassay was selected as a means of detecting anti-TNF activity because of its lack of species-specificity. TNFR-1 is considered to be less species specific than TNFR-2 (Zola, 1992) so that any feline anti-TNF activity detected in a bioassay using murine TNF is likely to be due to the presence of sTNFR -1.

3.4. Materials and Methods

All cell culture media, reagents and Nunc microtitre plates were obtained from Gibco (Paisley, UK) unless otherwise stated. Polypropylene tubes and tissue culture flasks were purchased from Greiner (UK) and petri dishes and universals from Sterilin (UK).

3.4.1. Source and preparation of samples

Immediately post mortem, the spleens were collected aseptically from three healthy specific pathogen free cats designated H41, H42 and H46. The tissue was deposited in sterile 50ml polypropylene tubes containing Dutch modification RPMI 1640 medium, supplemented with 2% heat inactivated foetal calf serum (HI-FCS), 2mM L-glutamine, 5 x 10^{-5}M 2-mercaptoethanol, 100IU/ml penicillin, 100μg/ml streptomycin and 10mM Hepes and kept on ice.

Once in the tissue culture hood, the spleens were placed in sterile petri dishes and overlain with further medium. Two scalpels were used to dice and tease apart the tissue, with initial cells being harvested after five minutes of maceration. Fresh medium was used to overlay the remaining tissue which was then dissected in a similar fashion for a further five minutes. At the end of this time, 15ml aliquots of cell suspension

were decanted into polypropylene tubes and carefully underlain with an equal volume of Histopaque 1077 (Sigma, UK).

After centrifugation for 15 minutes at 3000rpm, cells at the interface were removed with a pipette and diluted 1:2 with fresh medium to wash the cells. The resultant suspensions were re-centrifuged for 5 minutes at 1500rpm, the supernatant discarded and the cell pellet re-suspended in 5mls of medium. After determining cell numbers by counting in a haemocytometer, splenocytes were diluted to a concentration of $2.5 \times 10^6 ml^{-1}$ and replicate cultures of 20ml of cell suspension set up in $80cm^2$ tissue culture flasks. Spleens from each of the three cats were prepared in the same way.

3.4.2. Incubation procedure

Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Supernatants were harvested at time 0 and after 4, 8, 12, 24, and 42 hours. For each time point after time 0, duplicate flasks were incubated per cat, and one culture was stimulated with concanavalin A (Sigma) at a final concentration of $7.5 \mu g ml^{-1}$.

At the end of the incubation period, any adherent cells were released with a cell scraper and the medium from each flask was centrifuged for 5 minutes at 1500rpm. The cell-free supernatants were collected and stored at -20°C.

3.4.3. Concentration of supernatants

Samples were concentrated in an ultrafiltration unit (8050 Stirred Cell, Amicon, UK) using a filter membrane with a molecular weight cut off of 10kDa (Diaflo PM10, Amicon). Prior to use, the membrane was soaked for at least an hour in sterile distilled water (dH_2O) during which time the water was changed three times, and flushed with dH_2O once in the ultrafiltration chamber before applying the sample. Supernatant fractions of >10kDa were concentrated fourfold. The membrane was restored between samples with 0.1M NaOH then thoroughly flushed with dH_2O. No single membrane was used to concentrate more than ten samples.

Supernatants both greater than and less than 10kDa molecular weight and unfiltered supernatants were collected and stored at -20°C until analysed.

3.4.4. Initiation and maintenance of L929 cell line

Murine fibroblast L929 cells were obtained in a 1ml aliquot from the European Collection of Cell Cultures (ECACC no 85011425). The transported cells were washed in 10ml of complete medium (Dutch modification RPMI 1640 with 10% HI-FCS, 2mM L-glutamine, 5 x 10^{-5}M 2-mercaptoethanol, 100IU/ml penicillin and 100µg/ml streptomycin) to remove any dimethyl sulphoxide (DMSO) present in the transport medium and centrifuged for 5 minutes at 1000rpm. The cell pellet was re-suspended in 12ml of complete medium and incubated in an 80cm^3 flask at 37°C, 5%CO_2 in a humidified atmosphere until the cells were confluent. At confluency, cells were removed from the flask by the action of 0.01% trypsin in EDTA (Gibco, UK).

After trypsinisation, primary culture cells were washed in complete medium (CM), centrifuged, diluted tenfold in CM, and 10 replicate cultures of the diluted cells were set up. When these secondary cultures became confluent, cells were trypsinised, counted and stored in liquid nitrogen as secondary cultures in aliquots of 1ml in freezing down medium (45% HI-FCS, 45% CM, 10% DMSO, latter from Sigma) at a concentration of 2 x 10^7 cells/ml. The aliquots were frozen at -80°C for four hours before being transferred to liquid nitrogen.

At the same time as preparing the cells for storage, a 1:10 dilution of a secondary culture was set up in CM in an 80cm^3 flask to maintain the cell line as a working stock. Maintained cells were trypsinised weekly, diluted 1:10 and grown up in new flasks, for use as required in the bioassay.

3.4.5. L929 bioassay - TNF activity

The bioassay for TNF was performed as described by Wadhwa and others (Wadhwa et al. 1991) with slight modifications. L929 cells were taken in the log phase of growth, trypsinised, and diluted to 2 x 10^5 cells/ml in CM prepared as above. Aliquots of 100µl of cell suspension were put into each well of a 96 well microtire plate and the plates incubated at 37°C, 5% CO_2 in a humidified atmosphere for 24 hours. At the end of this time, the medium was replaced with 100µl of either sample or standard prepared in CM containing actinomycin D (Sigma, UK) at a concentration of 1µg/ml. Recombinant murine TNF-α (Cat no 410-MT, R + D Systems, UK) at an initial concentration of 1.25ng/ml was used for the standards. Serial twofold dilutions of

both standard and sample were made vertically down the microtitre plate. All standards, samples and positive and negative controls were plated in triplicate. The positive control was rmTNF-α in 6 two-fold serial dilutions from 2.5ng/ml in medium containing actinomycin D and the negative control was actinomycin D medium alone, as described in the original protocol. Plates were incubated at 37°C, 5% CO_2 in a humidified atmosphere for 24 hours.

After the second incubation, the medium was decanted and 100µl of naphthol blue black (NBB) stain (0.1% NBB, Sigma, 9% acetic acid, 0.1M sodium acetate) was added to each well. After 30 minutes, plates were fixed with 100µl fixative (9% acetic acid, 0.1M sodium acetate) per well for a further 15 minutes. After decanting stain and fixative, plates were washed with water and inverted till dry. The absorbance at 620nm was determined on a microtitre plate reader (MRX Microplate Reader, Dynex Technologies Inc., VA) using the 'endpoint' program after addition of 100µl of 0.1M NaOH per well and gentle shaking.

3.4.6. L929 bioassay - anti-TNF activity

The bioassay for TNF described in section 3.4.5. was adapted to detect anti-TNF activity. Twenty-four hour cultures of L929 cells grown in 96-well microtitre plates were prepared as above. Serial two-fold dilutions of supernatants were pre-incubated with rmTNF-α at a concentration of 0.0625ng/ml in CM supplemented with actinomycin D for 1 hour at 37°C. For this assay, rhsTNFR-1 (Cat no 225-B1-025, R + D Systems, UK) was used as a standard in two-fold dilutions from 1.25µg/ml to 0.02µg/ml and was also pre-incubated with rmTNF-α. After pre-incubation, 100µl aliquots of the incubated samples and standards were used in triplicate to replace the medium overlying the cells. A negative control was prepared with rmTNF-α at a concentration of 0.125ng/ml in CM with actinomycin D. The positive control was a sample that exhibited anti-TNF activity when used in this assay.

Prepared plates were incubated at 37°C, 5% CO_2 in a humidified atmosphere for 24 hours. At the end of this time, the medium was decanted and the plates were stained, fixed, dried and analysed as above. Soluble TNFR-1 levels were measured in µg/ml (human equivalent) based on the assumption that the cytotoxic activity detected was a result of sTNFR-1 activity.

3.4.7. Analysis

The Dynex Revelation 3.2 software (Dynex Technologies Inc, VA) was used to provide standard curves for both TNF and anti-TNF assays using the rmTNF-α and rhsTNFR-1 standards respectively. Each curve was plotted with logarithmic scaling on the x-axis and linear scaling on the y-axis using a sigmoid curve fit type.

Statistical analysis was performed using 'STATISTIX' Version 4.0 from Analytical Software, Tallahassee, Florida. Inter-group variation was analysed using one-way analysis of variance (Tukey's pairwise comparison), with significance determined as $p<0.05$. Unstimulated and stimulated groups of samples were compared using a two sample t test.

3.5. Results

3.5.1. TNF bioassay

L929 cells cultured with and without the presence of rmTNF-α are illustrated in figures 3.1.a. and 3.1.b. respectively. The standard curve obtained when using rmTNF-α in the bioassay is displayed in figure 3.2. with three sample curves superimposed. The standards produced a curve with a sigmoid best fit determined by the Revelation software ($r^2=0.9693$).

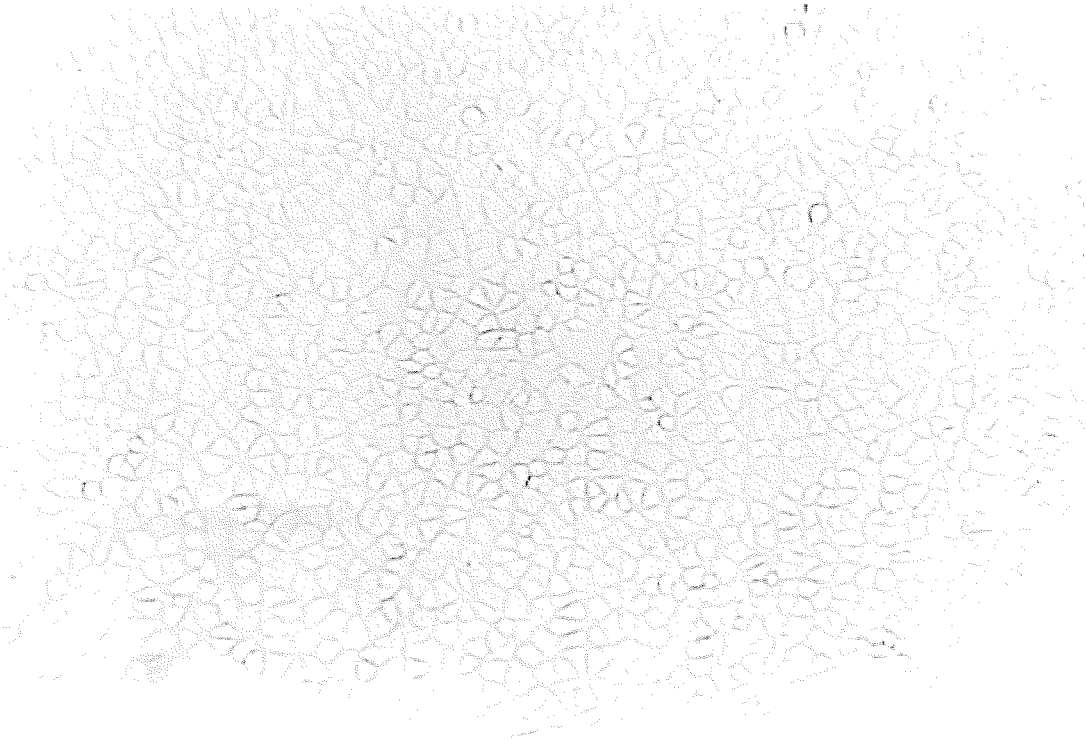
Only 2 of the 96 supernatant samples tested showed evidence of cytotoxic activity using the bioassay (illustrated in figure 3.2.). Both of these supernatants were from stimulated cultures and were unfiltered samples; one was from cat H41 and was taken after 24 hours of incubation, the other was from cat H42 and was taken after 42 hours of incubation. These samples gave a reduced absorbance consistent with cytotoxicity and their serial dilution curves showed some evidence of being parallel to the standard curve. When calculated from the least diluted results, the positive samples gave TNF concentrations of 0.037ng/ml and 0.085ng/ml. Recombinant human sTNFR-1 (R&D Systems), which was used in the anti-TNF assay, was also tested in the TNF assay and had no cytotoxic activity.

3.5.2. Anti-TNF assay

The standard and three sample curves for the anti-TNF assay are given in figure 3.3. The standards produced a curve with a sigmoid best fit determined by the Revelation

Figure 3.1. Photomicrograph of L929 cell monolayer cultured in complete medium a) without the addition of rmTNF-α and b) in the presence of rmTNF-α. The monolayer is disrupted as the cells die.

a)



b)

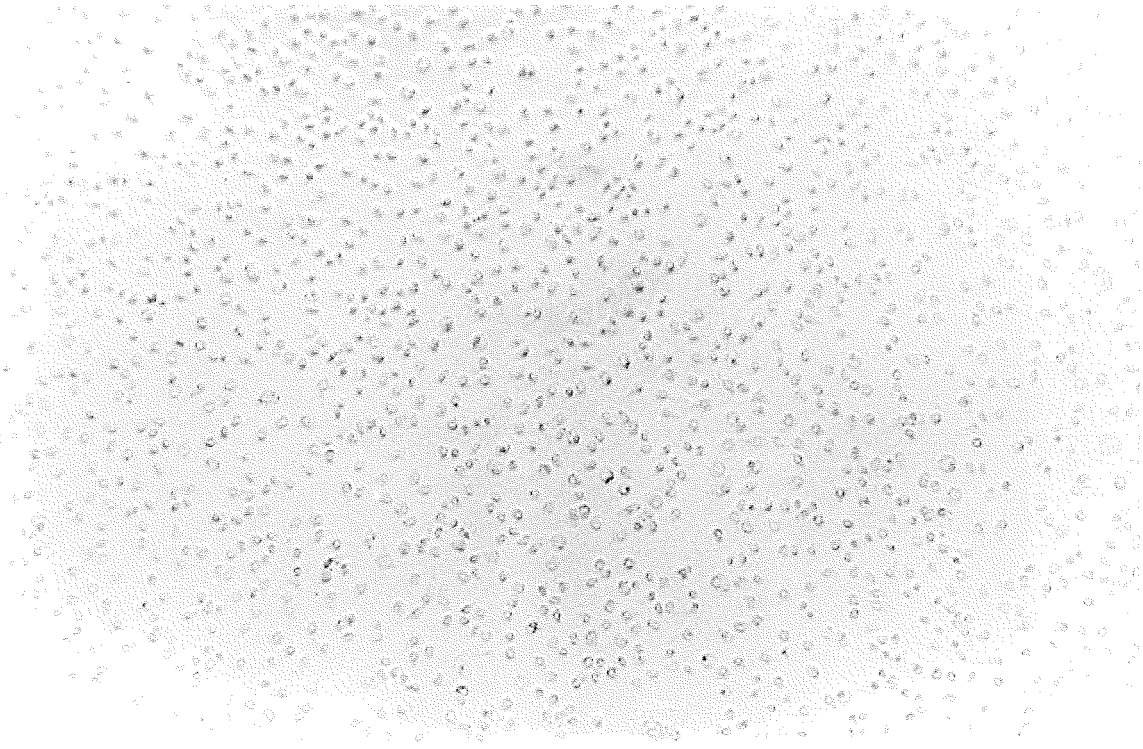


Figure 3.2. Standard curve for recombinant murine TNF-α using the L929 cell line (r^2 =0.9693). The titration curves of three samples have been superimposed on the standard curve. The standard (rmTNF-α) ranges in concentration from 0.0195ng/ml to 1.25ng/ml. Sample 1; negative sample, H46, stimulated, 24 hours and <10000, sample 2; positive sample, H42, 42 hours, unconcentrated and ConA stimulated, sample 3; positive sample, H41, 24 hours, unconcentrated and ConA stimulated.

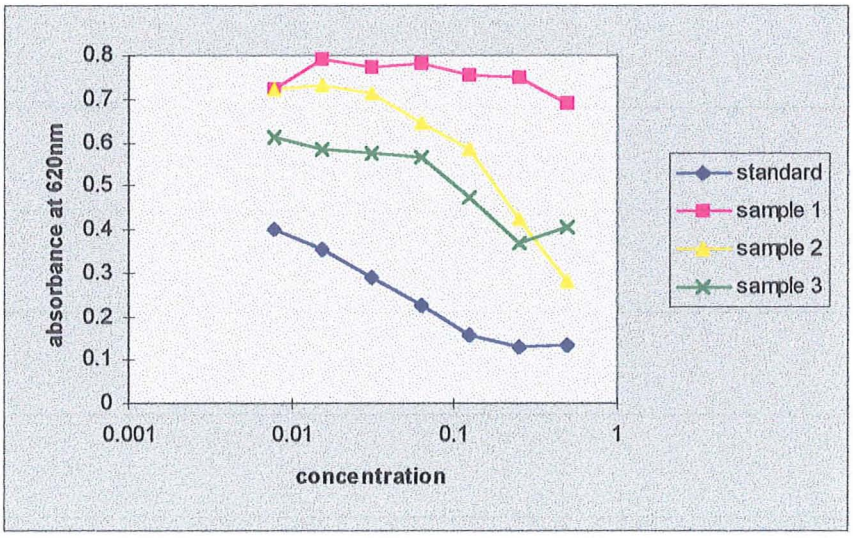
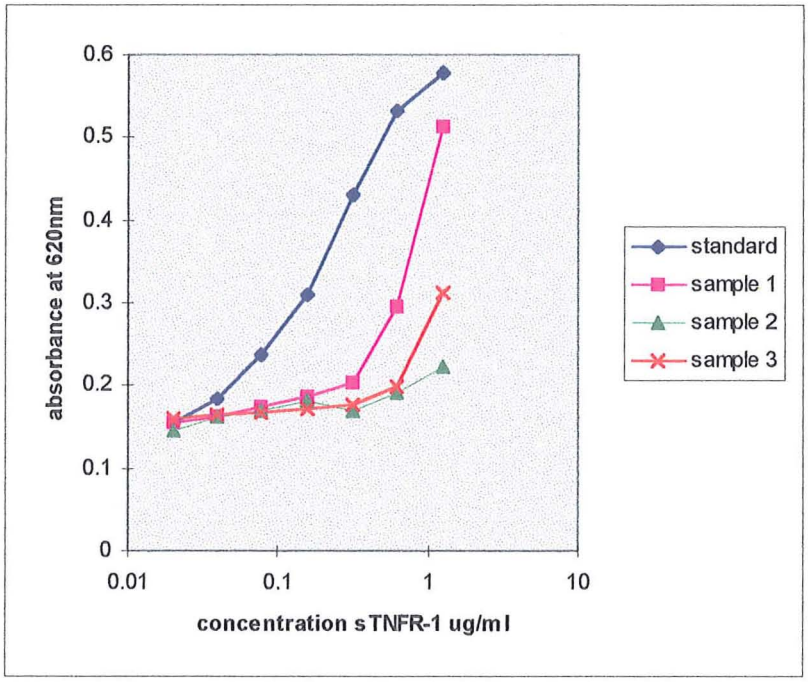


Figure 3.3. Standard curve (r^2=0.9945) for the anti-TNF activity of recombinant human soluble TNFR-1 using the L929 cell line. The titration curves of three samples have been superimposed on the standard curve. Note that the actual values on the x-axis apply only to the standard curve, other samples are plotted according to their dilution. Sample 1; H41, 4.5 hours, >10000 and unstimulated; sample 2; H41, 24 hours, <10000 and unstimulated; sample 3; H46, 12 hours, unstimulated and unconcentrated.



software (r^2=0.9945). The samples illustrated show evidence of high (0.55μg/ml), medium (0.15μg/ml) and low (0.073μg/ml) activity. Anti-TNF activity was demonstrated in all except 2 of the samples tested. Results are given as μg/ml (human equivalent) and the assumption is made that the detected activity is due to the presence of feline sTNFR-1. The maximum value recorded was greater than the maximum standard of 1.25μg/ml.

Groups of samples were analysed for differences between each time point, between unstimulated and stimulated supernatants and between concentrated and unconcentrated samples. There was no significant difference between results for any of the incubation times or between stimulated and unstimulated samples (figure 3.4.a and 3.4.b). However, a significant difference was detected between samples with a molecular weight cut off greater than 10kDa (median value 0.352μg/ml) and samples with a molecular weight cut off less than 10kDa (median value 0.116μg/ml). Although the median value of the unfiltered samples was intermediate between these two groups (0.2955μg/ml), this was not significantly different from either group (figure 3.4c.).

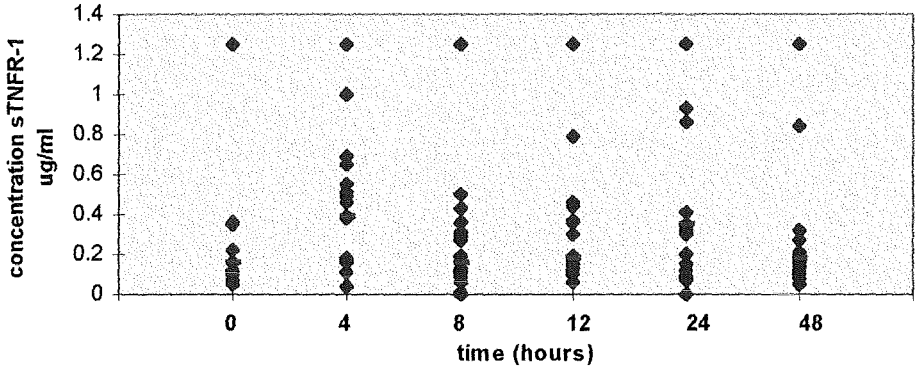
3.6. Discussion

The standard curves obtained in the assay for cytotoxicity illustrate the sensitivity of murine L929 cells to rmTNF-α. It is known that this cell line is sensitive to rhTNF-α (Wadhwa et al. 1991) and has been used to demonstrate cytotoxic activity in feline samples (Lawrence et al. 1995). However, little cytotoxic activity associated with TNF-α was demonstrated in the samples by this assay. It is likely that this was due to a combination of a lack of sensitivity to the very small concentrations of TNF-α that may be present, and the presence of soluble receptors that interfere with the bio-availability of TNF, rather than unresponsiveness of the murine cells to feline cytokines.

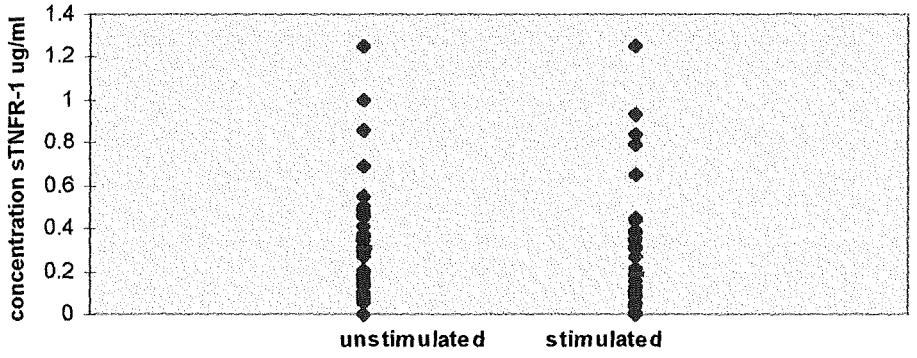
Previous studies measuring TNF-α activity using the L929 bioassay found detectable levels in plasma (Lawrence et al. 1992; Lawrence et al. 1995) rather than cell supernatants, although TNF-α activity has been detected in supernatants of LPS-stimulated feline monocyte cultures by ELISA and using the WEHI-164 bioassay (Lehmann et al. 1992). The latter study reported concentrations

Figure 3.4. a-c Graphs illustrating the distribution of results of anti-TNF activity (reported as μg/ml human equivalent) in different groups of cell culture supernatant samples. In each group the median values are represented by a red bar. a) Samples grouped into different time points, b) samples grouped into ConA stimulated and unstimulated, c) samples grouped into <10kDa, unconcentrated and >10kDa.

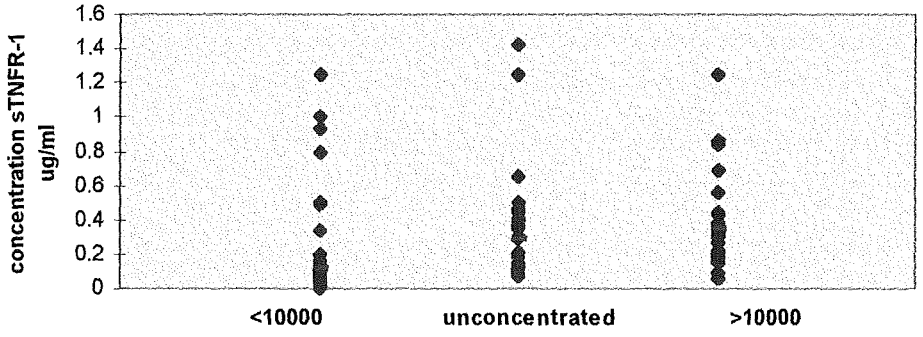
a)



b)



c)



of <10pg/ml to around 100pg/ml which is consistent with the level of cytotoxicity detected in the standards and two positive samples in this study. However the results reported here cannot be compared easily with those of Lawrence *et al* as they report TNF levels in international units. The use of concanavalin A rather than LPS to stimulate the cells may explain why so few samples displayed TNF activity.

Cross-reactivity between feline TNF-α and human anti-TNF-α antibody has been described, resulting in a neutralisation of the cytotoxic effect seen in the bioassay (Lehmann et al. 1992; Lawrence et al. 1995). Demonstration of inhibition of the activity described in the two positive feline splenocyte supernatants in this study by pre-incubating them with anti-human antibody might be used to confirm that the cytotoxicity detected was specific to this cytokine. The lack of cytotoxic activity of recombinant human sTNFR-1 confirms that this receptor is suitable for use as a standard in the assay of anti-TNF activity.

This is the first report of the demonstration of feline TNFR activity in feline cell culture supernatants. The protocol employed for the detection of anti-TNF activity in the supernatant samples was similar to that described previously for the assessment of the effect of the addition of sTNFR-1 purified from human urine (Engelmann et al. 1990) to the TNF bioassay.

There was no detectable difference between cultures stimulated with ConA and unstimulated cultures. Similarly, the length of incubation did not make a significant difference to levels of soluble receptor. TNFR-1 gene expression is thought to occur in a constitutive manner, with any increase in the soluble receptor resulting from cleavage from the cell surface rather than up-regulation of transcription and translation (Aggarwal and Reddy, 1994). The preparation of the splenic cell cultures may have induced the cleavage of receptors from cell membranes so that they were already present in supernatants removed at time 0. Subsequently, ConA may not have been a suitable stimulator of further receptor release or there may have been no membrane bound receptors remaining for cleavage. In human subjects after surgery levels of sTNFRs became elevated for at least 24 hours to a maximum of three-fold when compared to pre-operative levels (Neilson et al. 1996) which suggests that a decrease in receptor levels might not have been expected throughout the course of this experiment.

A significant difference was detected between the ultrafiltrate samples containing molecules smaller than 10kDa and concentrated samples that were retained by the PM10 filter and contained only larger molecules. This is consistent with the functional, soluble TNFR being retained during ultrafiltration as human sTNFR has a molecular mass of around 30kDa (Engelmann et al. 1990). The retention of some activity in the filtrate samples may reflect leakage through the PM10 membrane of molecules greater than 10kDa. This could have been investigated by the analysis of supernatant fractions by SDS-PAGE. Alternatively, there may be a different substance present that is exhibiting some anti-TNF activity. However, since the standard and sample curves are parallel, it is likely that the activity in each is due to the same molecule. Pre-incubation of samples with an inhibitory antibody to feline TNFR-1 would confirm that the detected activity was due to the soluble receptor. However an antibody reacting with the feline protein is not available.

The concentrations of receptor detected here (range 0.046 - >1.25μg/ml) were far greater than those previously described in human serum samples (range 0.86 - 3.54ng/ml) measured by enzyme-linked binding assay (Godfried et al. 1993) and monocyte culture supernatants (range 27 - 36pg/ml) measured by ELISA (Rimaniol et al. 1994). This result suggests that the binding of recombinant human sTNFR-1 and feline receptor in the supernatant samples to murine TNF-α are not directly comparable. In addition, using the results for two samples that were measured over repeated assays, the inter-assay coefficient of variation was calculated as over 50%, highlighting gross inconsistencies with the quantitative values provided by the bioassay. There was more consistency in the qualitative analysis of results, as samples that gave high readings compared to other samples on one assay date, produced a similar relative picture at a later date although the absolute value was quite different.

The two samples that had evidence of TNF activity had moderate anti-TNF activity which presumably was artificially low because of the presence of endogenous TNF.

3.7. Further studies

Following the study presented in this chapter, further work directed towards developing a species-specific immune based assay is likely to provide a more consistent

method of monitoring levels of feline sTNFR-1. Development of a neutralising antibody to feline sTNFR-1 would also allow confirmation that the anti-TNF activity detected by the bioassay is, indeed, attributable to feline sTNFR-1. Perhaps the most straightforward way to approach this would be to clone and sequence the cDNA encoding feline sTNFR-1, generate recombinant protein and raise antibody to it.

PCR-based methods of quantifying receptor expression are unlikely to be of value because serum levels of sTNFR-1 fluctuate as a result of protease activity on membrane bound receptors. Since human TNFR-1 is considered to be constitutively expressed in the manner of a house keeping gene, evaluating mRNA levels of the receptor in blood cells by real time RT-PCR will not provide a reflection of serum levels (Aggarwal and Reddy, 1994).

3.8. Conclusion

The L929 assay has been used here successfully to demonstrate the presence of anti-TNF activity in feline samples. This activity is likely to be due to the presence of feline sTNFR-1 but lack of neutralising antibody meant that this conclusion could not be confirmed. The inconsistency encountered with the quantitation of results means that the development of immune-based assays for feline TNFR-1 would be highly advantageous. As TNFR-2 is considered more species-specific, immune based methodology is also required for the analysis of feline sTNFR-2.

Chapter 4:
Cloning and Sequencing of Feline Tumour Necrosis Factor Receptors

4.1. Summary of Chapter

This chapter presents work designed to clone and sequence complementary DNA (cDNA) encoding tumour necrosis factor receptors 1 and 2 in the cat (TNFR-1, TNFR-2). The polymerase chain reaction (PCR) was used to amplify regions of the sequences coding for feline receptors using cDNA prepared from cat splenic tissue. PCR products were cloned into a standard TA-vector and sequenced. The expression of feline coding sequences homologous to both receptors for TNF that have been described in man were identified by this technique. The amplified regions demonstrated 85% and 77% homology at the nucleic acid level and 83% and 67% homology at the amino acid level to the corresponding regions of the human sequences for TNFR-1 and 2 respectively.

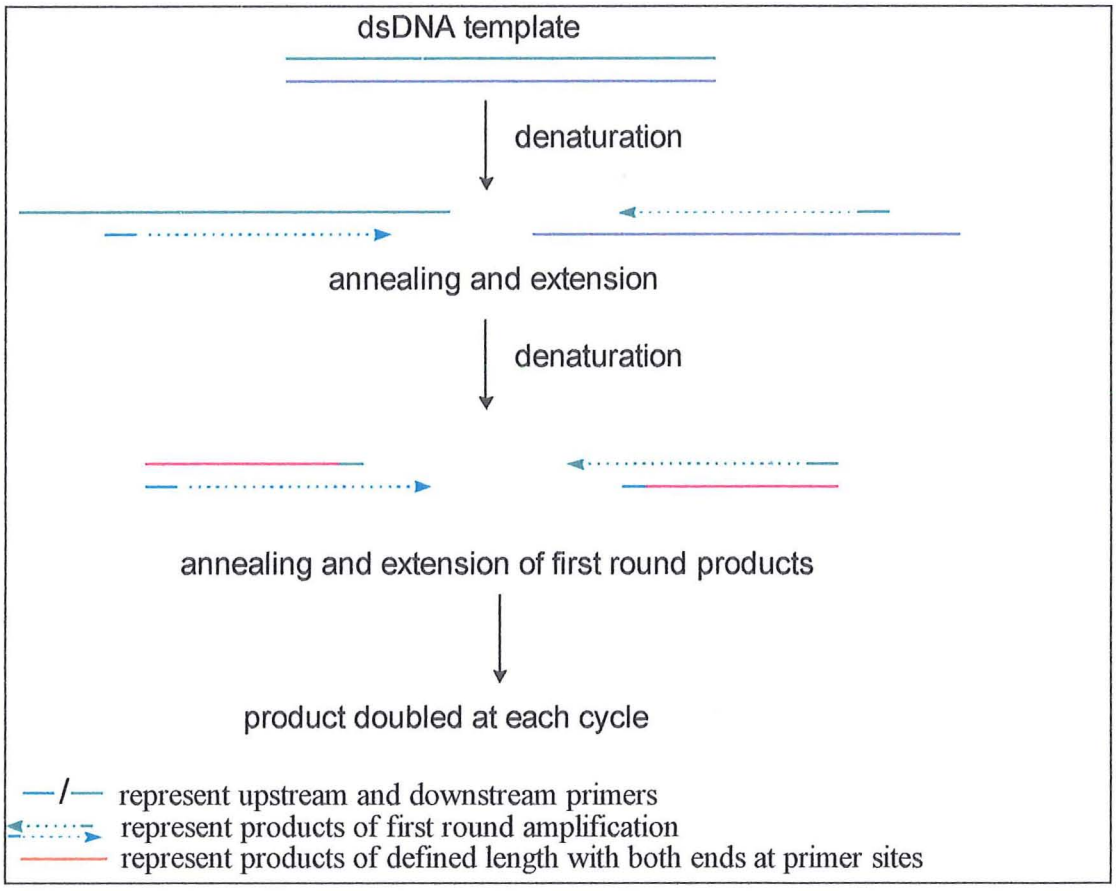
4.2. Background to Techniques Used

4.2.1. The polymerase chain reaction

The technique of PCR was originally described in the mid 1980's (Saiki et al. 1985) as an enzymatic means of amplifying regions of DNA by the extension of annealed primers. Initially the process was limited because the elevated temperatures required in the reaction denatured the enzyme, and fresh enzyme had to be added continually. With the incorporation of a thermostable polymerase into the protocol (Saiki et al. 1988), the enormous potential of PCR began to be realised. Simply, PCR is used to amplify a segment of DNA located between two areas of known sequence - the 'priming sites'.

Figure 4.1. illustrates a simple PCR protocol. In PCR, the double stranded DNA template is first denatured by heating, then the reaction temperature is reduced to permit the binding to each single DNA strand of a short oligonucleotide primer. Two oligonucleotides are used in each PCR, each complementary to opposite strands of template DNA, flanking the region to be amplified. They are added in molar excess

Figure 4.1. Diagrammatic representation of the polymerase chain reaction



of the template DNA to favour primer binding rather than template re-annealing. The annealed primers are then extended through the incorporation of deoxynucleoside triphosphates (dNTPs) by the action of a thermostable DNA polymerase to form a DNA strand complementary to the template. The temperature-dependent cycles of denaturation, annealing and extension are repeated around thirty times. The product doubles at every cycle until eventually a plateau of product is achieved mainly as a result of enzyme limitations and re-annealing of template in preference to primer binding. For the first few rounds of amplification, products are not of a distinct length, as only one end is determined by primer. In subsequent rounds, initial products with a primer-defined end become the template, so that a population of DNA products of specific length arises (see figure 4.1.).

PCR has been used for many different applications since its introduction. These include mutation detection, diagnosis of disease, generation of large amounts of DNA for probe production, cloning or sequencing, quantification of expression levels and identification of novel sequences, although this latter application requires a small amount of sequence or related sequence data to be available. The early problem of base mis-incorporation has been reduced by the development of the polymerases with 'proof-reading' or exonuclease activity which excise mis-matched nucleotides.

It is important to optimise each new PCR reaction for the primers being used. Primer design is perhaps the most critical parameter but the magnesium ion concentration and the annealing temperature employed during the reaction can also alter results dramatically. The balance is always to achieve maximum levels of the desired product with the minimum amplification of non-specific products. Magnesium ions allow dNTP incorporation, stimulate polymerase activity and increase the melting temperature of double stranded DNA and primer-template binding. Any change in dNTP concentration alters the availability of Mg^{2+}. The optimal annealing temperature for each primer is related to its length and its guanine/cytosine:adenine/thymine (G/C:A/T) content and should be similar for each primer pair designed.

4.2.2. Using PCR to determine novel sequence

PCR can be used to amplify previously unknown sequence using primers designed according to regions of homology in related sequences. Primers are either based on

one of the comparative sequences or incorporate degeneracy at sites where differences exist in the related sequences (Compton, 1990). Less stringent PCR conditions can be employed, although this practice may increase non-specific amplification, and bands of the desired size can be isolated, cloned and sequenced.

4.2.3. TNFR sequences in other species

Once the proteins TNFR-1 and TNFR-2 had been identified in man by their ability to inhibit the toxic effects of the cytokine, researchers began to unravel the cDNA encoding them. The nucleotide sequence for human TNFR-1 was originally described by different groups after detection using molecular techniques based on the amino acid sequence of the purified protein. These techniques included screening cDNA libraries with oligonucleotide probes to identify clones containing the appropriate insert (Engelmann et al. 1990b) (Schall et al. 1990) and designing degenerate primers for use in a reverse transcription-polymerase chain (Loetscher et al. 1990). The nucleotide sequence for human TNFR-2 was determined by expression screening of a cDNA library with radiolabeled TNF-α (Smith et al. 1990).

Cloning and sequencing of the murine TNF receptors and rat TNFR-1 soon followed using probes based on the human sequences to screen murine and rat cDNA libraries (Goodwin et al. 1991; Himmler et al. 1990; Lewis et al. 1991). Homologous regions between the human and mouse TNFR-1 sequences were used subsequently to design PCR primers for amplification of porcine TNFR-2 cDNA (Suter and Pauli, 1995).

The human TNFR-1 amino acid sequence shows 73% homology to the mouse and 74% homology to the rat, and the human TNFR-2 sequence shows 73% homology to the mouse. Murine and rat TNFR-1 sequences are 88% homologous.

4.3. Aim of Experimental Studies

The aim of the work presented in this chapter was to identify whether receptors for tumour necrosis factor homologous to those that have been described in man and the mouse are expressed in the cat. It was intended to use PCR to amplify fragments encoding the receptors in cDNA extracted from feline tissues and subsequently to characterise the amplified products by sequence analysis.

4.4. Materials and methods

4.4.1. Source of tissue

Feline splenic tissue was collected and prepared for cell culture as described in section 3.4.1. Cell cultures were incubated for 8 hours with ConA at a final concentration of 7.5μgml^{-1}. Any adherent cells were released into the culture medium with a cell scraper (Nunc, Gibco) and the medium was collected and centrifuged to provide a cell pellet for RNA extraction.

4.4.2. RNA extraction

All non-RNase free plasticware for RNA extraction was treated with diethyl pyrocarbonate (DEPC, (Sigma, UK)) and re-autoclaved prior to use. After quantifying the number of cells present in a haemocytometer, the pellet prepared in section 4.4.1. was lysed by the addition of 0.2ml RNAzol™B (AMS Biotechnology (Europe) Ltd.) per 10^6 cells. RNA extraction was performed according to the protocol supplied by the manufacturers. Briefly, 0.2ml of chloroform was added per 2ml of cell homogenate and the samples were shaken for 15 seconds, after which they were placed on ice for 5 minutes. The suspensions were centrifuged at 4°C for 15 minutes at 12000g in a refrigerated microfuge (Eppendorf), then the upper aqueous phase was removed to a fresh eppendorf tube, an equal volume of ice-cold isopropanol was added to precipitate the RNA and the samples were stored at 4°C for 15 minutes.

Precipitated RNA was collected by centrifugation at 4°C for 15 minutes at 12000g, washed with 75% ethanol and centrifuged for 8 minutes at 7500g at 4°C. All traces of ethanol were removed by pipette and the RNA pellet was allowed to dry in air. The dry pellet was dissolved in DEPC-treated dH_2O (0.5ml DEPC per 500ml dH_2O, autoclaved before use to destroy the DEPC) and its RNA concentration and quality assessed by spectrophotometry (GeneQuant, Pharmacia Biotech, UK) and by visualisation on a standard 1.5% agarose gel (see section 4.4.6.) after diluting 1:10 in dimethyl formamide and adding 2µl loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose). If the RNA was not to be reverse transcribed immediately, it was not reconstituted in dH_2O, but instead stored at -20°C under

ethanol. Following a similar protocol, murine RNA was extracted from L929 cells for use as a positive control.

4.4.3. Reverse transcription

Reverse transcription was performed in a total reaction volume of 25µl with a final concentration of 50mM Tris-HCl (pH8.3), 75mM KCl, 10mM dithiothreitol (DTT), 3mM $MgCl_2$, 0.5mM each dGTP, dATP, dTTP and dCTP, 0.25µl RNAisin (Promega) and 0.09 OD_{260} units of random primers (Gibco). Each reaction contained 2µg of total RNA which had been treated previously by heating to 65°C for 5 minutes and quenched on ice prior to addition to the reaction. Finally, 400 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) enzyme (Gibco, UK) were added to the reaction mix.

Reverse transcription was allowed to proceed at 37°C for 30 minutes and then at 42°C for one hour, after which the enzyme was denatured by heating to 95°C for 5 minutes. Aliquots of cDNA were stored at -20°C prior to use and at 4°C while in use. Murine RNA was reverse transcribed in the same way.

4.4.4. Primer design

Primers for use in PCR were designed according to regions of homology in previously published cDNA sequences in other species. At the time of primer design, human (Godfried et al. 1993; Loetscher et al. 1990; Schall et al. 1990), mouse (Lewis et al. 1991) and rat (Himmler et al. 1990) sequences were available for TNFR-1, and human (Smith et al. 1990) and mouse (Goodwin et al. 1991) sequences for TNFR-2. Sequence comparisons to identify regions of homology were performed using the Genetics Computer Group (GCG) software (see section 4.4.10). Figures 4.2 and 4.3 illustrate the primers that were designed to amplify feline TNFR-1 and TNFR-2 respectively. After successful amplification and sequencing of at least part of the feline TNFR-1 and TNFR-2 sequences, feline-specific primers were designed to use as positive controls when assessing further cDNA aliquots for the presence of the appropriate transcripts. For TNFR-1, these were eco-60 (5'gcatcgaattcatgggcctccccacc3') as the upstream primer and xba-60 (5'gctcatctagagaaccccgcgtggca3') as the downstream primer. Restriction sites were included in these primers for ease of cloning into an expression vector.

Figure 4.2 Siting and species homology of primers designed to amplify feline TNFR-1. The human cDNA sequence (Accession no. X55313) is shown by the blue line, with leader, extracellular (ECD), transmembrane (t-m) and intracellular (ICD) regions as indicated by the red bars. Primers were designed according to the human sequence (shown in blue) and nucleotide differences between that and the mouse sequence (M59377, shown in red) and the rat sequence (M63122, shown in green) are given. Upstream primers are marked *f* and downstream primers *r* and all are presented in 5'-3' orientation.

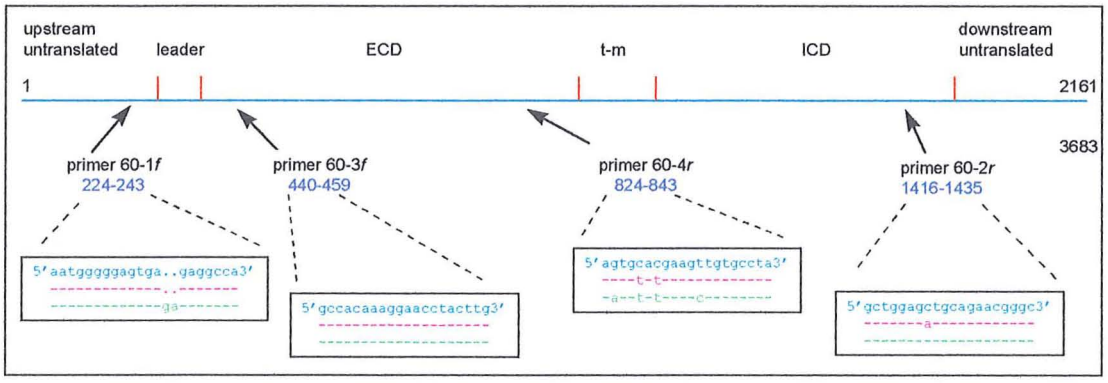
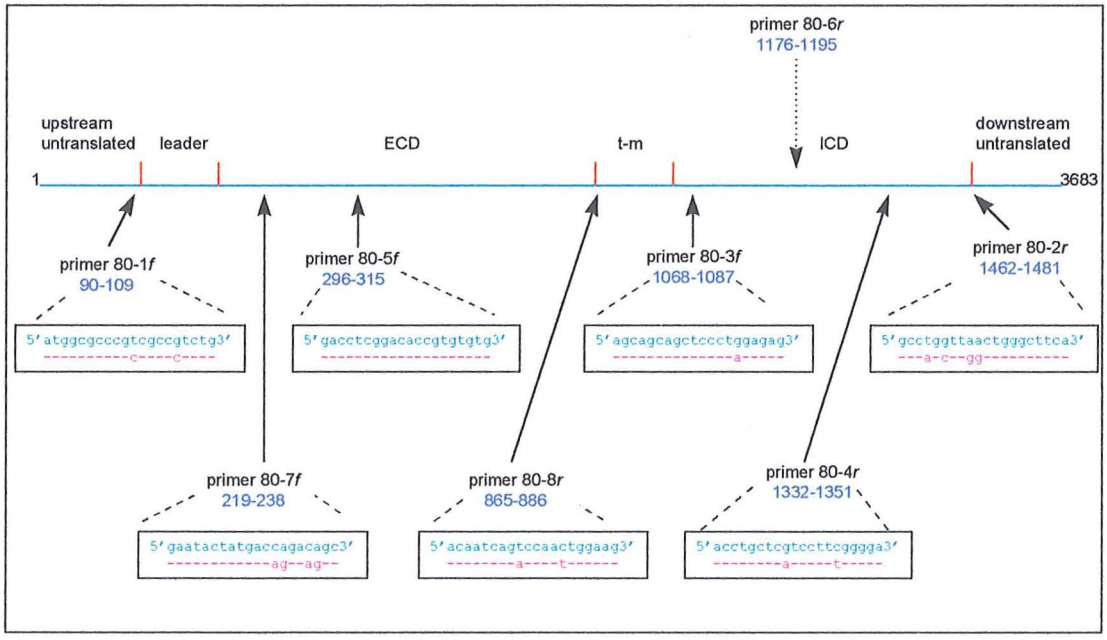


Figure 4.3 Siting and species homology of primers designed to amplify feline TNFR-2. The human cDNA sequence (Accession no. M32315) is represented by the blue line, with leader, extracellular (ECD), transmembrane (t-m) and intracellular (ICD) regions as indicated by the red bars. Primers were designed according to the human sequence (shown in blue) and nucleotide differences between that and the mouse sequence (M59378, shown in red) are given. Upstream primers are marked *f* and downstream primers *r* and all are presented in 5'-3' orientation. Primer 80-6*r* is feline specific; see section 4.4.4. for sequence.



For TNFR-2, only one feline specific primer was designed, 80-6*f* (5'tctgagctgctggagctggc3'), a downstream primer which was used in conjunction with 80-3 (see fig 4.3.).

Primers based on the human Abelson tyrosine kinase (ABL) housekeeping gene were used to check the quality of each cDNA aliquot. These primers were designed to span an intron in the genomic sequence and were designated ABL-1 (5' cagcggccagtagcatctgactt 3', position 452-474 in Genbank Accession number M14752) and ABL-2 (5' tgtgattatagcctaagacccggag 3' position 626-650).

Optimal melting temperatures (T_M) for each primer were calculated using the formula [T_M = (2xA/T) + (4xG/C)], and the initial annealing temperature used was calculated as 5°C lower than the T_M (Roux, 1995). A magnesium chloride titration was performed to determine the optimal magnesium ion concentration for each PCR reaction.

4.4.5. PCR

PCR was performed in thin-walled 0.5ml microcentrifuge tubes using a thermocycler (DNA Thermo Cycler 480, Perkin Elmer) in a total reaction volume of 50µl with a final concentration of 10mM Tris-HCl (pH8.8), 1.5mM $MgCl_2$ (or as otherwise suggested by $MgCl_2$ titration), 50mM KCl, 0.1% Triton X-100, 0.2mM of each dNTP and 0.4µM of each primer. After exposure of the reaction tubes to ultraviolet light for 10 minutes, 1 unit of thermostable DNA polymerase (DNAzyme, Finnzymes Oy, Finland) was added to each. Negative control reactions without template were prepared as above for each primer pair used. To the other tubes, 2.5µl of the cDNA template prepared as in section 4.4.3. was added. A drop of mineral oil was applied to the top of each reaction mix prior to application to the thermocycler.

PCR consisted of an initial denaturation step of 5 minutes at 95°C, followed by 35 cycles consisting of 1 minute each of denaturation at 94°C, annealing at the optimal temperature determined for the primers involved (see section 4.5.2.), and extension at 72°C. A final extension step of 30 minutes at 72°C was performed to facilitate cloning by ensuring the addition of 'A overhangs' (see section 4.4.8.).

4.4.6. Analysis of products by agarose gel electrophoresis

A 10µl aliquot of each PCR product was mixed with 2µl gel loading dye (composition as in section 4.4.2.), electrophoresed at 100V for 1 hour on 1.5% agarose gels in TBE buffer (90mM tris, 90mM boric acid, 2mM EDTA) in a minigel system (Hoefer HE 33 Mini Submarine Electrophoresis Unit, Pharmacia Biotech) and stained with ethidium bromide (3µl of 10mg/ml stock added to 100ml gel). Standard molecular weight markers were run in parallel with samples (Gibco BRL, Paisley, UK). Gels were examined over an ultraviolet light (T2201, Sigma or UVT 28-M, Herolab) and photographed using either a Polaroid camera or a digital imaging system (Cohu high performance charge coupled device (CCD) camera connected to a Sony monitor (SSM-930CE) and video graphic printer (UP 890CE)), recording onto film (Polaroid 665, Sigma) or printing paper (Sony Superior Density, UPP 110HA) respectively.

4.4.7. Purification of PCR products

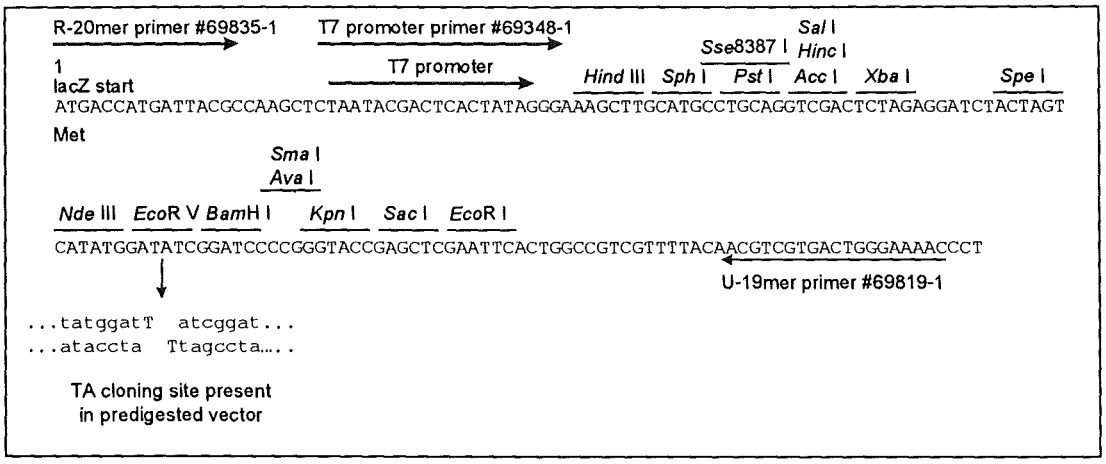
If PCR yielded a single band of the expected size, the PCR product was removed from underneath the mineral oil layer and cleaned by chloroform:isoamyl alcohol (CIAA) extraction. A volume of CIAA (24:1) equal to the reaction volume was added, the tube vortexed and then centrifuged at 12000g for 1 minute. The aqueous phase containing the DNA was collected and 2µl added to the ligation reaction.

When a single band was not observed after PCR, the remainder of the products was electrophoresed on an agarose gel and individual bands at the expected size were cut out with a sharp, clean scalpel blade, in as small a gel volume as possible. DNA was eluted from gel slices using a gel extraction kit (Qiaex II kit, Qiagen, UK) and quantified by spectrophotometry (GeneQuant, Pharmacia Biotech).

4.4.8. Cloning of PCR products

Purified PCR products were cloned into a standard TA-cloning vector (pT7Blue T-Vector, Novagen, Wisconsin) supplied as part of a kit (pT7Blue T-Vector Kit, Novagen, Wisconsin) containing all the reagents necessary for the cloning procedure. The vector is supplied pre-digested with *EcoR V* and with 3' dT residues at each end (figure 4.4.) so that PCR products obtained after amplification with a DNA polymerase that leaves 3'A-nucleotide overhangs can be readily ligated. Interruption

Figure 4.4 Diagram showing sequence flanking the cloning site of the pT7 vector (Novagen). The positions of the T7 and U19 primer binding sites and the cloning site are illustrated.



of the plasmid with insert disrupts an encoded β-galactosidase gene so that positive colonies appear white on medium containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-$_D$-galactopyranoside (X-gal, Sigma), whereas negative colonies appear blue.

The ligation reaction was prepared with 50ng vector, 0.2pmol PCR product (or 2μl of CIAA cleaned product), 5mM DTT, 0.5mM ATP and 0.5μl T4 DNA ligase (2-3 Weiss units) in ligase buffer (20mM Tris-HCl, pH 7.6, 5mM $MgCl_2$) in a total volume of 10μl and incubated overnight at 16°C. At the end of incubation, 1μl of the reaction was used to transform 20μl of NovaBlue competent cells (supplied with the kit) taken from -70°C and thawed on ice. Tubes were stirred gently, left on ice for 30 minutes, heated for 40 seconds at 42°C and returned to the ice for a further 2 minutes. After addition of 80μl of SOC medium (see Appendix 1) at room-temperature to each reaction, tubes were incubated in an orbital shaker at 37°C and spun at 200rpm for 1 hour.

Half (50μl) of each transformation reaction was spread on LB-agar (see Appendix 1) 82mm plates containing 50μg/ml ampicillin, 15μg/ml tetracycline, 35μl of 50mg/ml X-Gal and 20μl of 100mM isopropyl-β-thiogalactoside (IPTG, Sigma). Once the liquid had been absorbed, the plates were inverted and incubated overnight at 37°C.

The following morning, 10 white and one blue (control) colonies were picked off using a sterile pipette tip and dispersed in 2ml LB broth (see Appendix) with ampicillin at 50μg/ml (LB-amp). Colonies were incubated overnight at 37°C in an orbital shaker at 200rpm. Tubes showing evidence of growth (cloudiness) at the end of this incubation were tested for the presence of insert by PCR as follows. Fifty microlitres of culture medium were boiled for 5 minutes to lyse the cells which were then centrifuged at 12000g for 1 minute. Ten microlitres of the resultant supernatant were used as the template in a 50μl PCR reaction with 1 unit of thermostable DNA polymerase (DNAzyme, Finnzymes Oy, Finland), 10mM Tris-HCl (pH8.8), 1.5mM $MgCl_2$, 50mM KCl, 0.1% Triton X-100, 0.2mM of each dNTP and 0.4μM of each plasmid primer (upstream, T7 promoter primer 5'taatacgactcactataggg3', downstream U-19 primer 5'gttttcccagtcacgacgt3', see figure 4.4.). Without an insert, these primers are situated to give a product of 139 base pairs in length. If an insert is present, the primers amplify a product equivalent to the insert length plus 139 base

pairs of plasmid sequence. PCR products were analysed as described above (section 4.4.6.).

Stocks were made with 15% glycerol of any colony with evidence of an insert of the correct size and stored at -20°C. Plasmid DNA from the same colonies was purified using a kit (Plasmid Miniprep Kit, Qiagen, UK) with spin columns to bind the DNA. DNA was eluted from the columns in 40µl of dH_2O.

4.4.9. Sequencing

Three clones and 2 PCR products of the amplified region were sequenced bi-directionally using T7 and U19 primers or the PCR specific primers respectively. Samples were sent to the DNA Sequencing Service (King's College, London) for sequencing on an automated ABI DNA sequencer (Perkin Elmer, USA).

4.4.10. Analysis of results

Analysis of results was performed using the Wisconsin Sequence Analysis Package (Devereux et al. 1984) (Genetics Computer Group, Madison, Wisconsin). Sequencing data were analysed using 'SeqEd' and 'BestFit' (Smith and Waterman, 1981) programs and confirmation that the appropriate sequence had been amplified was achieved by comparison to a database (GenEMBL nucleotide sequence database) using the 'BLAST' and 'FastA' (Pearson and Lipman, 1988) programs. Novel sequence data were submitted to Genbank for release onto the database.

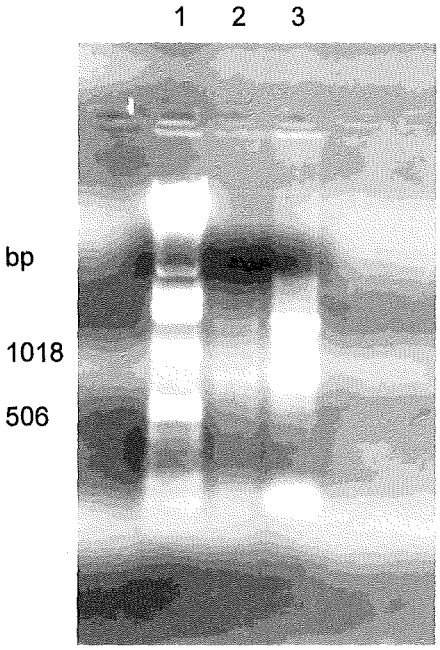
4.5. Results

4.5.1. RNA Assessment

A gel illustrating the products of two standard RNA extractions is illustrated in figure 4.5. By comparison to the size marker, the 28S and 18S ribosomal RNA bands can be visualised at 1500bp and 750bp respectively, with less intense, diffuse ethidium bromide staining between and around these bands. Generally, the extraction technique produced total RNA with an A_{260}:A_{280} greater than 1.6.

The reverse transcription step and DNA contamination of RNA were assessed by amplification of the cDNA with ABL primers. In cDNA these primers produced a

Figure 4.5. Agarose gel following electrophoresis of the products of 2 RNA extractions using the RNAzol™ B method. Lane 1: 1kb marker, lanes 2 and 3: products of two separate RNA extractions. Both RNA products were diluted 1:10 with formamide and 2μl loading mix added prior to running on the gel.



band of 198 base pairs, whereas in samples with genomic DNA contamination, an additional band of 800 base pairs was seen. Samples with genomic contamination or poor amplification with the control ABL primers were discarded.

4.5.2. PCR Amplification

Initial amplification was performed with primer pairs designed to amplify the majority of feline TNFR-1 and TNFR-2 sequences (60-1 and 60-2, 80-1 and 80-2) but these failed to yield any products. Subsequently primers were designed to amplify smaller, internal fragments of each sequence. Successful amplification was achieved with primer pairs 60-1 and 60-4, and later with eco60 and xba60 (figure 4.6.), and 80-3 and 80-4 (figure 4.7.). Both of these amplifications were optimal in the presence of 2.5mM $MgCl_2$ and with an annealing temperature of 58°C.

A feline specific primer (80-6 situated at bases 1175 to 1195 of the human sequence 5'3') was designed after sequencing of the 80-3 and 80-4 product. This produced the correct band as a single product when used for amplification with 80-3 (figures 4.7. and 4.8.) and gave a band of around 900bp when used in combination with 80-5. However, cloning and sequencing of the 80-5 and 80-6 product revealed 80-6 primer sequence followed by unknown sequence which showed no homology to other TNFR-2 sequences when run in the GCG FastA program.

A nested PCR approach was attempted by designing two further primers (80-7 and 80-8) where amplification with 80-7 and 80-6 was followed by amplification with 80-5 and 80-8, but this did not produce any bands of the desired size.

Figure 4.8. illustrates an $MgCl_2$ titration where higher concentrations of $MgCl_2$ are associated with an increase in non-specific bands. Addition of DMSO and formamide appeared to make little difference to the reaction whereas addition of glucose results in loss of the product altogether.

4.5.3. Analysis of sequence data

Consensus sequence information for the amplified regions of feline TNFR-1 and TNFR-2 is given in figures 4.9. and 4.10. The novel feline sequences were deposited in the GenBank database and given Accession numbers U72344 (TNFR-1) and U51429 (TNFR-2).

Figure 4.6. Agarose gel following electrophoresis of the products of PCR amplification of feline cDNA with primer pair 60-1 and 60-4. Lane 1: 100bp marker with bright band corresponding to 600bp, lane 2: 542bp fragment amplified with feline TNFR-1-specific primers eco-60 and xba-60, lane 3: 620bp fragment amplified with 60-1 and 60-4.

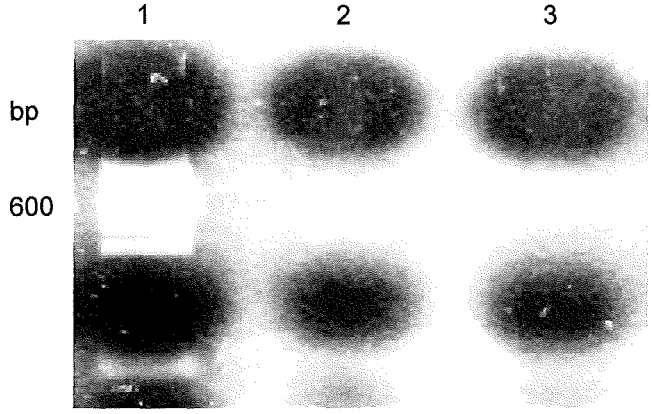


Figure 4.7. Agarose gel following electrophoresis of the products of PCR amplification of feline cDNA with primer pair 80-3 and 80-4. Lane 1, 100bp marker; lanes 2 and 3, 284bp fragment amplified from murine (lane 2) and feline (lane 3) cDNA with 80-3 and 80-4; lane 4, negative control with 80-3 and 80-4 primers; lanes 5 and 6, 130bp product and non-specific bands amplified from murine (lane 5) and feline (lane 6) cDNA with 80-3 and feline specific 80-6; lane 7: negative control with 80-3 and 80-6 primers, lanes 8 and 9: 180bp product amplified from murine (lane 8) and feline (lane 9) cDNA with ABL-1 and ABL-2; lane 10, negative control with ABL-1 and ABL-2 primers.

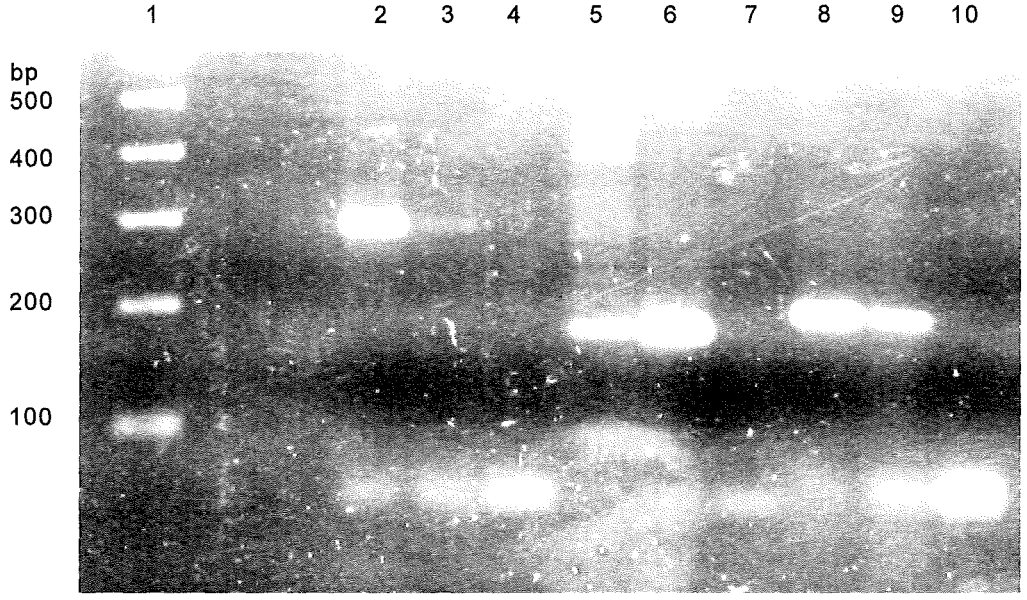


Figure 4.8. Agarose gel showing the effects of changing reaction conditions on PCR of feline cDNA with 60-3 and 60-6 primer pair. Lane 1, molecular weight marker; lanes 2-5 increasing concentrations of $MgCl_2$ in the reaction mix; lanes 6-8, formamide, DMSO and glucose respectively added to reaction mix; lane 9, positive control with ABL primers; lane 10, negative control. The arrow indicates the product of interest.

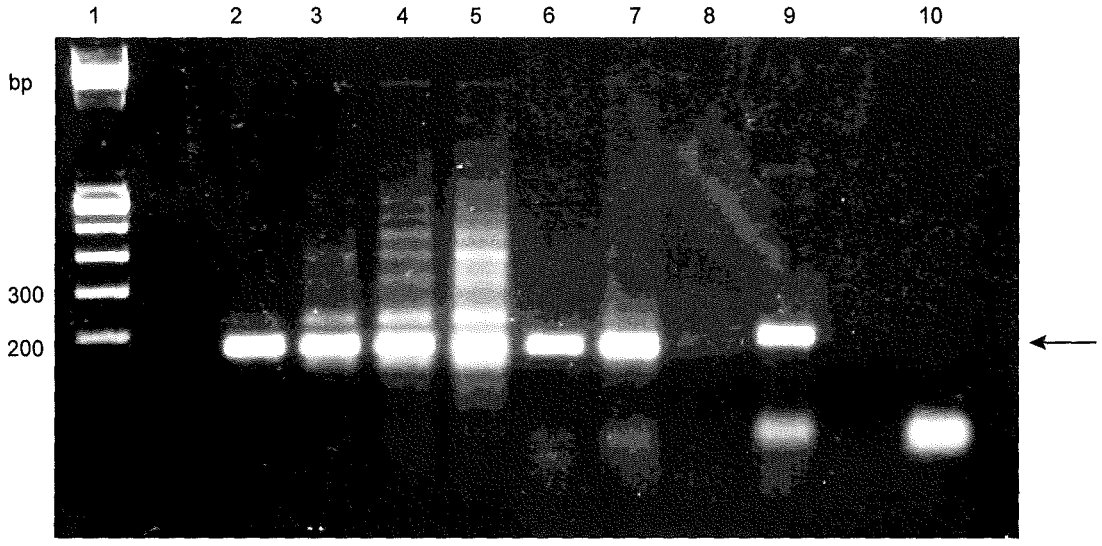


Figure 4.9. Nucleotide sequence and predicted translation of the amplified region of feline cDNA homologous to part of human TNFR-1, GenBank Accession number U72344. The coding region starts at nucleotide 14 and the sequence is flanked by 60-1 (upstream) and 60-4 (downstream).

```
        CAACTGGCCGGACATGGGCCTCCCCACCGTCCCTGGCCTGCTGCAGCCACTGGTACTTCT
      1 ---------+---------+---------+---------+---------+---------+
60
        GTTGACCGGCCTGTACCCGGAGGGGTGGCAGGGACCGGACGACGTCGGTGACCATGAAGA
                     MetGlyLeuProThrValProGlyLeuLeuGlnProLeuValLeuLe
                     M  G  L  P  T  V  P  G  L  L  Q  P  L  V  L  L

        GGCTCTGTTGGTGGAAATATACCCATTAAGGGTTACTGGACTGGTCCCTCACCTCAGGGA
     61 ---------+---------+---------+---------+---------+---------+
120
        CCGAGACAACCACCTTTATATGGGTAATTCCCAATGACCTGACCAGGGAGTGGAGTCCCT
        uAlaLeuLeuValGluIleTyrProLeuArgValThrGlyLeuValProHisLeuArgAs
         A  L  L  V  E  I  Y  P  L  R  V  T  G  L  V  P  H  L  R  D

        CCGGGAGAAGAGAGCTATTCCATGTCCCCAAGGAAAATATATTCACCCTCAAGATAATTC
    121 ---------+---------+---------+---------+---------+---------+
180
        GGCCCTCTTCTCTCGATAAGGTACAGGGGTTCCTTTTATATAAGTGGGAGTTCTATTAAG
        pArgGluLysArgAlaIleProCysProGlnGlyLysTyrIleHisProGlnAspAsnSe
         R  E  K  R  A  I  P  C  P  Q  G  K  Y  I  H  P  Q  D  N  S

        CATTTGCTGTACGAAGTGCCACAAAGGGACCTACCTGTACAATGACTGTGAGGGCCCAGG
    181 ---------+---------+---------+---------+---------+---------+
240
        GTAAACGACATGCTTCACGGTGTTTCCCTGGATGGACATGTTACTGACACTCCCGGGTCC
        rIleCysCysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysGluGlyProGl
         I  C  C  T  K  C  H  K  G  T  Y  L  Y  N  D  C  E  G  P  G

        GCTGGACACGGACTGCAGAGAATGTGAAAATGGCACATTTACTGCTTCAGAGAACTACCT
    241 ---------+---------+---------+---------+---------+---------+
300
        CGACCTGTGCCTGACGTCTCTTACACTTTTACCGTGTAAATGACGAAGTCTCTTGATGGA
        yLeuAspThrAspCysArgGluCysGluAsnGlyThrPheThrAlaSerGluAsnTyrLe
         L  D  T  D  C  R  E  C  E  N  G  T  F  T  A  S  E  N  Y  L

        CAGACAGTGCCTTAGCTGCTCCAAATGCCGAAAAGAAATGTACCAGGTGGAGATATCTCC
    301 ---------+---------+---------+---------+---------+---------+
360
        GTCTGTCACGGAATCGACGAGGTTTACGGCTTTTCTTTACATGGTCCACCTCTATAGAGG
        uArgGlnCysLeuSerCysSerLysCysArgLysGluMetTyrGlnValGluIleSerPr
         R  Q  C  L  S  C  S  K  C  R  K  E  M  Y  Q  V  E  I  S  P

        TTGCACAGTGTACCGGGACACCGTGTGTGGCTGCAGGAAGAACCAGTACCGGTATTACTG
    361 ---------+---------+---------+---------+---------+---------+
420
        AACGTGTCACATGGCCCTGTGGCACACACCGACGTCCTTCTTGGTCATGGCCATAATGAC
        oCysThrValTyrArgAspThrValCysGlyCysArgLysAsnGlnTyrArgTyrTyrTr
         C  T  V  Y  R  D  T  V  C  G  C  R  K  N  Q  Y  R  Y  Y  W

        GAGTGAAACCCATTTCCAGTGCCTAAACTGCAGCCTCTGCCTCAACGGCACGGTGCAGAT
    421 ---------+---------+---------+---------+---------+---------+
480
        CTCACTTTGGGTAAAGGTCACGGATTTGACGTCGGAGACGGAGTTGCCGTGCCACGTCTA
        pSerGluThrHisPheGlnCysLeuAsnCysSerLeuCysLeuAsnGlyThrValGlnIl
         S  E  T  H  F  Q  C  L  N  C  S  L  C  L  N  G  T  V  Q  I

        CTCCTGCAAGGAGACACAGAATACTGTATGCACCTGCCACGCGGGGTTCTTTCTAAGAGG
    481 ---------+---------+---------+---------+---------+---------+
540
        GAGGACGTTCCTCTGTGTCTTATGACATACGTGGACGGTGCGCCCCAAGAAAGATTCTCC
        eSerCysLysGluThrGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGl
         S  C  K  E  T  Q  N  T  V  C  T  C  H  A  G  F  F  L  R  G

        GAATGAATGCGTCTCTTGTGTTAACTGTAAGAAAAACACAG
    541 ---------+---------+---------+---------+-  581
        CTTACTTACGCAGAGAACACAATTGACATTCTTTTTGTGTC
        yAsnGluCysValSerCysValAsnCysLysLysAsnThr
         N  E  C  V  S  C  V  N  C  K  K  N  T
```

Figure 4.10. Nucleotide sequence and predicted translation of the amplified region of feline cDNA homologous to part of human TNFR-2, GenBank Accession number U51429. The sequence is flanked by 80-3 (upstream) and 80-4 (downstream).

```
        CTCAGGCAGCACCGCAGACGGGAGGGCGCCCACCGGGACCCGGCTGCCCGCACCAGGCAC
      1 ---------+---------+---------+---------+---------+---------+
60
        GAGTCCGTCGTGGCGTCTGCCCTCCCGCGGGTGGCCCTGGGCCGACGGGCGTGGTCCGTG
         SerGlySerThrAlaAspGlyArgAlaProThrGlyThrArgLeuProAlaProGlyTh
         S  G  S  T  A  D  G  R  A  P  T  G  T  R  L  P  A  P  G  T

        GGAGAAGGCCGCCGGGTCTGGGGAGGCCTGGGCCAGCTCCAGCAGCTCAGAGCCTTCCTC
     61 ---------+---------+---------+---------+---------+---------+
120
        CCTCTTCCGGCGGCCCAGACCCCTCCGGACCCGGTCGAGGTCGTCGAGTCTCGGAAGGAG
        rGluLysAlaAlaGlySerGlyGluAlaTrpAlaSerSerSerSerGluProSerSe
         E  K  A  A  G  S  G  E  A  W  A  S  S  S  S  S  E  P  S  S

        TGGCAGCCACGGGACCCAGGTCAACGTCACATGCATCGTGAATGTGTGCAGCGCCTCCGA
    121 ---------+---------+---------+---------+---------+---------+
180
        ACCGTCGGTGCCCTGGGTCCAGTTGCAGTGTACGTAGCACTTACACACGTCGCGGAGGCT
        rGlySerHisGlyThrGlnValAsnValThrCysIleValAsnValCysSerAlaSerAs
         G  S  H  G  T  Q  V  N  V  T  C  I  V  N  V  C  S  A  S  D

        CCACGGCTCTCAGTGCGCCTCCCAGGCCAGCTACACGATGGGGGACGTGGATGCCAGCTC
    181 ---------+---------+---------+---------+---------+---------+
240
        GGTGCCGAGAGTCACGCGGAGGGTCCGGTCGATGTGCTACCCCCTGCACCTACGGTCGAG
        pHisGlySerGlnCysAlaSerGlnAlaSerTyrThrMetGlyAspValAspAlaSerSe
         H  G  S  Q  C  A  S  Q  A  S  Y  T  M  G  D  V  D  A  S  S

        CTCCGGC
    241 -------  247
        GAGGCCG
        rSerGly
         S  G
```

When amplified products were compared to the GenBank database using the FastA program, the products from PCR with the TNFR-1 based primers showed most homology to human, pig, cow, mouse and rat TNFR-1 sequences, whereas those from PCR with TNFR-2 based primers showed most homology to cow, mouse, human and rat TNFR-2 sequences, confirming that the PCR products were homologues of the appropriate human protein. Homology in nucleotide and derived amino acid sequences between the cat and other species for which the corresponding part of the receptor has been published is illustrated in figures 4.11. and 4.12. For the regions amplified, TNFR-1 shows more inter-species homology than TNFR-2.

4.6. Discussion

The work presented in this chapter illustrates that part of the coding sequences for feline TNFR-1 and -2 have been successfully amplified by PCR. Comparison of the amplified region of feline TNFR-1 with soluble human TNFR-1 confirmed that a protein homologous to that in man exists in the cat with conservation of the cysteine rich areas which identify the human protein as a member of the nerve growth factor receptor super-family. Three disulfide cysteine bridges have been described in each cysteine rich domain of the human receptor which help to form a relatively rigid elongated structure (Banner et al. 1993). Optimal TNF binding is achieved when three of these receptors interact with the TNF trimer, holding the transmembrane and intracellular regions of neighbouring receptors in close proximity (Hilton, 1994). Here, three of the four areas with 6 cysteine residues have been completely amplified in the cat, as has the majority of the fourth.

In man, TNF receptors are fairly ubiquitously expressed, having been detected on all nucleated cells examined for their presence. For this reason, the starting material was assumed not to be critical and stimulated splenic cells were selected, as they were readily available and TNFR-2 predominates on activated leucocytes. Although failure to produce the correct band in a PCR might be due to lack of expression in the starting tissue, this was confirmed not to be the case by using the successful primers as positive controls.

One of the problems encountered when performing RNA extractions is contamination with RNases which are naturally rich in splenic tissue. This was minimised by using RNase-free disposable equipment or treating with DEPC. Total

Figures 4.11.and 4.12. Homology of nucleotide (nt) and derived amino acid (aa) sequences of the amplified regions of feline TNFR-1 and TNFR-1 and the corresponding regions in the other species in which they have been published.

Table 4.1.

Species and Accession no.	TNFR-1 nt (%)	TNFR-1 aa[1] (%)
human X55313	85	83
mouse M59377	78	74
rat M63122	78	72
cow U90937	80	74
pig U19994	84[2]	79

Table 4.2.

Species and Accession no.	TNFR-2 nt (%)	TNFR-2 aa (%)
human M32315	77	67
mouse M59378	73	60
rat U55849	76[3]	46[4]
cow AF031589	78[5]	66

[1] aa sequence is compared for the coding region only

[2] the pig nt sequence starts 4 bases later than the amplified cat sequence

[3] amplified cat and rat sequences only overlap over 172 base pairs

[4] homology between cat and rat derived aa sequence was assessed over 28 aa residues (BestFit program)

[5] this region of the bovine sequence is 3 nts (1aa) shorter than the cat sequence

Figure 4.13. Aligned predicted amino acid sequences for much of the extracellular domains of feline TNFR1 and the complete extracellular region of the human receptor. Residues where the feline sequence differs from the human are indicated. The four conserved cysteine rich repeat areas are under- or over- lined, the cysteine residues shown in red and the putative site for cleavage of the human soluble receptor shown in green type. (~ represent areas of the extracellular domain of feline TNFR1 not amplified and sequenced.)

```
          1                                                  50
feline    ---P---G-- Q-----A--- E---LR-T-- ----R----- AIP-------
human     MGLSTVPDLL LPLVLLELLV GIYPSGVIGL VPHLGDREKR DSVCPQGKYI

          51                                                 100
feline    ---D------ -------------- --E---L--- -----N-T-- ----Y--Q--
human     HPQNNSICCT KCHKGTYLYN DCPGPGQDTD CRECESGSFT ASENHLRHCL

          101                                                150
feline    ----------Y -----P---Y ----------- ---Y----TH ---L------
human     SCSKCRKEMG QVEISSCTVD RDTVCGCRKN QYRHYWSENL FQCFNCSLCL

          151                                                200
feline    ----QI--K- T---------- -----G---- --V----NT~ ~~~~~~~~~~
human     NGTVHLSCQE KQNTVCTCHA GFFLRENECV SCSNCKKSLE CTKLCLPQIE

                              201         211
                  feline      ~~~~~~~~~~ ~
                  human       NVKGTEDSGT T
```

RNA was extracted in preference to messenger RNA because of the ease of the technique. The A_{260}:A_{280} of total RNA after extraction should generally be between 1.8 and 2. Lower ratios make the quantification of RNA more difficult and suggest contamination with glyco-polysaccharides, or an RNA pellet that is not totally soluble. High ratios suggest that the RNA has become degraded.

Although RNA extraction was straightforward, DNA contamination was regularly detected despite exercising great care when removing the aqueous phase containing the RNA from the interphase and organic phase which contain DNA and protein. This is a recognised problem with RNA extraction protocols (Bauer et al. 1997) and is best tested for by performing a reverse transcription reaction without enzyme. Any product after subsequent PCR must be due to genomic DNA contamination of RNA which can be removed by treatment with DNase I. DNA contamination is a serious problem particularly when assessing selective expression or quantifying expression levels. In this work, samples with contamination were discarded to prevent genomic DNA interfering with PCR amplification of cDNA.

Despite designing multiple primers for both receptors, attempts to amplify either of the complete cDNA transcripts were disappointing. After initially trying to obtain each transcript in a single PCR, internal primers were designed to amplify shorter, overlapping stretches of each receptor. Primers were unlikely to have been exactly complementary to the feline template but any discrepancies between the available sequences were not permitted towards the 3' end of designed primers. In addition, complementarity of the 3' prime ends was avoided both *inter* and *intra* individual primers to reduce the formation of 'primer-dimers'.

While some of these internal primers were successful, many were not, although it is not completely clear why not. Several explanations were considered. Because there was complete homology between human and murine sequences for primer 80-5, it was assumed that this was likely to apply to the cat sequence, but this may not be true and lack of homology may have been a causative factor in primer failure. In some cases, inequality of each of the bases in a primer may help to explain the problems encountered. For instance, primer 60-2 was fairly homologous throughout the species but had a G/C content of 70%. For the product derived from amplification with the 80-5 and 80-6 primer pair, sequencing showed that 80-6 acted as both forward and reverse primers.

Although not the ideal positive control because the primers were based upon human sequences, murine cells were readily available and murine cDNA was useful for testing new primer pairs. Altering the reaction conditions through magnesium ion concentration or the addition of DMSO, formamide or glucose did not improve the performance of primers that failed to amplify the correct product.

By the end of this project, TNFR-1 sequences from the pig and cow and TNFR-2 sequences from the rat and cow had been released onto the database. Alignment of all known sequences may identify further conserved regions for which primers can be designed. Introducing degeneracy into these primers to cover base differences across the species might be a way of amplifying more of the sequences. Alternatively, because partial sequence information is now available for the cat, a rapid amplification of cDNA ends (RACE) protocol could be employed (Frohman et al. 1988) or a feline cDNA library screened with a feline-specific probe to elucidate the remainder. RACE is a PCR-based technique in which gene-specific primers are designed on the sequence data available and used in combination with primers that anneal to oligonucleotides ligated to each end of the cDNA transcript (Fig 4.14.). The products of PCR can then be sequenced by a standard method.

Three clones and 2 PCR products for each transcript were sequenced in an attempt to filter out any PCR mis-incorporations or sequencing errors that may have arisen. Whereas degeneracy of the genetic code caused by wobble at the third position of the triplet codon suggests that amino acid sequence might be more conserved between species than nucleotide sequence, it can be noted that the opposite is true in the sequences presented here with less homology at the amino acid level. This has also been described for feline IL-2 when compared with human, bovine, rat and murine IL-2 but is not true for feline TNF which is 91% homologous to human TNF at the nucleotide level and 92% homologous at the amino acid level (comparison using BestFit program of sequences with Accession nos. M92061 and M10988). The high level of homology found between feline and human TNFR sequences, particularly TNFR-1, suggests that these proteins are likely to have a similar function in the cat to that in the human.

In this study, the cDNAs encoding the extracellular regions of the receptor were of particular interest because it is these portions which are released from cells and exist as soluble proteins. This region of feline TNFR-1 has been amplified

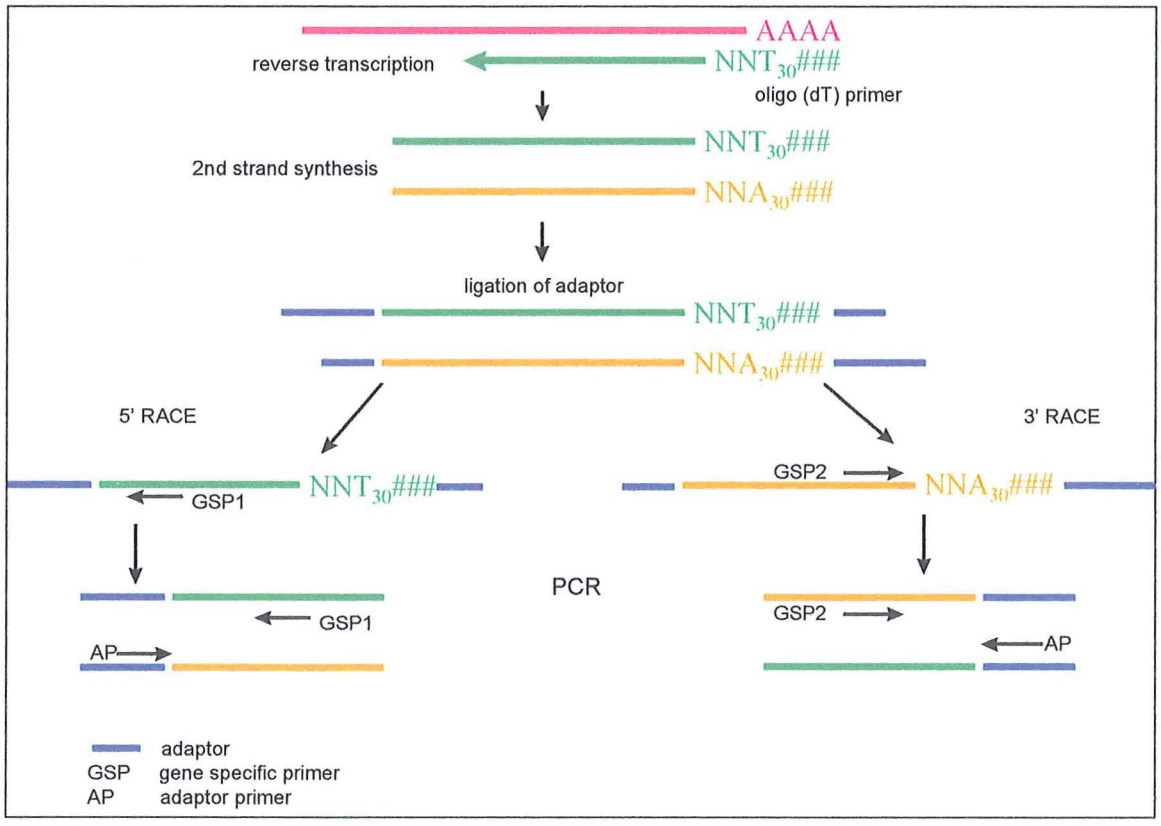
successfully and the information gained can be used to generate antibody for the development of a species-specific assay to allow quantification of the soluble receptor in clinical samples. Antibody production may be undertaken in a number of ways including DNA vaccination and expression of recombinant protein or peptide synthesis for inoculation into a foreign host.

In contrast, the only part of feline TNFR-2 that was amplified is located within the intracellular region so further sequence has to be elucidated before work to develop an assay for this soluble receptor can progress. It is desirable to continue this work because the value of human soluble TNFR-2 as a marker of disease progression in HIV is clear (Aukrust et al. 1996) and the situation is likely to be similar in FIV.

4.6. Conclusion

The majority of the extracellular region of feline TNFR-1 was successfully amplified by PCR, sequenced and cloned. This success will allow work to progress on the development of an assay for the protein, feline soluble TNFR-1. The ultimate objective is to be able to monitor levels of the protein in serum samples from cats infected with FIV. Although part of feline TNFR-2 has also been amplified, sequenced and cloned, this is situated in the intracellular region and further work is required to elucidate extracellular sequence before progression in the development of an assay for this receptor can be made.

Figure 4.14. Diagrammatic representation of RACE



Chapter 5:
Expression of Feline Soluble TNFR-1

5.1. Summary of Chapter

This chapter presents work designed to express, as a recombinant protein, feline soluble tumour necrosis factor receptor type 1 (sTNFR-1). Complementary DNA encoding feline sTNFR-1 as determined in chapter 4 was ligated into two different bacterial expression vectors (pGEX 4T-3, Pharmacia Biotech and pET33b(+), Novagen). Although both systems gave disappointing results when synthesising recombinant protein, the system expressing the receptor as a fusion with glutathione-S-transferase yielded sufficient protein for antibody production in a sheep.

5.2. Introduction to Protein Expression

Previous work using Western blotting failed to detect any cross-reactivity between commercially available anti-human TNFR-1 antibody and feline samples (see section 3.3). Consequently, species-specific antibody was required for the development of an immunological assay for feline TNFR-1. To this end, expression of feline sTNFR-1 was undertaken to provide recombinant protein to immunise a sheep for polyclonal antibody production.

Cell-free and cell-associated systems are available for protein expression. The technique of cell-free protein synthesis has the advantage of producing protein with a minimum of background from translation of endogenous RNA and can be very rapid, but is limited by the quantity of protein that it can produce. The *in vivo* expression of cloned gene products is an important method of protein production on a larger scale for use in research. It is used in the study of protein structure and function, protein-protein and protein-nucleic acid interactions, antibody production and mutagenesis.

Both eukaryotic and prokaryotic *in vivo* expression systems can be used to provide protein for subsequent antibody production. Bacterial expression is generally the most straightforward and least costly method and can give high yields with closely regulated expression (Goeddel, 1990). Although eukaryotic systems can also give high yields and are more likely to allow the post-translational modifications

necessary to make the protein functional, this is generally not required for antibody production. One of the problems associated with prokaryotic systems is the tendency to over-express protein, resulting in it being stored in insoluble inclusion bodies which hampers purification. However, since bacterial expression is generally straightforward, it remains a very attractive technique when the initial goal is antibody production.

In studies to examine the functional properties of recombinant human (Schall et al. 1990), murine (Lewis et al. 1991) or rat (Himmler et al. 1990) TNFR-1, expression was performed in either mammalian or insect (Moosmayer et al. 1994) cell systems. However, commercially available assays for human and murine TNFR-1 utilise antibody raised against recombinant protein expressed in *E coli* (R&D Systems, Minneapolis, USA) and recombinant feline TNF produced in a bacterial expression system (FLAG, International Biotechnologies Inc) was used successfully to inoculate chickens for subsequent antibody production (Otto et al. 1997).

5.2.1. Bacterial Expression Systems

5.2.1.1. Glutathione-S-transferase fusion proteins

The glutathione S-transferase (GST) gene fusion system (pGEX vectors, Pharmacia Biotech) has been designed to allow protein expression where the recombinant protein is fused to GST from the parasite *Schistosoma japonicum* (Smith, 1993). Such fusion proteins can be rapidly purified by chromatography because of the high affinity that GST has for glutathione (Smith et al. 1986) and are also easily detected by exploiting the biochemical or immunological reactions of GST which is important when expressing a novel protein for which no other reagent exists. GST-protein fusion systems have been used successfully in molecular immunology (Toye et al. 1990), vaccine production (Johnson et al. 1989) and in the study of DNA-protein (Chittenden et al. 1991) and protein-protein (Kaelin et al. 1992) interactions. The pGEX system has also been used to express the cytokine, feline stem cell factor (Dunham, 1997) as a functional protein.

The pGEX vectors carry the *tac* promoter which is induced by the lactose analogue isopropyl β-$_D$-thiogalactopyranoside (IPTG), and allows transcription to be readily regulated. The *tac* promoter is a hybrid of two promoters, *lac* and *trp*, and is more efficient than either parent. It is controlled by the *lac* repressor which is readily

de-repressed by IPTG, resulting in high level expression (Wong, 1996). Expressed proteins often, though not always (Frangioni and Neel, 1993), remain soluble and do not require to be denatured during their purification (Smith and Johnson, 1988) so are more likely to retain their antigenicity (Smith and Corcoran, 1997). The GST moiety generally does not interfere with the antigenicity of the expressed protein (Toye et al. 1990).

5.2.1.2. His-tag fusion proteins

The pET system (Promega) is a powerful method of producing recombinant proteins in which expression is under transcriptional control of a bacteriophage T7 promoter (Studier and Moffatt, 1986). Vector transcription remains silent until expression of a chromosomal copy of T7 RNA polymerase is induced by IPTG activation of the *lac*-controlled T7 RNA polymerase gene promoter (Rosenberg et al. 1987). Cells carrying the pLysS plasmid inhibit background expression prior to induction because they express low levels of T7 lysozyme which is a natural inhibitor of T7 RNA polymerase (Novagen, 1995). Upon induction, the overwhelming production of T7 RNA polymerase counteracts the effects of lysozyme. This stringent regulation of expression ensures that toxic inserts can be established in the BL21(DE3)pLysS host and still be expressed to high levels (Promega, 1996).

The pET33b(+) vector encodes both N- and C- terminal histidine-tag sequences allowing easy purification of expressed protein by metal chelation chromatography. The vector also carries a kanamycin rather than an ampicillin resistance gene to allow selection. Ampicillin selection can be lost in cultures as the drug is degraded by secreted β-lactamase or by a reduction in pH as a result of bacterial fermentation.

5.3. Aim of Experimental Studies

When the sequence for the extracellular portion of feline TNFR-1 had been elucidated (chapter 4), the goal became to raise antibody against it to allow the development of an immunological assay for the protein. To this end, the aim of the work described in this chapter was to use the available sequence to express the protein in a bacterial expression system, and subsequently to inoculate sheep with

purified expressed fusion protein to generate antiserum for use as a reagent in the assay.

5.4. Materials and Methods

5.4.1. Glutathione-S-transferase fusion protein

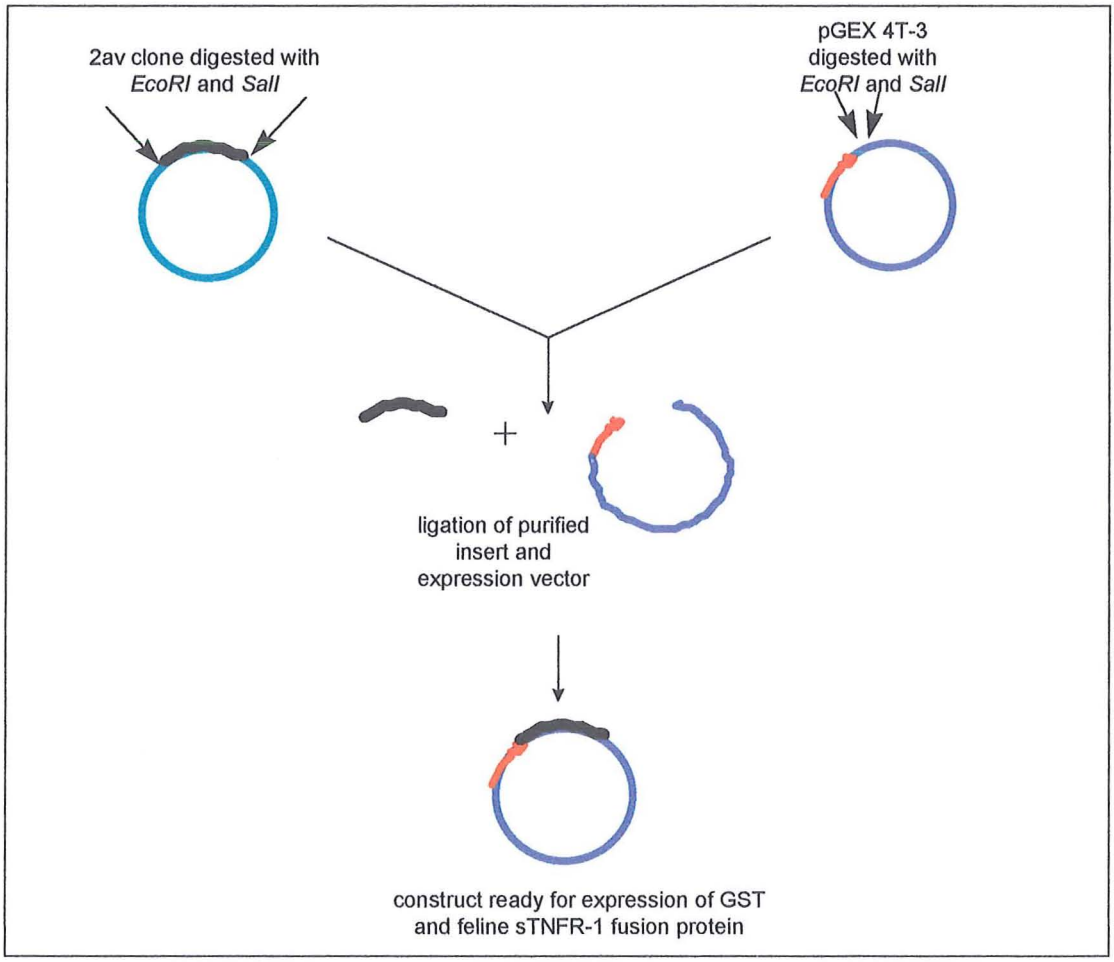
Construction of the plasmid and insert for expression is illustrated in figure 5.1.

5.4.1.1. Preparation of insert DNA

A glycerol stock (designated clone 2av) of the pT7Blue T-Vector (Novagen, Wisconsin) containing the PCR product obtained after amplification with primers 60-1 and 60-4 as an insert (see section 4.4.8.) was used to inoculate 5ml LB broth containing 50μg/ml of ampicillin (LB-amp, Appendix 1). The culture was grown overnight at 37°C in an orbital shaker at 200rpm. At the end of incubation, the cells were collected by centrifugation at 1800rpm for 10 minutes and plasmid DNA purified using a commercial kit (Qiagen). Purified DNA was quantified (GeneQuant, Pharmacia Biotech) and sequenced bi-directionally with T7 promoter (5' taatacgactcactatagg) and U19 (5' gttttcccagtcacgacgt) primers (DNA Sequencing Service, Kings College, London) to detect orientation of the insert and ensure an open reading frame throughout.

Ten μg of plasmid DNA were digested for 1 hour at 37°C in a total reaction volume of 100μl with a final concentration of 50mM Tris-HCl (pH8), 10mM $MgCl_2$ and 100mM NaCl and with 50 units each of the restriction enzymes *EcoRI* and *SalI* (GibcoBRL, Paisley, Scotland). Digestion products were electrophoresed on a 0.8% agarose gel at 100V for 1 hour and the 680bp insert band was excised. DNA was electro-eluted from the gel in dialysis tubing (Cellulose membrane, Sigma) for 10mins at 150V and precipitated in 0.1M sodium acetate and 50% ethanol. After storage at -20°C overnight, DNA was collected by centrifugation and washed with 70% ethanol prior to drying. Lyophilised DNA was dissolved in 20μl dH_2O.

Figure 5.1. Diagrammatic representation of cloning procedure. DNA insert encoding feline sTNFR-1 is shown in black, whereas that encoding GST is shown in red. The pT7 and pGEX 4T-3 vectors are coloured green and blue respectively. Selection of the appropriate vector for expression ensures the insert is ligated in-frame. The construct was used initially to transform JM109 cells.



5.4.1.2. Preparation of pGEX vector

Two μg of pGEX 4T-3 plasmid DNA (Pharmacia Biotech) were digested for 1 hour at 37°C in a total reaction volume of 20μl with a final concentration of 50mM Tris-HCl (pH8), 10mM $MgCl_2$ and 100mM NaCl and with 5 units each of the restriction enzymes *EcoRI* and *SalI*. The products of digestion were electrophoresed and linear plasmid DNA was excised and purified as above (section 5.4.1.1.).

5.4.1.3. Ligation of insert to pGEX plasmid

Linearized pGEX DNA and insert DNA were ligated at a molar ratio of 1:5 in a total reaction volume of 20μl with 1mM ATP, 10mM Tris acetate, 10mM magnesium acetate, 50mM potassium acetate and 10 units of T4 DNA ligase (all reagents from Pharmacia Biotech). Ligation was allowed to proceed for 2 hours at room temperature.

5.4.1.4. Preparation of competent cells

Both JM109 (Promega) and BL21 (Pharmacia Biotech) strains of *E. coli* were purchased; the former for maintenance of the plasmid and the latter for expression. To make the cells competent, they were grown in 5ml cultures of LB broth without antibiotic and incubated overnight in an orbital shaker at 200rpm. The following morning a 1:50 dilution of the overnight culture was prepared in broth and growth was continued until the A_{650} reached 0.6 (Beckman spectrophotometer). At this time, the cells were collected by centrifugation at 2000rpm for 10 minutes at room temperature and re-suspended in a half volume of ice-cold 0.1M $CaCl_2$. After incubation on ice with occasional shaking for 20 minutes, the cells were centrifuged as before and suspended in a tenth of the former volume of ice-cold 0.1M $CaCl_2$. If to be used immediately, competent cells were kept on ice until required. Alternatively, an equal volume of sterile 50% glycerol was added and the cells were stored in aliquots at -70°C.

5.4.1.5. Transformation reaction

Ten μl of the ligation mix were added to 500μl of competent JM109 cells and kept on ice for 30 minutes. Cells were heated to 42°C for 2 minutes and quenched on ice

for a further 2 minutes. Five hundred µl of SOC medium (Appendix 1) were added and the tubes were incubated in an orbital shaker for 1 hour at 37°C. At the end of this time, the cells were collected by centrifugation for 10 minutes at 1800rpm and re-suspended in 0.5ml LB-amp broth before being plated onto LB-amp agarose plates and incubated overnight at 37°C. Negative control plates with cells transformed with undigested and digested vector with no insert were also prepared.

5.4.1.6. Recovery of plasmid DNA

Single colonies were picked off and used to inoculate 3ml aliquots of LB-amp broth. These cultures were incubated at 37°C overnight. The following morning 15% glycerol stocks were made of each culture and plasmid DNA was recovered using a standard miniprep (Qiagen, UK). To check for the presence of an insert, 5µl of each colony was digested with *EcoRI* and *SalI* in a total reaction volume of 20µl as described in section 5.4.1.2. Glycerol stocks were stored at -80°C.

5.4.1.7. Transformation of BL21 cells

Cells were transformed with plasmids with and without the TNFR-1 insert. Aliquots of 200µl of BL21 cells were transformed as described in section 5.4.1.5. with 0.5µl of plasmid DNA as prepared in section 5.4.1.6. Transformed cells were plated onto agarose, colonies grown in LB broth with ampicillin and glycerol stocks made as described previously. Again, integrity of an open reading frame was confirmed by sequence analysis using the primers 5'pGEX Sequencing Primer (869 5'gggctggcaagccacgtttggtg 3') and 3' pGEX Sequencing Primer (1040 5'ccgggagctgcatgtgtcagagg 3') (Pharmacia Biotech).

5.4.1.8. Screening for protein expression

Glycerol stocks were used to inoculate 10mls of LB-amp broth and the cultures were incubated overnight at 37°C with vigorous agitation. The following morning, cultures were diluted 1:3 and grown under the same conditions until reaching an A_{600} of 0.6-0.8. At this time, IPTG was added to a final concentration of 0.2mM, and the incubation continued for a further 2 hours. Duplicate cultures were maintained with no added IPTG. Different incubation times (1, 2, 4 and 6 hours), IPTG

concentrations (0.1, 0.2, 0.5 mM) and incubation temperatures (37°C and room temperature) were used and the effect on expression levels was monitored.

Post-induction, 1ml of each culture was collected, the bacteria were pelletted by centrifugation and the pellet was re-suspended in 167µl phosphate buffered saline and 33µl treatment buffer (0.06M Tris-HCl, 1.7%SDS, 6% glycerol, 0.1M dithiothreitol, 0.002% bromophenol blue). Each sample was boiled for 5 minutes before analysing by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (see section 5.2.1.9.).

5.4.1.9. SDS-PAGE

SDS-PAGE was performed using a mini-gel system (BioRad) with 12.5% polyacrylamide gels (Laemmli, 1970) (Appendix 2). Ten microlitres of each sample were loaded into wells in the gels and electrophoresis carried out in tank buffer (25mM Tris, 192 mM glycine, 0.1% w/v SDS, pH 8.3) at 20mA per gel until the dye front reached the bottom. The gels were stained overnight with 0.025% Coomassie Brilliant Blue R-250 in 40% methanol and 7% acetic acid and de-stained in the same solvents. Molecular weight standards were run simultaneously (Wide Range Color Markers, Sigma, St Louis, USA).

Stained gels were photographed using a digital imaging system (Cohu high performance charge coupled device (CCD) camera connected to a Sony monitor (SSM-930CE) and video graphic printer (UP 890CE)) and recorded onto printing paper (Sony Superior Density, UPP 110HA) or dried between cellulose sheets (Pharmacia Biotech) in a warm air drier (Hoeffer). Gels used for Western blotting were left unstained.

5.4.1.10. Western blotting

Proteins were transferred from polyacrylamide gels to nitrocellulose membrane in Towbin transfer buffer (25mM Tris, 192mM glycine, 20% methanol and 0.1%SDS) using a Trans-Blot Cell (Bio-Rad) with cold water cooling at 200mA for 2 hours. After transfer, nitrocellulose membranes were blocked overnight by incubating in 5% (w/v) dried skimmed milk powder in 50mM Tris-HCl buffered saline (TBS), pH7.4. The blocked membrane was incubated with goat anti-GST antibody (Pharmacia Biotech) diluted 1:100 in TBS with 1% dried skimmed

milk powder and 0.05% Tween-20 (TBS-Tween-milk) for 1 hour at room temperature and washed 3 times for 10 minutes in TBS-Tween-milk.

Donkey anti sheep/goat IgG conjugated to horseradish peroxidase (Scottish Antibody Production Unit, Law Hospital) was used as the second antibody at a concentration of 1:1000 in TBS-Tween-Milk, and the membrane was incubated in this buffer for a further hour. After 3 washes carried out as before, the membrane was stained with 3,3'-diaminobenzidine (Sigma Fast DAB tablets, Sigma) before washing with dH_2O.

5.4.1.11. Large scale protein expression

Large scale protein expression in 5 litres of culture medium was performed three times. Two litres of LB-amp broth were inoculated with 100µl glycerol stock and incubated overnight as 500ml aliquots in 2 litre flasks. The following morning the aliquots were diluted 1:3 and induced with IPTG when an A_{600} of 0.6-0.8 was reached in a similar manner to smaller volumes. After induction, the culture was transferred to 500ml centrifuge containers and centrifuged at 7700 x g for 10 minutes at 4°C (Beckman JA10 rotor in a centrifuge). Bacterial pellets were re-suspended in 0.01 volume of ice-cold PBS containing protease inhibitors (Protease Inhibitor Cocktail, Sigma) and the cells disrupted by sonication for three bursts of 30 seconds. To assist solubilization of the fusion protein, 20% Triton X-100 was added to the sonicate to a final concentration of 1% before mixing gently for 30 minutes. At the end of this time, the supernatant was collected after centrifugation at 12000 x g for 10 minutes at 4°C (Beckman JA-20 rotor).

Aliquots of the pre-sonicate suspension and the post-sonicate solution and cell debris pellet were taken for analysis by SDS-PAGE.

5.4.1.12. Purification of expressed proteins

Expressed proteins were purified by binding to glutathione sepharose slurry prepared according to the accompanying protocol (Pharmacia Biotech). For 5 litre cultures, the sonicate was allowed to flow through a column of 2mls (bed volume) of glutathione sepharose. Bound protein was washed with 10 x bed volumes of PBS and then eluted by three additions of 2mls of Elution Buffer (10mM glutathione, Pharmacia).

5.4.1.13. Thrombin cleavage

Thrombin cleavage was performed by adding 10µl of thrombin solution (10 cleavage units, Pharmacia Biotech) per mg of fusion protein in the eluant and the mixture left at room temperature overnight. The following morning the reaction was dialysed against 2000 volumes of PBS with stirring for 2 hours and then purified on a glutathione column as in section 5.4.1.12. Aliquots of eluant immediately after addition of thrombin and prior to dialysis were saved for analysis along with the products of purification.

5.4.1.14. Quantification and preparation of expressed proteins

Purified expressed protein was analysed by SDS-PAGE and quantified by scanning densitometry of stained gels at 600nm (Chromoscan, Joyce-Loebel) and by assay with Bradford reagent (Sigma) for total protein. The eluants from the three 5 litre cultures were pooled and concentrated in a stirred cell concentrator (Amicon 8050 mini-system) with a 43mm PM10 membrane pre-soaked in dH_2O. Concentrated protein was electrophoresed on an SDS-PAGE gel, stained with Coomassie blue and the appropriate band was cut out and macerated with a scalpel. Four aliquots of 100µg of protein contained in the gel were used by the Scottish Antibody Production Unit (SAPU, Law Hospital, Lanarkshire) for injection into a sheep at 4 four-weekly intervals.

5.4.1.15. Analysis of antiserum

Pre-immune serum and antiserum taken after the second, third and fourth injections of antigen were returned from SAPU and examined by western blotting as described above. Prior to use in the blot, any cross-reaction with *E. coli* proteins had to be removed from the antiserum by absorption. For this, *E. coli* cells containing vector without an insert were cultured, re-suspended in PBS and then ruptured by sonication. Antiserum was added to this suspension at a ratio of 1ml antiserum to 9ml culture and the mixture was left at 4°C overnight. Cell debris was removed by centrifugation and sodium azide was added to a final concentration of 10mM. Absorbed antiserum was diluted 1:5 in TBS-T-M for use as the initial antibody in a western blot.

5.4.2. Expression in pET33b(+)

The vector pET33b(+) (Novagen, US) was also used to express feline TNFR-1. In a similar manner to that described for pGEX, both pET33b and the 2av clone were digested with *EcoRI* and *SalI* and the purified insert was ligated into the expression vector. The vector was maintained in pMOS blue cells (Amersham,) and expressed in BL21 DE3 pLys(S) (Promega) cells. Aliquots of 20mls of LB broth with kanamycin and chloramphenicol were inoculated with culture which was induced with 1mM IPTG for 1, 2, 3 and 4 hours when the A_{600} reached 0.6. Analysis of cell lysates was performed on SDS-PAGE gels as in section 5.4.1.9.

5.5. Results

5.5.1. Glutathione-S-transferase system

5.5.1.1. Preparation of plasmid with insert

Digestion of plasmid DNA from clone 2av and pGEX 4T-3 plasmid with *EcoRI* and *SalI* produced a 680 base pair insert and linear plasmid (figure 5.2.) with cohesive ends. Insert and plasmid were readily ligated and the ligation reaction successfully transformed *E coli* cells. Positive clones were identified by the presence of an insert after re-digestion (figure 5.3.). The 4T-3 plasmid allowed the insert to be ligated in-frame and this was confirmed by sequence analysis. Numerous colonies grew after transformation of JM109 cells with vector ligated to insert. When digested vector alone was used in the transformation reaction, 3 colonies grew. It is likely that these 3 colonies arose from re-ligated vector after digestion with only one enzyme.

5.5.1.2. Protein expression

Analysis of the bacterial lysates prepared by boiling showed induction of GST in the colonies that contained vector alone, but no evidence of fusion protein expression in colonies containing vector with the insert. Purification identified a weak band of about 50kDa which was produced by the colonies containing vector with an insert but not in the colonies containing vector alone (figures 5.4. and 5.5., column 2). Confirmation that this band represented the fusion protein was obtained by its disappearance after thrombin cleavage (figure 5.5.) and also by its reaction with anti-GST antibody in a western blot (figure 5.6.).

Figure 5.2. Agarose gel following electrophoresis showing pGEX 4T-3 and plasmid DNA from clone 2av after digestion with *EcoRI* and *SalI*. Lane 1, 1kB marker; lane 2, 680bp insert from 2av DNA; lane 3, linear 4T-3.

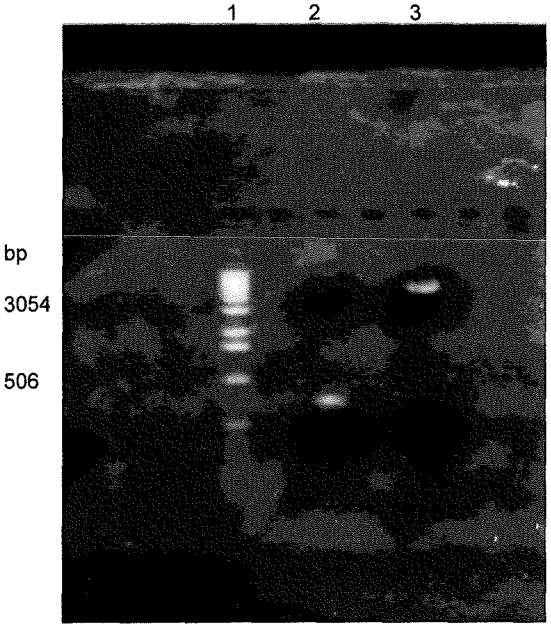


Figure 5.3. Negative image of an agarose gel showing successful ligation of 4T-3 and insert. Plasmid DNA from transformed BL21 cells has been digested with *EcoRI* and *SalI* and the presence of an insert demonstrated by electrophoresis. Lane 1, 1kB marker; lanes 2-5, 680bp insert from different clones after digestion of 4T-3; lane 6 negative control sample of vector without insert that has been digested in a similar manner. The upper arrow shows digested plasmid and the lower arrow shows insert.

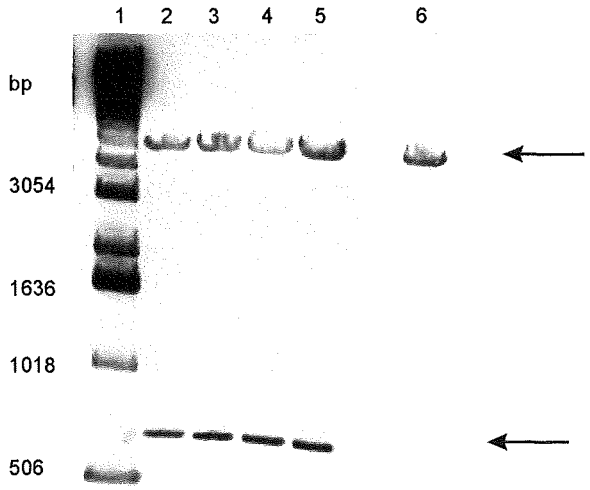


Figure 5.4. SDS-PAGE gel stained with Coomassie blue illustrating bacterial expression of GST. Lane 1 molecular weight marker; lane 2, cell lysate from a colony of cells containing pGEX 4T-3 plasmid without an insert; lane 3, purified lysate used in lane 2. The arrow indicates the GST protein band (29kDa).

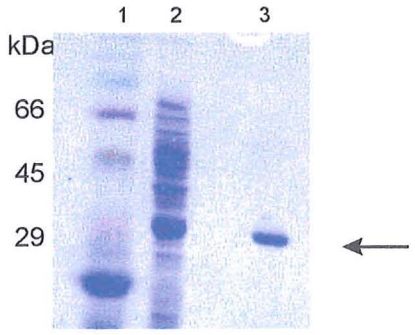


Figure 5.5. SDS-PAGE gel stained with Coomassie blue illustrating loss of fusion protein band after thrombin cleavage. Lane 1, molecular weight marker; lane 2, purified cell lysate from a colony of cells containing plasmid with feline TNFR-1 insert; lane 3, purified lysate from lane 2 after thrombin cleavage; lane 4, fusion protein cleaved with thrombin after GST had been removed by purification on a glutathione column. The arrow indicates the band that is present in colonies containing vector with an insert but not in colonies containing vector only that disappears after thrombin cleavage.

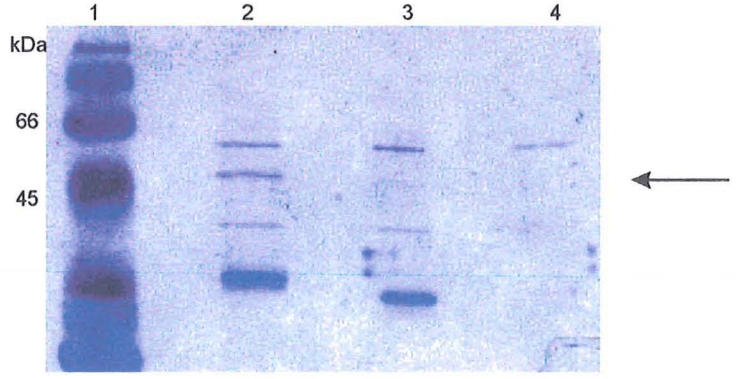
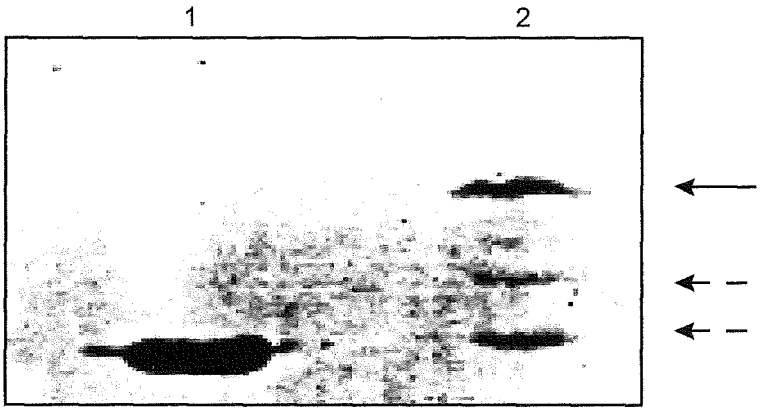


Figure 5.6. Western blot using anti-GST antibody illustrating breakdown of fusion protein. Lane 1, purified GST from 4T-3 without an insert; lane 2, purified fusion protein from pGEX 4T-3 with feline TNFR-1 insert. The upper band (solid arrow) is around 50kDa and represents intact protein whereas the smaller bands (broken arrows) reflect protein cleavage.



Quantification of the fusion protein after electrophoresis using a scanning densitometer by comparison with dilutions of albumin (figure 5.7.) showed that 400μg had been produced from the three 5 litre cultures and, after separating from contaminating *E coli* proteins on a polyacrylamide gel (fig 5.8.). This material was divided into 4 equal aliquots and sent to SAPU for antibody production.

Alterations in time or temperature of incubation, or in the concentration of IPTG did not improve the production of fusion protein. Analysis of the post-sonicate pellet and glutathione column washes showed that expressed protein was not lost at either of these stages.

5.5.1.3. Analysis of antiserum

Pre-immune sera from the inoculated sheep showed no cross reaction with recombinant protein when examined by western blotting. Analysis of antiserum returned after the fourth inoculation with antigen showed binding to the expressed protein from a colony containing the vector with insert but not one containing vector alone (fig 5.9.). Two bands stained on the western blot at 25kDa, both about half the size of the expected 50kDa and staining was weak. Expressed protein from colonies containing vector with or without insert reacted with anti-GST antibody, although again the bands in both were around the same size (fig 5.9.).

The antiserum did not react with any proteins in the region of the size of the protein band excised for inoculation and the reaction between the antiserum and the smaller band could not be reproduced consistently.

5.5.2. Expression in the pET33b system

The pMOS blue cells used for maintenance were readily transformed with the ligation reaction and plasmid prepared from these cultures successfully transformed the BL21 cells as confirmed by plasmid digestion and sequence analysis. However, there was no evidence of expression of protein by the pET33b vector when induced cell lysates were analysed by SDS-PAGE.

Figure 5.7. SDS-PAGE gel stained with Coomassie blue used to quantify fusion proteins. Lane 1 molecular weight marker; lanes 2 and 3, sample of fusion protein after purification from two 5 litre cultures, with the arrow indicating the band of interest; lanes 4-8, 10, 25, 50, 75 and 100μg/ml samples of bovine serum albumin respectively.

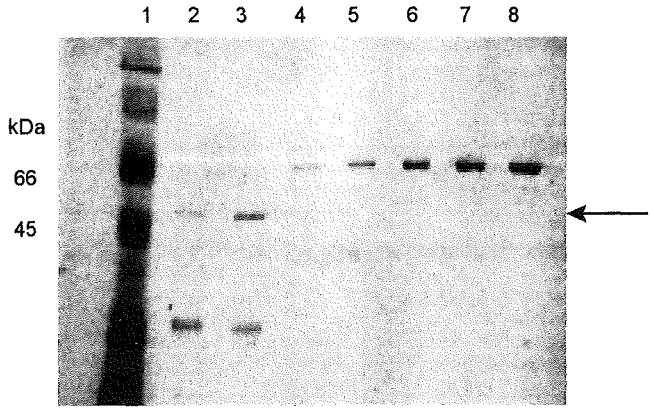


Figure 5.8. SDS-PAGE gel stained with Coomassie blue illustrating the band excised for inoculation of a sheep. The molecular weight marker can be seen on the left and concentrated purified fusion protein has been run in the other lanes. The arrow shows where the band identified by Western blotting and thrombin cleavage has been excised from the gel.

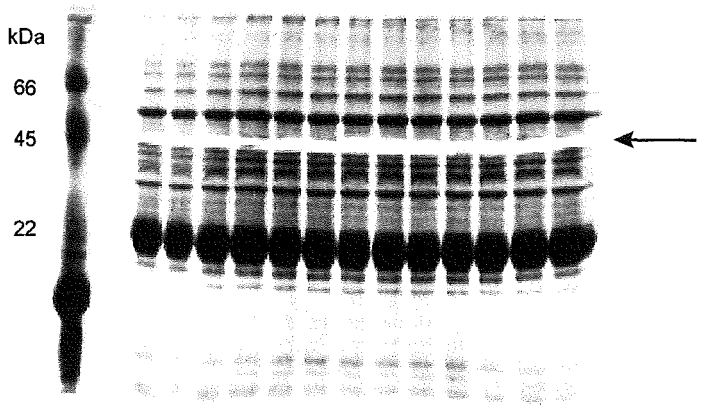
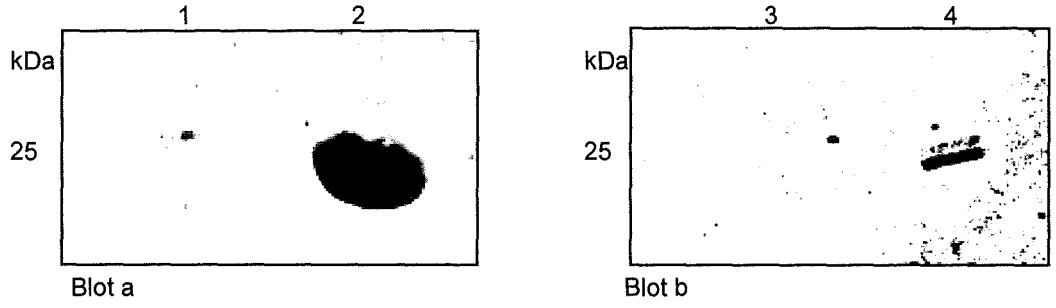


Figure 5.9. Western blots using anti-GST antibody (blot a) and antiserum taken from the sheep after the final inoculation and absorbed with *E coli* proteins (blot b). Lanes 1 and 4 contain purified fusion protein and lanes 2 and 3 contain purified expressed GST. Protein of around 25kDa in lanes 1 and 4 has reacted with both anti-GST antibody and the antiserum suggesting breakdown of the fusion protein whereas GST alone only reacts with specific anti-GST antibody.



5.6. Discussion

The work presented in this chapter describes the cloning and expression of the amplified region of feline TNFR-1 described in chapter 4. Expression in *E. coli* as a GST fusion protein produced only sufficient protein for the inoculation of one sheep. Although the vector for expression was readily ligated to the feline TNFR-1 sequence, the amount of fusion protein recovered after purification was disappointing. Only 400μg of protein were obtained from 15 litres of culture, considerably less than the 1-10 mg/litre suggested by other reports of use of the pGEX gene system (Dunham, 1997). Although only a small amount was obtained, this protein was used to inoculate a sheep which provided sufficient antiserum for use in the development of an assay for the protein in clinical samples. To eliminate the possibility of an idiosyncratic response in a single animal, it may have been preferable to use the small amount of protein to inoculate two rabbits (Harlow and Lane, 1988b) and accept the accompanying reduction in antiserum produced.

Small yields from expression systems may be a result of either poor protein expression or loss of protein during purification. It is suggested that a pre-purification yield may be in the order of 10-100μg of fusion protein per ml of culture (Frangioni and Neel, 1993), although 60-70% of protein may be lost at the elution step. With larger proteins the saturation binding level of glutathione agarose is much reduced so that proteins greater than 100kDa are extremely poorly bound. The fusion protein expressed here was only 50kDa, so should be retained by the column. SDS-PAGE analysis of post-sonicate supernatant and column fractions did not detect significant amounts of fusion protein.

The lack of protein expression was unlikely to be due to the protein being insoluble and in inclusion bodies since it could be detected in neither cell lysate nor purified fractions. Analysis of pre-sonicate and post-sonicate cell debris, the wash phases and glutathione column post-elution did not reveal a stage at which the protein might have been lost during these procedures. Attempting to improve protein recovery by selecting a smaller portion of feline receptor for expression, perhaps omitting the hydrophobic or highly charged regions might have been a useful alternative strategy if solubility had been a problem. This approach was not attempted

because there was no evidence of expressed protein in cell lysate fractions when analysed by SDS-PAGE.

It has been reported that fusion protein expression may be toxic to cells (Otto et al. 1995). If this were true, and β-lactamase production by cells carrying the plasmid depleted ampicillin in the culture, then antibiotic selection would be disrupted resulting in overgrowth by cells from which plasmid had been lost. This could explain the continued turbidity of the culture concurrent with the lack of protein expression. It may have been possible to test for this by performing a plasmid stability test in which log phase culture of transformed cells are plated onto plates containing antibiotic only, antibiotic and IPTG, IPTG only and no additives, and subsequent growth is analysed. A small proportion only of the cells (2%) should not contain plasmid and grow on the plate containing IPTG. If overgrowth had been identified by this method, addition of 2% glucose to the pre-induction culture medium may have been sufficient to limit basal expression in further cultures.

A plasmid turbidity test was not carried out, however changing to expression in JM109 cells or altering the induction time, temperature or concentration of IPTG did not affect expression levels. In cells containing the pET33b(+) plasmid with kanamycin selectivity, there should be a reduced incidence of overgrowth of cultures by cells that have lost the plasmid (Novagen, 1995).

Purification on a glutathione column did not result in a single protein band detectable by SDS-PAGE as there was simultaneous purification of *E. coli* proteins. This may be due to over-zealous use of the sonicator. Definitive analysis of the purified proteins was required. No antibody specific to feline TNFR-1 was available to check the production of expressed protein by Western blotting. Instead, anti-GST antibody was used to identify protein containing the GST sequence and a band of around 50kDa was detected consistent with the fusion protein. The anti-GST antibody also reacted with some smaller bands, suggesting some breakdown of the fusion protein. Subsequently, anti-proteases were added to cultures during purification.

The only feline TNFR-1 available to test the antiserum for cross-reactivity was expressed protein. As the level of expression was very poor, this was not entirely satisfactory, and results from the last aliquot of antiserum suggested that some proteolysis of the fusion protein had occurred. Excessive sonication can result in

protein disruption as well as the simultaneous purification of *E coli* proteins, so this may also have occurred. BL21 cells used for expression are protease deficient and thus should not contribute to protein degradation.

Protein expression by the GST fusion system was poor and it made identification of the expressed protein difficult since bacterial proteins were present even after purification. Determination of molecular radius by SDS-PAGE, identification by Western blotting with anti-GST antibody and disappearance after thrombin cleavage were relied on as identification techniques. However, it was intended to confirm protein production by amino acid sequencing. This did not prove possible because of the low quantities of protein generated and because of the loss after thrombin cleavage. The GST moiety was expressed on the N-terminus and thus has to be cleaved before any useful sequence information can be gained. Protein loss after thrombin cleavage may have been as a result of thrombin degradation of the expressed protein as well as release of the GST moiety (Sun and Budde, 1995) or due to such small amounts being present originally.

The aim of raising antiserum to soluble feline TNFR was to develop a species-specific assay for the receptor. Although the antiserum did detect expressed protein in Western blots, the result was poor and inconsistent. The development of an assay based on this antiserum is likely to give unreliable results. Consequently, the sheep used in this experiment was maintained without further inoculation whilst other methods of antibody production were assessed.

Since expression in the bacterial system had been poor, it became necessary to consider other methods of antibody production. One possibility was expression of protein in a mammalian system which, in addition to providing protein, is likely to produce functional receptor. However this was not undertaken due to lack of time. A further possibility, as sequence but not protein was available for the receptor of interest, was to raise antibody to peptides based on the known sequence. It would also be possible to vaccinate an animal with naked DNA of the cloned sequence of interest to provide antiserum in a similar manner to that used for protection from disease (Johnson et al. 1989). One of the problems with this latter approach is that although the host's immune response may be protective, cell mediated immunity is likely to dominate and antibody production may be minimal. This may be overcome

by the simultaneous injection of DNA encoding a cytokine selected to drive a humoral response.

5.7. Further Studies

The results of expression of feline TNFR-1 in two bacterial expression systems were disappointing. However, the sequence of feline sTNFR-1 is now available and the clear way forward is to immunise animals with appropriate peptides based on the sequence. An alternative would be to re-boost the sheep, or other animals with recombinant protein perhaps after expression in a mammalian system. This is a more time-consuming option, as would be DNA vaccination. In addition, peptide synthesis provides a source of antigen to assess specific antibody production in the inoculated animal. Further analysis of the antiserum raised in the current sheep is difficult because of the poor expression of recombinant protein and the lack of reactivity between the antiserum and rhTNFR-1.

5.8. Conclusion

Feline sTNFR-1 was purified after expression in *E. coli* using the pGEX 4T-3 vector. Antibody raised against the purified receptor reacted weakly with recombinant feline but not recombinant human protein. This reaction was inconsistent and the antiserum was therefore not used in the development of an assay for the protein.

Chapter 6:
Immunological Detection of Feline Soluble TNFR-1

6.1. Summary of Chapter

This chapter presents work designed to detect feline soluble tumour necrosis factor receptor type 1 (sTNFR-1) by immunological assay. Feline-specific antisera were generated by inoculating sheep with synthetic peptides based on the feline TNFR-1 sequence described in chapter 4. Cross-reactivity of the antisera with the peptides, human sTNFR-1 and feline TNFR-1 was assessed using western blotting, enzyme-linked immunosorbent assay (ELISA) and immunocytochemistry. An attempt was also made at the development of the ELISA to detect sTNFR-1 in feline samples.

6.2. Introduction

Attempts to generate antiserum to recombinant feline sTNFR-1 expressed in *E. coli* were very disappointing because of poor protein production by clones containing the gene for this receptor (see Chapter 5). However a specific antibody was required before an effective assay for the receptor could be developed. To this end, antiserum was produced against synthesised peptides based on the feline sTNFR-1 sequence. An advantage of this approach is that a plentiful supply of antigen is available to assess antisera.

6.2.1. Selection of antigenic peptides

Antigenic determinants are surface features of proteins and are frequently found on exposed areas of the molecule. Stretches of charged, hydrophilic amino acid side chains are common features of antigenic regions which tend to have few hydrophobic residues (Hopp and Woods, 1981). These properties have been used to devise computer programs which will predict the location of regions of antigenicity in proteins (Hopp and Woods, 1983) and their hydropathic character (Kyte and Doolittle, 1982).

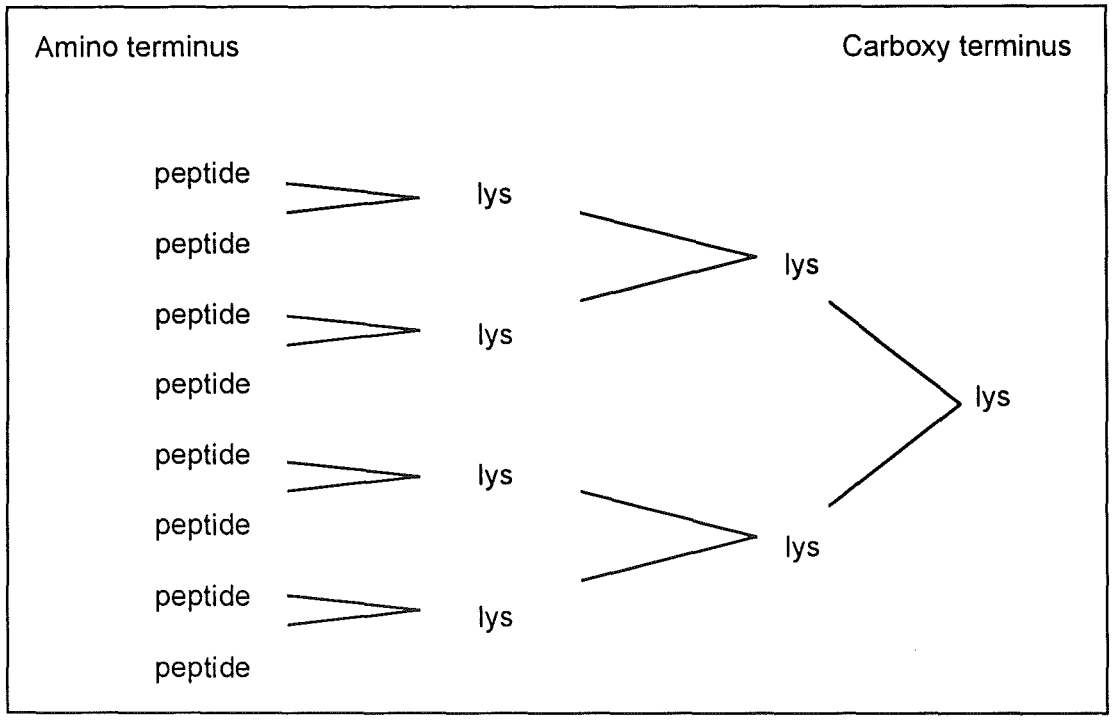
When designing peptides for antibody production, it is obviously important to select an area of the protein predicted to be immunogenic. In addition, it is wise to

choose a region that has poor homology with the homologous protein of the species used for raising antibody so that the animal recognises the peptide as foreign. For these reasons, a number of peptides are often synthesised to increase the chance of detecting one that elicits a good immune response. To improve their immunogenicity, peptides are either conjugated to a carrier protein, often keyhole limpet hemacyanin or bovine serum albumin, or are constructed into a larger molecule prior to inoculation. These larger molecules are known as multiple antigenic peptides (MAPs) and have a simple scaffolding of a low number of sequential levels of an amino acid as the core matrix on the C-termini of copies of the peptide, forming a macromolecule with a high density of peptide antigens (figure 6.1.) (Tam, 1988). Although the amino acid core is non-immunogenic, the immunoreactivity of MAPs has been found to be superior to that of monomeric peptides conjugated to a protein carrier (Tam and Zavala, 1989).

Linear peptides can be synthesised independently in a single chain to provide antigen for analysing the immune response in an animal inoculated with a MAP when recombinant or purified protein is not available and to assist with purification of the resultant antiserum if required. Thus, anti-peptide activity can be detected readily although this will not necessarily reflect activity against the native protein. The absence of activity against the native protein is one of the major problems encountered with anti-peptide antibodies (Harlow and Lane, 1988).

MAPs have been used successfully to generate antiserum in sheep to peptides based on the third variable (V3) loop of FIV (Rigby et al. 1996) and to generate antiserum in rabbits to feline γ-interferon (Argyle, 1995). In these studies, inoculations were made with complete Freund's adjuvant for the initial vaccination and incomplete Freund's adjuvant thereafter. Adjuvants are non-specific stimulators of the immune response and are particularly necessary when using soluble antigens to induce an immune response. Freund's adjuvant contains mineral oil which creates a depot and protects the antigen from rapid catabolism (Burrin and Newman, 1991) and killed *Mycobacterium tuberculosum* for immune stimulation (Harlow and Lane, 1988). Incomplete Freund's adjuvant does not contain the bacteria and is used for boosting vaccinations to avoid the side effect of persistent granuloma.

Figure 6.1. Structure of an octameric MAP/peptide complex with a poly-lysine core.



6.2.2. ELISA protocol

The construction of an ELISA for the assay of antibody generally relies on antigen immobilised on a solid phase (antibody capture) (Achord et al. 1991), whereas an ELISA for the assay of antigen relies on immobilised antibody. An inhibition ELISA uses antigen immobilised on a solid phase, and antigen in test samples is measured by inhibition of antibody binding (figure 6.2.).

6.3. Aim of Experimental Studies

The aim of the work presented in this chapter was to develop an immunoassay for feline soluble TNFR-1 using antiserum generated against peptides based on the predicted amino acid sequence for the receptor.

6.4. Materials and Methods

6.4.1. Peptide design

The 'Peptide Structure' program of the Genetics Computer Group (GCG, Wisconsin, USA) was used to predict antigenic regions within the derived amino acid sequence for feline TNFR-1 (Accession no. U72344, see chapter 4). This programme shows potential glycosylation sites, determines hydrophilicity, surface probability, chain flexibility, secondary structure and provides an antigenicity index for each amino acid (Wolf et al. 1988). Appendix 3 shows the 'PeptideStructure' output demonstrating the antigenic profile of the available feline TNFR-1 sequence.

Two areas with high antigenicity indices that did not contain potential glycosylation sites were selected for peptide synthesis. The synthesised peptides were designated peptide A and peptide B. Peptide A contained 16 amino acid residues (residues 28-43, Accession number U72344) and peptide B contained 15 (residues 175-189, Accession number U72344). Each differed from human, mouse and cow sequences as indicated in figures 6.3. and 6.4. Peptide A was located close to the leader sequence and peptide B was at the N-terminal end of the known sequence, in the region immediately upstream from the transmembrane domain.

Figure 6.2. Diagram illustrating the principle of the ELISA with capture antigen or antibody bound to the solid phase. a) the antibody capture ELISA designed to detect the presence of antibody, b) the antigen capture ELISA designed to detect the presence of antigen and c) shows the antibody capture ELISA where inhibition of antibody binding is caused by the presence of free antigen in test samples.

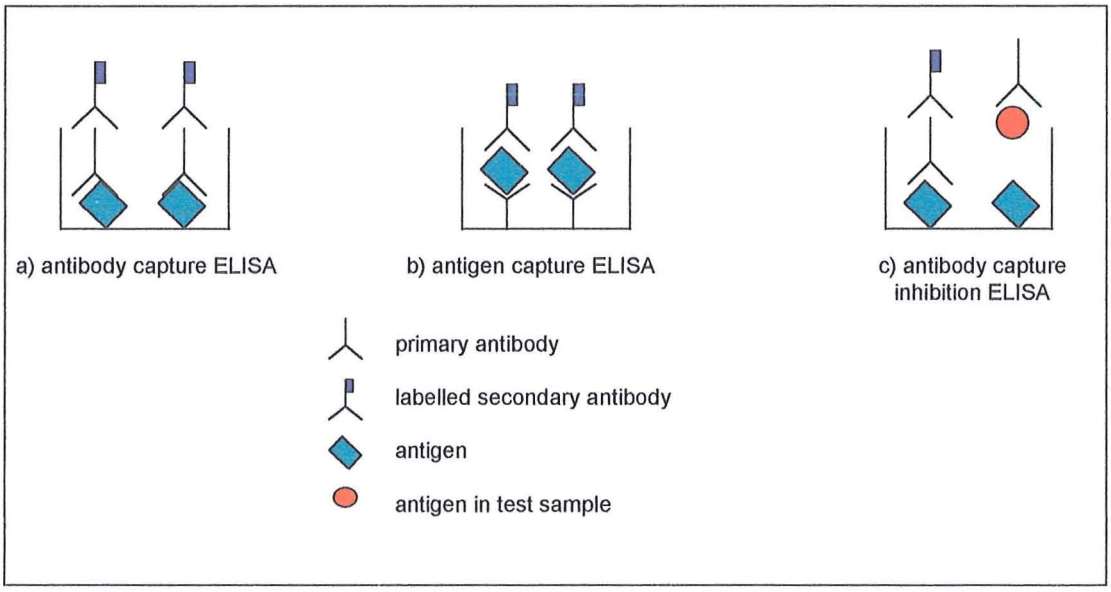


Figure 6.3. Diagram showing feline, human, murine and bovine amino acid sequences for the region represented by peptide A. The dashes represent consensus residues between the cat and other species.

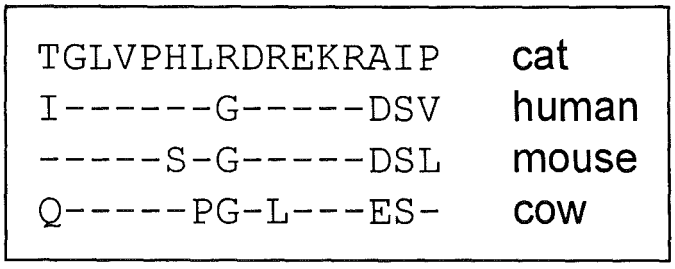
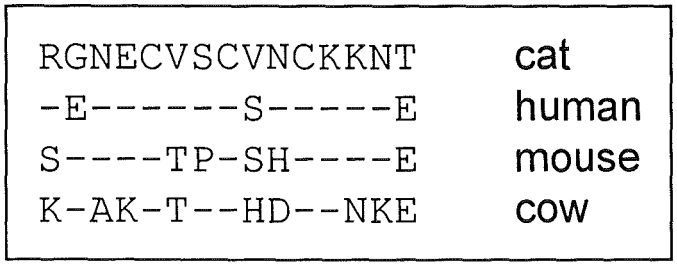


Figure 6.4. Diagram showing feline, human, murine and bovine amino acid sequences for the region represented by peptide B. The dashes represent consensus residues between the cat and other species.



6.4.2. Peptide synthesis

Both linear (150mg) and MAP (15μmole) peptides with N-terminal acetylation were synthesised commercially (Alta Bioscience, University of Birmingham, UK). To remove impurities, MAPs were purified by solubilising in 5ml of 10% acetic acid-8M urea, and dialysing against 5 changes of 5% acetic acid over 48 hours using a membrane with a 2000 Da cut-off (D7884, Sigma). After dialysis, the peptide was recovered by lyophilising and was re-dissolved in 4ml of saline.

6.4.3. Inoculation of sheep

The protein concentration of each solution was determined on an Axon discrete biochemical analyser (Bayer Diagnostics, Basingstoke, UK) using the standard Biuret method. Four aliquots of 0.5mg (0.5ml) were submitted to the Scottish Antibody Production Unit (SAPU, Law Hospital, Lanarkshire) for each sheep to be inoculated.

Inoculations were made on 4 occasions at intervals of 4 weeks. Blood samples were taken prior to initial inoculation and 7 days after subsequent injections. The serum was collected after coagulation and stored at -20°C prior to use. Peptide A was injected into sheep A, peptide B into sheep B and both A and B into sheep C, which had previously been used to raise antibody against recombinant GST-feline sTNFR-1 fusion protein (see chapter 5). Initial inoculations of sheep A and B were made with complete Freund's adjuvant. All subsequent inoculations and inoculations of sheep C were made with incomplete Freund's adjuvant.

6.4.4. Assessment of antiserum by western blotting

The linear peptides proved very difficult to dissolve, despite attempts using various buffers of different composition and pH. Consequently, for western blotting, peptides were dissolved in dimethyl sulphoxide (DMSO) before the gradual addition of treatment buffer and loading onto the gels. Antiserum at a dilution of 1:100 in TBS-Tween-milk was used as the first antibody but otherwise the western blots were prepared as previously described (chapter 5) except that the percentage of acrylamide in the gels was increased to 15% to improve the separation among the lower molecular weight peptides.

6.4.5. ELISA

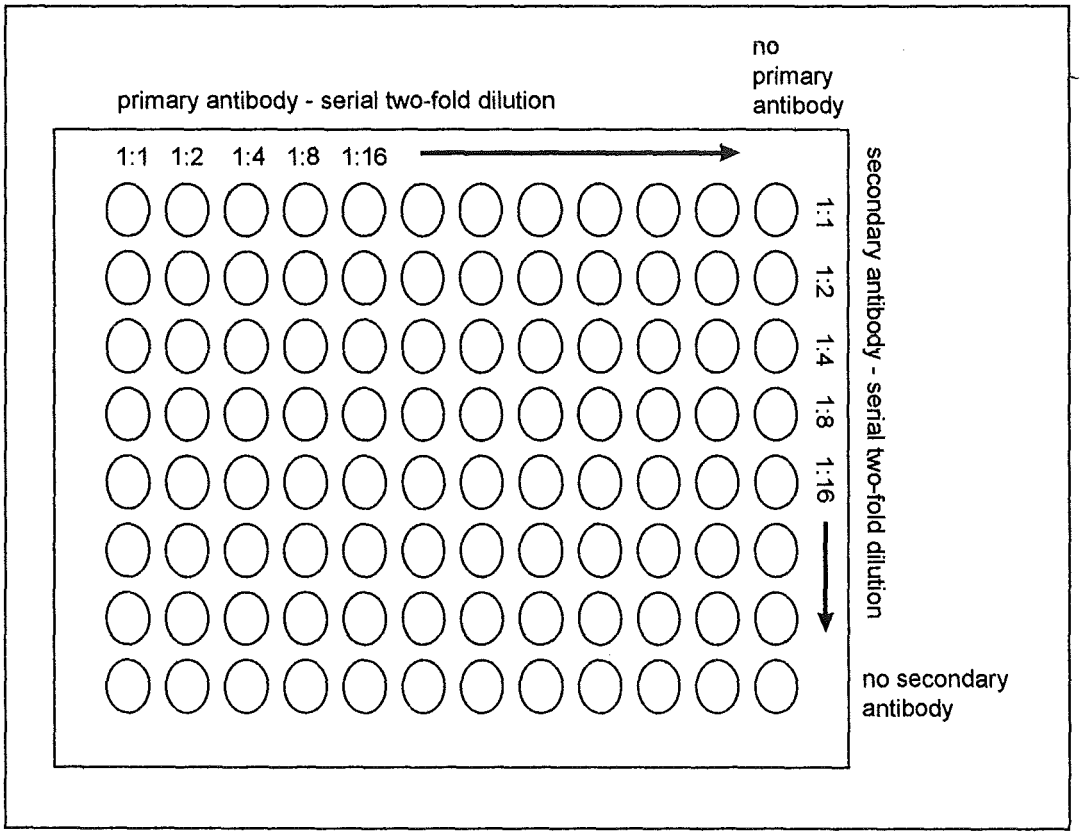
A kit providing a starter buffer pack was used for the ELISA (Pierce, Rockford, Illinois). A two dimensional serial dilution protocol was used to optimise the assay (figure 6.5.). This protocol is designed so that primary antibody is diluted serially across the plate and secondary antibody is diluted serially down the plate, allowing the optimal combinations of both reagents to be determined. All incubations were performed at room temperature.

Linear peptide was applied to microtitre plates at a final concentration of 10μg/ml by adding 95μl of coating buffer (1 packet of BupH™ Carbonate in 500ml distilled water) to 5μl of peptide at a concentration of 0.2mg/ml in DMSO. The antigen was allowed to bind to the plate for 1 hour, after which each well was washed 3 times with 100μl wash buffer (2.5ml Surfact-Amps™ Tween® 20, 5ml Blocker™ BSA in PBS and 1 packet BupH™ Dulbecco's PBS made up to 500ml with distilled water). The plate was then incubated with 100μl blocking buffer (10ml Blocker™ BSA in PBS made up to 100ml with BupH™ Dulbecco's PBS) per well for 1 hour. After blocking, 100μl of primary antibody diluted in blocking buffer was applied to each well and the plate was incubated for a further hour.

When analysing the binding of antisera to the peptides, the optimal concentration was determined by performing serial two-fold dilutions of primary antibody across the plate. To achieve this, 100μl wash buffer was added to all wells and 100μl antibody solution at a dilution of 1:500 in blocking buffer was added to each well in column 1. The contents of column 1 were mixed by filling and emptying the wells with a pipette 4 times, then transferring 100μl from each well in column 1 to the adjacent well in column 2, mixing and repeating across the plate. The final column was left free of primary antibody as a negative control.

After incubation with the primary antibody, the plate was emptied and each well was washed 3 times with 100μl wash buffer. A horseradish peroxidase-labelled secondary antibody (anti sheep/goat IgG, SAPU, Scotland, UK) diluted 1:500 in blocking buffer was applied in a similar manner to the primary antibody with the dilutions being made from row to row rather than between columns. The final row was left without any secondary antibody as a negative control. Incubation with

Figure 6.5. Diagrammatic representation of the two-dimensional serial dilution ELISA protocol used to optimise primary and secondary antibody concentrations.



secondary antibody was terminated after 1 hour, the wells were washed 3 times with100µl wash buffer and 100µl wash buffer was left in the wells for 5 minutes. The plate was then emptied by inversion and any residual fluid was removed by tapping the plate on a paper towel.

The enzyme substrate solution was made by dissolving one 3,3',5,5'-tetramethylbenzidine dihydrochloride tablet (1mg TMB tablets, Sigma) in 10ml of 0.05M phosphocitrate buffer (25.7ml 0.2M dibasic sodium phosphate, 24.3ml 0.1M citric acid and 50ml de-ionised water, pH5) and adding 2µl fresh 30% hydrogen peroxide immediately prior to use. One hundred microlitres of this solution was added to each well and the plate was left until a strong blue colour developed in the wells with the highest antibody concentrations (about 20 minutes). The reaction was stopped by the addition of 50µl 2M H_2SO_4, which caused the chromogen to change to yellow and the absorbance was read at 450nm using a microtitre plate reader (MRX Microplate Reader, Dynex Technologies Inc, VA).

6.4.6. ELISA with rhsTNFR-1

To determine the presence of any cross-species reactivity, the ELISA was repeated as described using recombinant human sTNFR-1 (rhsTNFR-1) as the capture antigen at a concentration of 10µg/ml in coating buffer.

6.4.7. Inhibition ELISA

An inhibition ELISA was developed to detect any reaction between the antiserum and rhsTNFR-1 or feline sTNFR-1 in clinical samples. Peptide A was bound to the microtitre plate at 10µg/ml as described. The primary antibody was pre-incubated with recombinant human soluble TNFR-1 (rhsTNFR-1) or feline clinical sample for 2 hours in a separate microtitre plate at room temperature before transfer to the ELISA plate. Initially, varying concentrations of anti-peptide A antiserum and rhsTNFR-1 were analysed to determine the optimal concentrations of each to detect inhibitory activity.

6.4.8. Immunocytochemistry

Immunocytochemistry was performed by the Department of Veterinary Pathology at the University of Glasgow. Antiserum to peptide A was used as the primary antibody

and biotinylated rabbit anti-goat IgG (E0466, DAKO, Cambridge, UK) as the secondary antibody. Sections of healthy feline liver were stained using multiple dilutions of primary antibody to determine the optimal concentration to give minimal background yet retain specific staining. The optimal concentration of primary antibody was then used to stain slides which had been treated with trypsin and by microwave and pressure cooking in an attempt to enhance antigen retrieval. Slides prepared without the addition of a primary antibody were used as negative controls.

6.5. Results

6.5.1. Western blotting

Although difficulty was encountered in dissolving the linear peptides, western blotting demonstrated that antisera from sheep A and sheep C reacted with peptide A with bands stained over a range less than 29kDa in size. Pre-immune serum taken from sheep A and C prior to inoculation with peptide did not react. Antisera from sheep B and sheep C reacted with peptide B with bands staining over a similar range to peptide A. The reaction of both peptides with antiserum from sheep C is shown in figure 6.6. This illustrates that both peptides induced an immune response in the inoculated sheep which resulted in the production of peptide-specific antibody.

6.5.2. ELISA - Serial dilution protocol for antiserum from sheep A

Figure 6.7. shows the antibody dilution curves using peptide A as the capture antigen and antiserum from sheep A taken after the final inoculation of peptide. The antiserum showed strong binding activity against linear peptide A when tested by ELISA. This activity was not detected when pre-immune serum from sheep A was used as the primary antibody. After performing the two dimensional serial dilution protocol, it was found that a primary antibody dilution of 1:8000 and a secondary antibody dilution of 1:1000 gave an A_{450} reading of approximately half of the maximum value on the microtitre plate reader, (1.157 compared to 2.008). These antibody dilutions were considered optimal and were used subsequently in the ELISA.

Figure 6.6 Digital image of western blot showing the reaction of antiserum taken from sheep C against the linear peptides. Lane 1; peptide A, lane 2; peptide B.

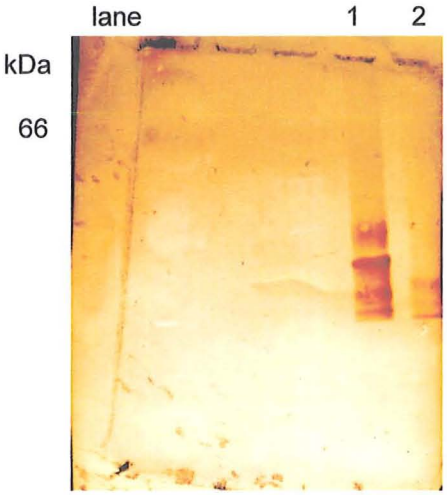
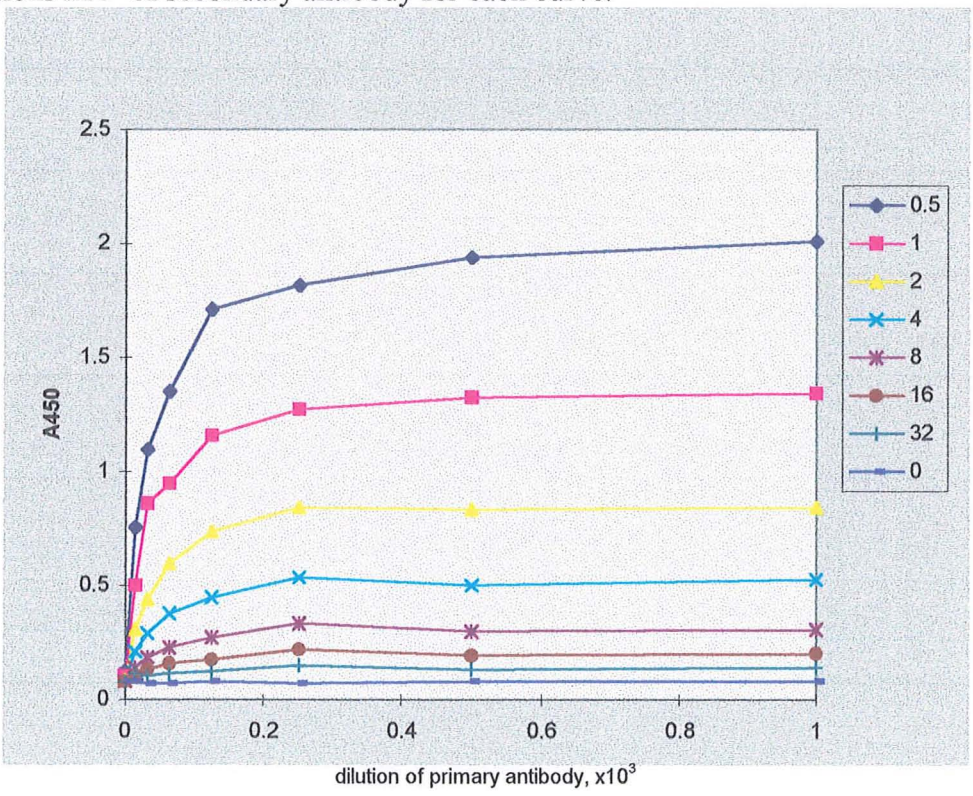


Figure 6.7. Antibody dilution curves for the ELISA using peptide A as the capture antigen and antiserum from sheep A as the primary antibody. Serial dilutions of primary and secondary antibody were used in the assay. The key shows reciprocal dilutions x10^3 of secondary antibody for each curve.



6.5.3. ELISA - Serial dilution protocol for antiserum from sheep B

Antiserum from sheep B taken after the final injection of peptide showed little increase in activity against peptide B above background levels (determined by wells coated with DMSO or coating buffer without capture antigen). For this reason, further assessment of antiserum from sheep B was not pursued.

6.5.4. ELISA for rhsTNFR-1

Antiserum from sheep A that had anti-peptide activity showed a strong reaction against rhsTNFR-1. However pre-immune serum from sheep A also demonstrated this activity (figure 6.8.).

6.5.5. Inhibition ELISA

Initial results of the inhibition ELISA demonstrated that pre-incubation of anti-peptide A antibody with rhsTNFR-1 resulted in interference with primary antibody binding to the capture antigen (figure 6.9.). However, this reduction in antibody binding could not be reproduced consistently and reduction of the concentration of capture peptide to 2.5μg/ml or increase of rhsTNFR-1 concentration to 5μg/ml did not improve the results. In addition, no inhibition could be demonstrated by pre-incubation of anti-peptide A antiserum with feline splenic supernatant samples that exhibited sTNFR-1 like activity detected by bioassay (chapter 3) prior to use in the ELISA.

6.5.6. Immunocytochemistry

Titration of primary antibody determined that a dilution of 1:3000 was optimal to demonstrate specific activity with no background staining. Pre-treatment of the liver sections by microwave cooking or by incubation with trypsin for 30 minutes gave the best demonstration of anti-peptide A antiserum binding to receptor on hepatocytes (figure 6.10.).

Figure 6.8. Graph illustrating the reaction of pre-immune and post-inoculation anti-sera with bound rhsTNFR-1 when used in the ELISA.

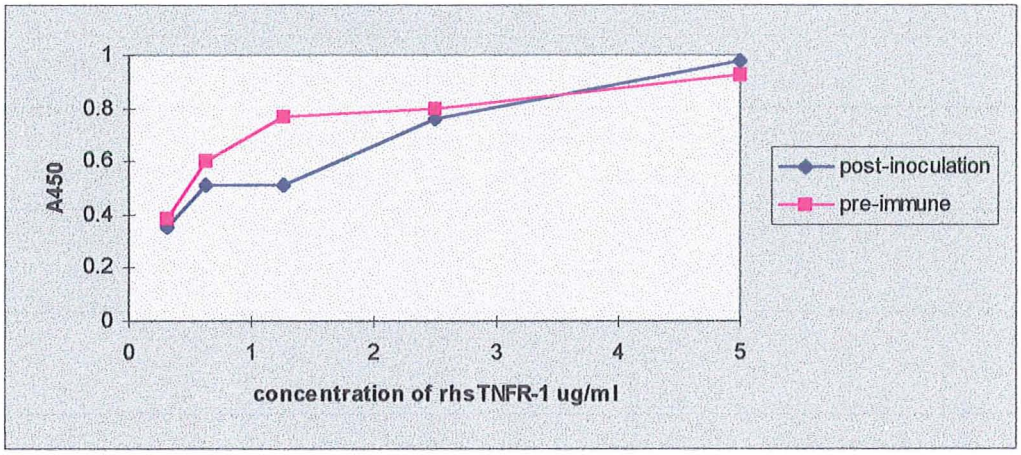


Figure 6.9. Dilution curves for the inhibition ELISA using peptide A as the capture antigen and antiserum from sheep A as the primary antibody. Serial dilutions of primary antibody were pre-incubated with serial dilutions of recombinant human soluble TNF receptor -1 (rhsTNFR-1) from 0.018 to 1.25μg/ml. The key shows reciprocal dilutions $x10^3$ of primary antibody for each curve.

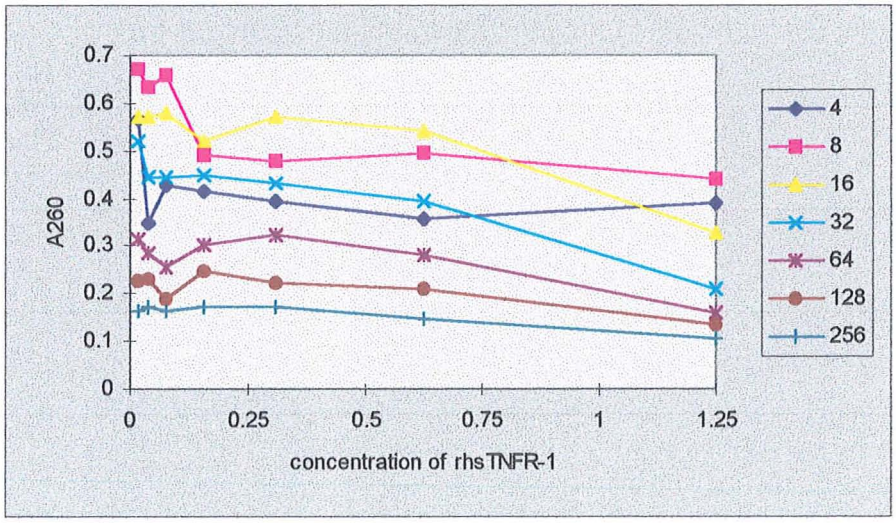
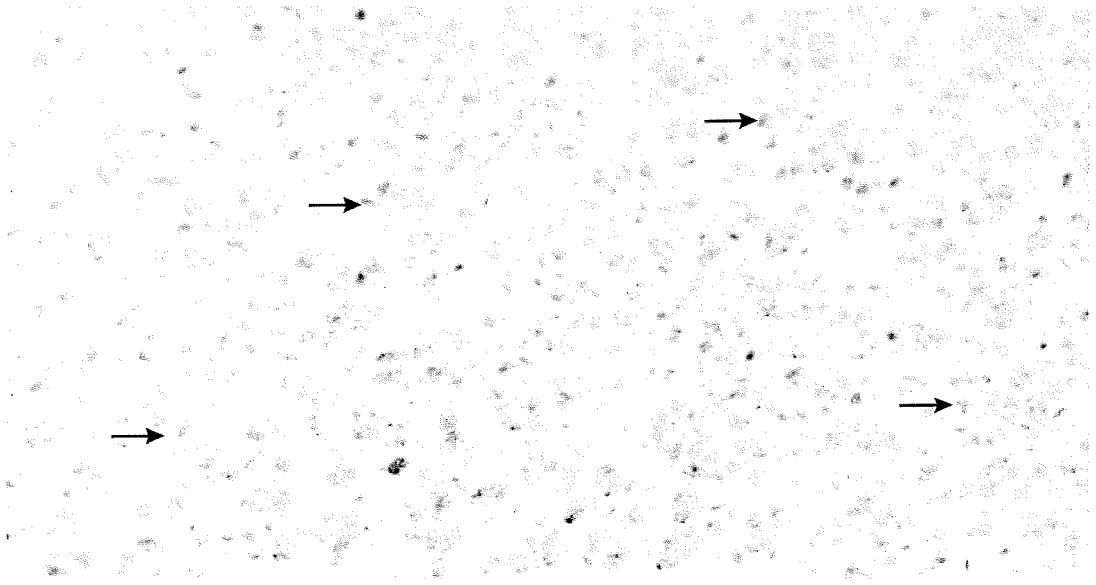


Figure 6.10. Immunocytochemistry slide of a liver slice which has been pre-treated by microwave cooking then stained with anti-peptide A antiserum. Magnification = x500. The arrows highlight some areas of positive staining.



6.6. Discussion

The work presented in this chapter demonstrates that antiserum raised in sheep to peptides A and B contained within feline sTNFR-1 recognised the corresponding peptides in western blot (peptides A and B) and ELISA (peptide A only). However, development of an immunoassay system was not possible in the time available. Although the antiserum recognises linear peptide when that is used as an antigen, lack of availability of native protein still prevented full characterisation of the antiserum and assessment of its value when monitoring clinical samples. Analysis by western blotting of supernatant samples that exhibited strong anti-TNF-α activity using the L929 bioassay (chapter 3) may illustrate activity against native protein although these samples did not inhibit the binding of anti-peptide A antiserum to peptide A in an ELISA.

Although antiserum taken from sheep A after the final inoculation showed a strong reaction to rhsTNFR-1, pre-immune serum also reacted. Given the findings in chapter 5 when pre-immune serum from sheep C reacted against *E coli* proteins, it is likely that the present findings represent a similar phenomenon, because the human protein was produced by expression in *E coli*. Presumably, the sheep had previously encountered and mounted an immune response against bacterial proteins. To fully assess the activity of anti-peptide A antiserum against the human receptor, both pre- and post-immune serum could be absorbed against *E coli* proteins and re-analysed. In addition, it would be interesting to assess whether there is any cross-reactivity between polyclonal anti-human sTNFR-1 antibody and peptides A and B.

The inhibition ELISA appeared to demonstrate interaction between antiserum to peptide A and rhsTNFR-1 resulting in reduced binding of the primary antibody to the bound antigen. However, these results could not be reproduced consistently, despite reducing the amount of bound antigen and increasing the concentration of rhsTNFR-1 incubated with primary antibody. Treatment of the rhsTNFR-1 with a mild detergent prior to incubation with antiserum might result in better exposure of the peptide site to antibody and thus greater consistency. However, as the peptides that were selected for inoculation into sheep were from areas rich in interspecies variation with the aim of promoting the production of an immune response, it would not be surprising if the resulting antiserum did not recognise the human protein. Alternatively the reaction between the antiserum and *E coli* antigens in rhsTNFR-1

may have interfered with the inhibition ELISA and contributed to the inconsistent results.

MAP-containing peptides show a better binding capacity to plastic surfaces than monomeric peptides (Tam and Zavala, 1989) although linear peptides were used to analyse antiserum in the work presented here because they were readily available. The MAPs were easily dissolved in buffer prior to their purification so it is likely that their use in analysing antiserum may have been preferable to the use of linear peptides. Rigby and others (Rigby et al. 1996) used a MAP as the target antigen to detect antibody raised to peptide by an ELISA. The antiserum to peptide B may be behaving poorly because of poor binding of peptide B to the microtitre plate. Peptides that contain bulky and hydrophobic side chains and those that can form amphiphatic helixes or β sheets may bind better than others to solid plastic surfaces.

In the work described in chapter 5, a sheep was selected to generate antibody to recombinant feline sTNFR-1 to allow an ample volume of antiserum to be generated for the development of an assay for the receptor. For the same reason, the decision was made to raise anti-peptide antibodies in sheep. Although the sequence for ovine TNFR-1 has not been reported, the degree of homology between the sequences for the cow and the cat (see table 4.1.) is similar to that between the mouse and the cat. Consequently, the species are equally likely to recognise the selected peptides as foreign. The problem encountered previously with the very small quantity of immunogen available in the form of recombinant protein would not be a problem with synthesised peptides.

Little reaction was seen when using peptide B as the capture antigen in the ELISA. This may be a result of a poor immune response in sheep B, either because of poor immunogenicity of the peptide or because of a problem with the individual sheep. However, since immunoreactivity was detected by Western blotting, it is perhaps more likely that there was poor binding of this peptide to the microtitre plate. Linking the peptide to a carrier such as bovine serum albumin may assist coating of the plate with antigen.

The activity demonstrated against the peptides by Western blotting was not to a single low molecular weight band as might be expected, but instead over a range less than 29kDa. The poor solubility of the linear peptides may have been

affecting their electrophoretic migration. A range of sizes can be caused by truncated peptides arising during synthesis but these would have been expected to be at a much smaller molecular weight than the bands detected. Antiserum taken from sheep C immediately prior to inoculation with peptide did not react with either peptide A or B in a Western blot, suggesting that there is no cross-reaction between the anti-GST-fusion protein antiserum and the peptides.

The immunocytochemistry study illustrates binding of anti-peptide A antiserum to hepatocytes. Since the immunocytochemistry was performed on hepatocytes, it is a possibility that the apparent positive staining could result from intracellular granulation. However, the antigen retrieval processes of microwave and trypsin treatment enhanced antibody binding, and the negative controls did not show this binding, supporting the interpretation that the positive staining is real. Confirmation could be achieved by staining a different tissue with the same antiserum.

6.7. Further Studies

The initial aim of further studies would be to determine whether the antiserum to peptide A recognises native protein. This could be assessed by examining by western blotting supernatants shown by bioassay to exhibit anti-TNF activity. Cross-reactivity between the antiserum absorbed with *E coli* proteins and rhsTNFR-1 should be assessed. The use of anti-rhsTNFR-1 antibody against peptide A in a western blot might help to confirm cross-species reactivity. If activity of antiserum from sheep A against the native feline protein were demonstrated, then the next step would be to return to the inhibition ELISA and to develop it to assess samples. It may be possible to use serial dilutions of peptide rather than rhsTNFR-1 as standards for pre-incubation with primary antibody.

The anti-peptide A antiserum could be used in further immunocytochemical studies to examine expression of membrane bound receptor in other tissues, both from healthy and diseased cats.

Another method of detecting antiserum activity against the native protein would be using flow cytometry. Since receptors for TNFR-1 are widespread, white blood cells could be used for this analysis. Anti-peptide antisera could be used as the

primary antibody, binding to membrane-bound receptor and an anti-sheep FITC conjugated antibody used as the secondary antibody.

6.8. Conclusion

Antiserum was raised in sheep to two peptides based on the feline TNFR-1 sequence available. Antiserum to peptide A recognised the linear peptide when it was used as a capture antigen in an ELISA. However, the ability of the antiserum to recognise native protein could not be assessed *in vitro* because of lack of availability of native protein. This problem has to be addressed before the ELISA can be developed to measure soluble receptor in feline samples.

Chapter 7:
Conclusion

The work presented in this thesis is directed towards the identification of markers of inflammation that may be useful when monitoring feline disease. Initially, the measurement of alpha-1 acid glycoprotein (AGP) was determined to be of benefit as an aid to the diagnosis of feline infectious peritonitis. This is of value to the clinician while a definitive diagnostic test for this condition remains unavailable. The AGP assay is straightforward and likely to be of benefit when monitoring other inflammatory conditions in the cat and when assessing response to therapy. It is now used routinely as part of an 'FIP' screen in the feline virus unit.

A recent publication supports our findings that AGP and haptoglobin (Hp) act as moderate acute phase proteins in the cat (Kajikawa et al. 1999). The authors measured both proteins using radial immunodiffusion assay kits (Saikin Kagaku, Japan) and presented a reference range for AGP concentration in healthy cats (0.148-0.340g/l) comparable to that presented in chapter 2 (0.1-0.48g/l). The reference range that they determined for Hp (0.49-0.783g/l) was somewhat narrower than that described in chapter 2 (0.04-3.84g/l). After the induction of inflammation, the maximum levels of protein were found after 48 hours and the magnitude of rise was similar to that detected in the work presented in this thesis. Serum amyloid A (SAA) and C-reactive protein (CRP) were also measured in the study. Like AGP and Hp, SAA was found to act as a moderate acute phase responder, but levels of CRP did not vary significantly between clinically normal cats and hospitalised cats or those with induced inflammation.

The fluctuations in levels of inflammatory cytokines that have been recorded over the asymptomatic phase of infection with feline immunodeficiency virus (FIV) (Lawrence et al. 1995) suggest that there may be corresponding changes in serum concentrations of acute phase proteins. This was found not to be the case, but the need for identifying prognostic markers in FIV was apparent. Although the decline in CD4 positive lymphocytes has been shown to occur over the asymptomatic phase of the disease (Novotney et al. 1990), the technology involving flow cytometry is not readily available to the clinician and analysis of samples over 24 hours old is

inaccurate. In HIV, an increase in viral load has also been associated with disease progression, however the development of an assay that will identify markers of disease progression in FIV and can be easily applied to samples received from the field will be of great benefit when expensive equipment, technical expertise and funding are not readily available for lymphocyte subset analysis and real time PCR. Soluble forms of the TNF receptors continue to be quoted as useful markers of disease progression and response to therapy in HIV (Aukrust et al. 1999). Thus the decision to develop assays for feline soluble TNF receptors and to determine if these give useful prognostic information in cats with FIV infection remains sound. The initial approach used here was the L929 bioassay to detect anti-TNF activity which may be attributable to the presence of feline soluble TNFR-1. Although anti-TNF activity was readily demonstrated by this means, the assay results were inconsistent and it was subsequently decided that the development of an immunologically-based assay may be more reliable.

Cloning and sequencing of the feline soluble TNF receptors was undertaken using PCR-based methodology utilising primers based on regions of homology in published sequences from different species. Whilst this was successful for feline sTNFR-1, only part of the intracellular portion of feline TNFR-2 could be amplified using this method. Cloning of feline sTNFR-1 was followed by expression of the recombinant protein as a fusion to glutathione-S-transferase. Expression levels were extremely poor and analysis of antiserum raised to the expressed protein was hampered by the lack of suitable antigen. Expression in an alternative *E. coli* vector did not improve results.

As the results of analysis of the antiserum to feline sTNFR-1 fusion protein were disappointing, the decision was made to design 2 peptides based on the known feline sequence and use these for the generation of anti-peptide antibody. Antiserum to one of the peptides showed very strong anti-peptide reactivity by ELISA, but its activity against native protein could not be assessed because of the lack of availability of the latter. Initially, human soluble TNF receptors were purified from pools of 300 litres of urine (Engelmann et al. 1989). It was not feasible to collect such a large amount from cats.

To continue the work of this thesis in the design of assays for soluble feline TNF receptors, it is intended to screen a feline thymic cDNA library using the

amplified portion of feline TNFR-2 as a probe. Any clones identified by the probe will be analysed to obtain the sequence for the remaining portions of the receptor. Once this is achieved, as for feline TNFR-1, it is likely that expression as a GST-fusion protein will be undertaken and antiserum raised to the expressed protein.

As discussed in chapter 6, the problems encountered with TNFR-1 mean that an alternative approach has to be tried for the development of an assay to this receptor. The main stumbling block has been the lack of native protein for the analysis of generated antiserum. One way to attempt to circumvent this would be to attempt expression in a mammalian system, which should produce functional protein. Although DNA vaccination is a technique that may give appropriate antiserum, it will not solve the problem of the deficiency of suitable antigen with which to analyse antiserum and any vector to be used for DNA vaccination should initially be tested for expression in mammalian cells in culture. A slightly different approach would be to clone a smaller portion of the receptor and attempt expression of this in an *E coli* system to see if expression levels improve. This would be fairly easily performed and negative or positive results should be rapidly obtained.

It may also be useful to analyse the anti-feline TNFR-1 antiserum produced against both recombinant protein and peptides by flow cytometry. Any reactivity with peripheral blood mononuclear cells would provide evidence of recognition of the native protein. Any antibody reacting positively could then be used to monitor the cell surface expression of the receptor and detect whether a down-regulation in cell surface receptor is associated with an up-regulation in soluble receptor.

APPENDIX 1

LB Broth

10g tryptone
5g yeast extract
10g NaCl
Dissolve to 1litre in dH_2O and autoclave.
For LB-agar, add 1.2-1.5% agarose prior to autoclaving.

SOC Medium

20g tryptone
5g yeast extract
0.5g NaCl
Dissolve to 1litre in dH_2O and autoclave.
After autoclaving, add 1ml of sterile $MgCl_2$, 1ml of sterile 250mM KCl and 2.78ml sterile 2M (36%) glucose per 100 mls of medium.

APPENDIX 2

Monomer Solution

60g acrylamide (FW 71.08)
1.6g bisacrylamide (FW 154.2)
Add ddH_2O to 200ml

4x Running Gel Buffer (1.5M Tris-Cl, pH8.8)

36.3g tris (FW 121.1)
Add 150ml ddH_2O, adjust pH to 8.8 with HCl and add ddH_2O to 200ml

4x Stacking Gel Buffer (0.5M Tris-Cl, pH6.8)

3.0g tris (FW121.1)
Add 40ml ddH_2O, adjust pH to 6.8 with HCl and add ddH_2O to 50ml

12.5% Running Gel Recipe

12.5ml monomer solution
7.5ml 4x running gel buffer
0.3ml 10% SDS
9.6ml ddH_2O
150µl 10% ammonium persulfate
10µl TEMED

Stacking Gel Recipe

0.88ml monomer solution
1.66ml stacking gel buffer
66µl 10% SDS
4.06ml ddH_2O
33.4µl 10% ammonium persulfate
3.3µl TEMED

APPENDIX 3

PEPTIDESTRUCTURE of: fcu72344.pep check: 2441 from: 1 to: 189
TRANSLATE of: fcu72344.gb_om check: 3951 from: 14 to: 581
Hydrophilicity (Kyte-Doolittle) averaged over a window of: 7
Surface Probability according to Emini
Chain Flexibility according to Karplus-Schulz
Secondary Structure according to Chou-Fasman
Secondary Structure according to Garnier-Osguthorpe-Robson
Antigenicity Index according to Jameson-Wolf

Pos	AA	GlycoS	HyPhil	SurfPr	FlexPr	CF-Pred	GORPred	AI-Ind ..
1	M	.	-0.925	0.468	1.000	.	.	-0.450
2	G	.	-0.600	0.528	1.000	.	.	-0.450
3	L	.	-1.200	0.307	1.000	.	.	-0.450
4	P	.	-0.800	0.479	1.000	.	.	-0.450
5	T	.	-0.471	0.479	1.030	.	.	-0.450
6	V	.	-1.071	0.479	1.032	.	.	-0.450
7	P	.	-1.071	0.256	1.030	.	.	-0.450
8	G	.	-0.800	0.307	1.017	.	B	-0.450
9	L	.	-0.671	0.639	1.009	.	B	-0.450
10	L	.	-0.614	0.341	1.007	H	B	-0.450
11	Q	.	-1.443	0.256	1.003	H	B	-0.450
12	P	.	-2.043	0.256	0.996	H	B	-0.600
13	L	.	-2.043	0.256	0.976	H	B	-0.600
14	V	.	-1.757	0.149	0.952	H	B	-0.600
15	L	.	-2.800	0.080	0.931	H	H	-0.600
16	L	.	-3.571	0.080	0.917	H	H	-0.600
17	A	.	-3.629	0.080	0.914	H	H	-0.600
18	L	.	-2.529	0.167	0.917	H	H	-0.600
19	L	.	-2.629	0.142	0.918	H	H	-0.600
20	V	.	-1.900	0.220	0.920	H	H	-0.600
21	E	.	-1.414	0.413	0.921	H	B	-0.600
22	I	.	-1.414	0.413	0.923	H	B	-0.600
23	Y	.	-0.229	1.089	0.926	.	B	-0.150
24	P	.	-0.229	0.467	0.933	.	B	-0.300
25	L	.	-0.629	0.961	0.946	B	B	-0.600
26	R	.	0.071	0.607	0.962	B	B	0.300
27	V	.	-0.657	0.324	0.981	B	B	-0.600
28	T	.	-1.486	0.291	0.993	B	B	-0.600
29	G	.	-0.714	0.230	0.991	B	B	-0.600
30	L	.	-0.900	0.422	0.978	B	B	-0.600
31	V	.	-0.843	0.241	0.962	B	B	-0.600
32	P	.	-0.300	0.477	0.955	h	B	-0.300
33	H	.	0.143	0.966	0.962	h	.	0.300
34	L	.	1.329	2.548	0.980	h	.	0.750
35	R	.	2.429	2.854	1.006	h	T	1.300
36	D	.	2.757	4.195	1.030	h	T	1.300
37	R	.	2.943	9.962	1.048	h	T	1.300
38	E	.	3.229	5.138	1.054	h	T	1.300
39	K	.	1.943	2.157	1.039	h	T	1.300
40	R	.	1.671	1.703	1.011	h	T	1.300
41	A	.	0.671	0.527	0.978	h	T	1.000
42	I	.	0.400	0.408	0.960	h	.	0.300
43	P	.	0.343	0.360	0.971	t	.	0.500
44	C	.	-0.243	0.353	1.006	t	.	0.050
45	P	.	0.571	1.007	1.052	T	T	1.700
46	Q	.	1.400	1.020	1.080	T	T	1.700
47	G	.	0.529	1.334	1.073	T	T	1.700
48	K	.	1.343	1.174	1.041	.	T	1.300
49	Y	.	1.343	1.048	1.001	.	T	1.300
50	I	.	1.343	1.835	0.986	.	.	0.750

51	H	.	1.786	1.532	0.999	.	.	0.750
52	P	.	1.729	1.573	1.034	t	T	1.500
53	Q	.	1.657	3.006	1.067	T	T	1.700
54	D	.	1.657	1.549	1.068	T	T	1.700
55	N	.	0.843	0.537	1.052	T	T	1.550
56	S	.	0.257	0.166	1.011	t	T	1.050
57	I	.	-0.143	0.144	0.973	.	T	0.100
58	C	.	-0.086	0.179	0.963	.	T	0.100
59	C	.	-0.943	0.071	0.957	.	T	-0.200
60	T	.	-0.600	0.139	0.971	t	T	0.000
61	K	.	0.600	0.517	0.986	t	T	1.200
62	C	.	1.014	0.955	0.994	.	T	1.000
63	H	.	1.471	0.955	1.015	t	T	1.350
64	K	.	1.557	0.748	1.030	T	T	1.550
65	G	.	0.457	1.151	1.033	T	T	1.400
66	T	.	1.000	1.326	1.019	B	T	1.300
67	Y	.	1.043	1.066	0.992	B	T	1.150
68	L	.	0.986	1.799	0.976	B	T	1.150
69	Y	.	0.571	0.668	0.972	B	T	1.000
70	N	.	0.971	0.739	0.988	B	T	1.000
71	D	.	0.843	0.886	1.016	t	T	1.350
72	C	.	1.614	0.875	1.031	t	T	1.350
73	E	.	1.486	0.538	1.049	t	T	1.350
74	G	.	0.443	0.266	1.056	t	.	0.650
75	P	.	0.443	0.828	1.052	T	T	1.250
76	G	.	0.900	0.690	1.050	T	T	1.550
77	L	.	0.900	1.164	1.047	.	T	1.300
78	D	.	0.486	0.404	1.047	.	T	0.850
79	T	.	0.900	0.799	1.047	T	T	1.550
80	D	.	1.343	1.678	1.043	T	T	1.700
81	C	.	1.529	0.538	1.029	t	T	1.350
82	R	.	1.529	0.646	1.019	T	T	1.550
83	E	.	1.929	0.622	1.019	T	T	1.550
84	C	.	1.486	1.149	1.028	.	T	1.300
85	E	.	1.943	0.846	1.051	T	T	1.550
86	N	G	0.900	0.423	1.062	T	T	1.550
87	G	.	0.500	1.139	1.056	.	T	1.000
88	T	.	0.600	0.665	1.037	.	.	0.750
89	F	.	0.214	0.554	1.013	.	.	0.450
90	T	.	0.214	0.969	1.005	.	.	0.450
91	A	.	0.657	1.080	1.013	.	.	0.900
92	S	.	0.743	1.955	1.021	.	.	0.900
93	E	.	0.600	1.117	1.023	T	T	1.700
94	N	.	1.143	2.165	1.011	T	T	1.700
95	Y	.	1.900	2.798	0.996	B	T	1.150
96	L	.	1.429	0.866	0.985	B	T	1.000
97	R	.	0.386	0.444	0.979	B	T	0.700
98	Q	.	0.000	0.380	0.970	B	T	0.700
99	C	.	-0.543	0.247	0.956	B	T	-0.200
100	L	.	0.114	0.169	0.956	B	T	0.700
101	S	.	0.029	0.195	0.964	t	T	0.900
102	C	.	-0.829	0.195	0.980	t	T	0.000
103	S	.	0.171	0.463	1.005	T	T	1.250
104	K	.	1.271	0.691	1.021	T	T	1.550
105	C	.	1.657	2.234	1.028	t	T	1.500
106	R	.	1.743	1.650	1.026	.	T	1.300
107	K	.	1.814	1.293	1.014	.	T	1.300
108	E	.	1.757	4.176	0.986	.	T	1.150
109	M	.	1.514	1.582	0.957	B	T	1.150
110	Y	.	1.371	1.370	0.939	B	T	1.150
111	Q	.	0.171	0.555	0.932	B	T	0.700
112	V	.	-0.214	0.751	0.947	B	.	-0.300
113	E	.	0.286	0.741	0.973	B	.	0.300
114	I	.	-0.257	0.229	0.996	B	.	-0.300
115	S	.	-0.657	0.446	1.010	.	.	-0.450

116	P	.	-0.657	0.191	1.006	.	T	-0.050
117	C	.	-0.971	0.427	0.986	B	T	-0.200
118	T	.	0.314	0.625	0.969	B	T	0.700
119	V	.	0.700	0.675	0.962	B	T	1.000
120	Y	.	0.571	1.816	0.971	B	T	1.150
121	R	.	0.329	0.934	0.989	B	T	0.700
122	D	.	-0.129	0.675	0.993	B	T	0.100
123	T	.	0.529	0.426	0.984	B	T	1.000
124	V	.	-0.014	0.117	0.962	B	T	0.100
125	C	.	-0.014	0.137	0.947	B	T	0.100
126	G	.	0.043	0.190	0.954	t	T	0.900
127	C	.	0.443	0.411	0.980	t	T	0.900
128	R	.	1.543	1.327	1.016	t	T	1.500
129	K	.	2.086	2.100	1.042	T	T	1.700
130	N	.	2.671	7.675	1.042	T	T	1.700
131	Q	.	3.214	6.140	1.018	B	T	1.300
132	Y	.	2.757	4.810	0.978	B	T	1.150
133	R	.	2.329	3.145	0.940	B	T	1.150
134	Y	.	1.943	2.434	0.923	B	T	1.150
135	Y	.	1.943	2.690	0.928	B	T	1.150
136	W	.	1.857	1.982	0.958	B	T	1.150
137	S	.	1.671	1.721	0.993	.	.	0.750
138	E	.	1.086	0.951	1.010	.	T	1.150
139	T	.	1.400	1.567	1.004	.	T	1.300
140	H	.	0.914	0.627	0.974	B	T	1.000
141	F	.	0.257	0.298	0.942	B	T	0.700
142	Q	.	0.257	0.333	0.920	B	T	0.700
143	C	.	-0.200	0.131	0.915	B	T	0.100
144	L	.	-0.543	0.203	0.925	B	T	-0.200
145	N	G	-0.686	0.097	0.933	B	T	-0.200
146	C	.	-1.543	0.097	0.940	B	T	-0.200
147	S	.	-1.729	0.097	0.943	B	T	-0.200
148	L	.	-0.686	0.097	0.948	B	T	-0.200
149	C	.	-1.129	0.178	0.964	B	T	-0.200
150	L	.	-0.671	0.192	0.989	B	T	-0.200
151	N	G	-1.386	0.173	1.014	B	T	-0.050
152	G	.	-0.343	0.558	1.027	B	T	0.250
153	T	.	-0.629	0.474	1.020	B	T	-0.050
154	V	.	0.029	0.395	0.997	B	B	0.300
155	Q	.	-0.829	0.214	0.971	B	B	-0.600
156	I	.	-0.329	0.297	0.966	B	B	-0.300
157	S	.	0.071	0.692	0.974	.	B	0.300
158	C	.	0.771	0.577	1.000	.	B	0.750
159	K	.	0.771	1.425	1.041	.	T	1.300
160	E	.	1.914	1.711	1.071	.	T	1.300
161	T	.	1.900	4.605	1.092	.	T	1.300
162	Q	.	1.657	1.709	1.096	t	T	1.500
163	N	.	0.743	0.529	1.068	t	T	1.350
164	T	.	0.343	0.529	1.027	B	T	0.850
165	V	.	-0.114	0.164	0.978	B	B	-0.300
166	C	.	-0.157	0.139	0.935	B	B	-0.300
167	T	.	-0.914	0.097	0.915	B	B	-0.600
168	C	.	-0.957	0.129	0.906	B	B	-0.600
169	H	.	-0.757	0.209	0.910	h	B	-0.600
170	A	.	-0.800	0.125	0.916	h	T	-0.200
171	G	.	-1.443	0.193	0.920	h	T	-0.200
172	F	.	-0.443	0.278	0.931	h	T	-0.200
173	F	.	-0.843	0.272	0.954	h	T	-0.200
174	L	.	-0.086	0.442	0.989	h	T	0.100
175	R	.	0.357	0.884	1.027	t	T	1.050
176	G	.	0.400	0.547	1.049	t	T	1.050
177	N	.	0.200	0.492	1.044	t	T	1.050
178	E	.	0.857	0.337	1.014	t	T	1.350
179	C	.	-0.143	0.182	0.969	B	T	0.100
180	V	.	-0.800	0.084	0.936	B	T	-0.200

181	S	.	-0.800	0.078	0.918	B	T	-0.200
182	C	.	-1.657	0.078	0.913	B	T	-0.200
183	V	.	-0.743	0.211	0.932	B	T	-0.200
184	N	.	0.414	0.314	0.957	B	T	0.700
185	C	.	0.800	0.943	0.994	B	T	1.000
186	K	.	1.257	1.834	1.000	t	T	1.500
187	K	.	2.167	1.458	1.000	t	T	1.500
188	N	.	1.900	3.477	1.000	t	T	1.500
189	T	.	3.000	2.222	1.000	.	T	1.300

GLOSSARY

A	adenine
A:G	albumin:globulin
Ab	antibody
ABL	abelson tyrosine kinase
AGP	α-1 acid glycoprotein
AIDS	acquired immunodeficiency syndrome
APP	acute phase protein
C	cytosine
CD	cluster of differentiation
cDNA	complementary DNA
CIAA	chloroform:isoamyl alcohol
CM	complete medium
ConA	concanavalin A
CRP	C-reactive protein
CV	coefficient of variation
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphates
DPR	differential positive rate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetate
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FCoV	feline coronavirus
FIP	feline infectious peritonitis
FIV	feline immunodeficiency virus
G	guanine
GST	glutathione-S-transferase
HI-FCS	heat inactivated foetal calf serum
HIV	human immunodeficiency virus
Hp	haptoglobin
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl β-$_D$-thiogalactopyranoside
kDa	kiloDaltons
LPS	lipopolysaccharide
MAP	multiple antigenic peptide
MHC	major histocompatability complex
MMLV	Moloney murine leukaemia virus
NF-κB	nuclear factor κB
NO	nitrous oxide
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction

pig-MAP	pig major acute phase protein
RACE	rapid amplification of cDNA ends
rh/m	recombinant human/ murine
RNA	ribonucleic acid
RT-PCR	reverse transcription-PCR
SAA	serum amyloid A
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
SPF	specific pathogen free
sTNFR	soluble TNFR
T	thymine
TEMED	N,N,N',N',-tetramethylethylenediamine
Th	T helper cell
Tm	melting temperature
TNF	tumour necrosis factor
TNFR	TNF receptor
TP	total protein
TRAF	TNFR-2 associated factors

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