Studies on Ovine Tumour Necrosis Factor-Alpha.

by
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Dedication:-

This work is dedicated to the memory of R.E.Green (1926-1991), without whom it would not have been possible.

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Declaration:-

I hereby certify that this thesis and the work it describes were written and performed by myself, except where I have stated otherwise.

Signed,

18/6/93

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ABSTRACT.

Tumour necrosis factor alpha (TNFα), a mediator of inflammatory responses and pathologies of a wide variety of diseases, has been extensively studied in humans and mice. However, little has previously been known about this cytokine in the sheep, a species of value not only to the agricultural industry, but also as a laboratory animal.

In this work, the cDNA encoding ovine TNFα has been amplified, cloned, sequenced and used to express recombinant ovine TNFα (rovTNFα). The latter has been partially purified, characterised and used to raise both poly- and mono- clonal antibodies.

The sequence of ovine TNFα shows a high degree of homology to those of other species. Certain regions, which are known to be structurally or functionally important to the mRNA and/or protein, are particularly well conserved. Consequently, rovTNFα displays several biological activities previously noted for TNF'sα of other species, including cytotoxicity, enhancement of thymocyte and fibroblast proliferation and cartilage-degrading and anti-viral activities. However, whilst rovTNFα is active in assays on ovine cells at concentrations comparable to those observed in similar assays for other species, it is 1000 fold less active than recombinant human TNFα (rhTNFα) in cytotoxicity assays on TNF-sensitive murine (L929) cells, whose general lack of species specificity allows their use in detecting TNF'sα from many sources.

A monoclonal antibody raised to rovTNFα detects a glycoprotein of appropriate size for mature ovine TNFα in the supernatants of stimulated ovine cell cultures. As in other species both ovine TNFα mRNA and protein are rapidly inducible. Such supernatants repeatedly have no activity in cytotoxicity assays (sensitive to 30pg rhTNFα/ml) on L929 cells, in spite of many containing >1ng ovine TNFα/ml. At least one of these supernatants displays biological activity attributable to TNFα (through its neutralisation by a polyclonal antiserum raised to rovTNFα) in an assay on ovine cells. By comparing the amino acid sequences of TNF'sα from many species and using knowledge gained from structure/function studies on human TNFα, possible explanations for the apparent species specificity of ovine TNFα are proposed.

Finally, preliminary investigations have been performed to examine the rôle that TNFα plays in Maedi-Visna (MV) disease, a chronic lentivirally-induced ovine disease. RovTNFα can differentially regulate MV viral expression in different cell types in vitro, whilst the native protein is produced in MV-infected cultures of adherent ovine lung cells. Ovine TNFα may therefore play a complex rôle in MV disease, both by contributing to its pathogenesis and influencing the viral life-cycle.

CHAPTER 1:- TUMOUR NECROSIS FACTOR ALPHA AND ITS POTENTIAL ROLES IN OVINE DISEASE: AN INTRODUCTION.

1.1. TNFα: historical aspects:-

In the second half of the 19th century, several workers observed the spontaneous regression of some human tumours following episodes of bacterial infection and attempted to exploit this phenomenon by deliberately infecting selected cancer patients (Busch, 1866; Fehleisen, 1882; Coley, 1893). Though partial responses were sometimes obtained, such therapy was not surprisingly marred by undesirable reactions. Coley (1906) later found that some bacterial extracts could also be effective and although the use of 'Coley's toxins' was still associated with iatrogenic toxicity, it became one of the few recognised medical treatments for neoplasia in the early 1900's.

In attempts to isolate the active fraction(s) from Gram-negative bacteria, Shear et al. (1943) succeeded in purifying the component now known as endotoxin, or lipopolysaccharide (LPS). Unfortunately however, whilst LPS could induce haemorrhagic necrosis of transplanted tumours in mice, a 'shock-inducing' toxic activity co-purified with this fraction (Shear, 1944).

In 1962, O'Malley et al. demonstrated that the anti-tumour effect of heat-stable LPS was indirect, since it induced a heat-labile agent(s) in the sera of treated mice, which had tumour-necrotising activity when transferred to other tumour-bearing mice. The existence of an endogenous mediator was confirmed by Carswell et al. (1975), who extended the observation of tumour necrosis activity in vivo to a direct, in vitro cytotoxicity of such sera for cells of the transformed, murine, L cell line. This provided a simpler assay for the component they dubbed 'Tumour Necrosis Factor' (TNF).

TNF was subsequently purified (Aggarwal et al., 1985b) and cDNA's encoding TNF in several species were cloned and sequenced (see chapter 2), whereupon it became apparent (Pennica et al., 1984) that TNF shared significant homology with another cytotoxic factor, lymphotoxin (LT), a product of mitogen-stimulated T-lymphocytes (Gray et al., 1984). Though antigenically distinct, it was shown that TNF and LT

bind to the same cell surface receptors (Aggarwal et al., 1985a), generally evoking similar responses. This led to the renaming of TNF and LT as TNFα and TNFß, respectively.

Sequencing also demonstrated that TNFα was identical to 'cachectin' (Beutler et al., 1985a). Cachectin had been studied for several years by Cerami et al. as a result of investigations into the cachexia suffered by trypanosome-infected cattle and rabbits. These rabbits developed an hypertryglyceridaemia, linked to a deficiency of the enzyme lipoprotein lipase (LPL) (Rouzer & Cerami, 1980). Similar derangement of lipid metabolism could be induced by LPS-treatment of endotoxin -sensitive, but not -resistant, strains of mice (Kawakami & Cerami, 1981) and the presence of an endogenous mediator, which they later named cachectin because of its likely rôle in the metabolic disturbances of cachexia, was confirmed by inter-strain transfer of serum.

The identity of TNF and cachectin defined TNFα as a pleiotropic molecule and fuelled searches for other actions. Michalek et al. (1980) had previously demonstrated that in addition to its tumour-necrotising action, the toxic effects of LPS were also indirect. It was soon found that TNFα could induce a shock-like state itself (Tracey et al.,1986) and furthermore, prior, passive immunisation with antibodies to TNFα could confer protection against LPS-induced lethality (Beutler et al.,1985c). Thus TNFα was identified not only as an essential mediator of LPS toxicity but also as 'much more than a tumour necrosis factor,' as will be further illustrated below.

1.2. TNFα: cell sources and inducers:-

In their early work, Carswell et al. (1975) found that mice required priming with an agent such as BCG before LPS treatment induced sufficient TNFα for detection. Since BCG was known to induce hyperplasia of the lymphoreticular system, cells of monocyte/ macrophage lineage were considered possible sources of LPS-induced TNFα. Such suspicions were subsequently confirmed by several workers using isolated cell populations (Männel et al., 1980; Matthews, 1978, 1981a,b; Zacharchuk et al., 1983), whilst Kawakami and Cerami (1981) had likewise found that macrophages were a major source of cachectin. TNFα does not exist in stored form in these cells, nor is it secreted

by resting monocyte/macrophages (Beutler et al., 1986a). However, their potential for producing large amounts of TNFα de novo on activation was demonstrated by Beutler et al. (1985b), who estimated that TNFα comprised as much as 2% of the total secreted product of LPS-stimulated RAW 264.7 cells (a macrophage cell line).

Whilst macrophages from all tissues studied can produce TNFα (Decker et al., 1987), the potential to induce TNFα varies between different forms of LPS and shows some correlation with the pathogenicity of their source (Loppnow et al., 1990). LPS derived from non-pathogenic bacteria, or incomplete forms of LPS, may even block the induction of TNFα by other forms (Männel & Falk, 1989).

Many other agents have also been tested for their ability to induce TNFα, occasionally with conflicting results. Amounts of TNFα produced in response to these alternative stimuli are often much lower than those induced by endotoxin. Hence, because of its potency and widespread presence in vitro, possible contributions by contaminating LPS should always be considered in such studies.

Several other types of bacteria or their products have now been shown to be capable of inducing TNFα from monocyte/macrophages. These include some products of Gram-positive bacteria, for example polysaccharide f from Streptococcus mutans and toxic shock syndrome toxin 1 from Staphylococcus aureus (Benabdelmoumene et al., 1991; Fast et al., 1988; Jupin et al., 1988), as well as organisms with a predominantly intracellular lifestyle, such as Listeria monocytogenes (Havell, 1987), Mycobacterium tuberculosis (Rook et al., 1987) and Mycoplasmae (Sugama et al., 1990). With regard to the latter group, it has also been noted that phagocytosis of undigested particles, such as zymosan, can lead to sustained TNFα release (Stein & Gordon, 1991).

Since cachectin was originally discovered following research into trypanosomiasis, it is not surprising that other protozoa, such as Plasmodium, or their soluble antigens, have proven to be TNFα inducers (Bate et al., 1988). Macrophages also produce TNFα on encountering assorted viruses, including Encephalomyocarditis virus, Vesicular Stomatitis virus, Adenovirus 2, Herpes simplex virus and even viral features such as double-stranded RNA (Wong & Goeddel, 1986). TNFα production in response to infection with Human Immunodeficiency virus (HIV) has also been documented (Merrill et al., 1989), though this has

been the subject of some debate (see 1.8.5). Aderka et al. (1986) found that Sendai virus can be as potent a stimulus as LPS, whilst Influenza A virus not only stimulates TNFα production but also markedly potentiates its induction by LPS (Nain et al., 1990). In summary, TNFα can be induced by a wide range of aetiological agents.

Numerous endogenous inducers of TNFα have also been described. These include specific pathological products, such as urate crystals (diGiovine et al., 1991), components of tumour cells (Jänicke & Männel, 1990) and advanced glycosylation end-products, found in ageing tissues (Vlassara et al., 1988), as well as products of the immune system, such as IgG immune complexes (Warren et al., 1991) and C5a, formed during activation of complement (Okusawa et al., 1988). The second group also includes several cytokines:- GM-CSF (Chantry et al., 1990); IL-2 (Economou et al., 1989a); IL-1 and TNFα itself (Philip & Epstein, 1986).

TNFα was once believed to be exclusively produced by monocyte/ macrophages. However, more sensitive detection methods have now identified its production in many other cell types and though such cells generally produce much smaller amounts of TNFα in cell-for-cell comparisons with macrophages, they can represent an important source of TNFα on account of their overall tissue mass.

Most known subsets of lymphocytes can make TNFα in addition to TNFβ. First reports of TNFα production by lymphocytes involved Natural Killer (NK) cells, from which it can be induced by NK -sensitive but not -resistant cell targets (Degliantoni et al., 1985; Peters et al., 1986) and yeast cells (Djeu et al., 1988). Both $CD4^+$ and $CD8^+$ subsets of T-lymphocytes secrete TNFα in response to mitogens, but not LPS (Cuturi et al., 1987) and Van Kooten et al. (1991) have further suggested that memory and naïve sub-divisions of $CD4^+$ cells have similar capacities for TNFα production. Lymphokine-activated killer (LAK) cells secrete TNFα in response to IL-2 (Espevik et al., 1988), whilst immature T-cells, in the form of thymocytes (Ranges et al., 1988), and B-cells (Sung et al., 1988b) also produce TNFα when induced to proliferate. Furthermore, TNFα transcripts have been observed in the latter following infection with Sendai virus (Goldfeld & Maniatis, 1989).

Neutrophils make and release TNFα in response to LPS (Dubravec et al., 1990) and yeast (Djeu et al., 1990), whilst mast cells appear to be unique amongst TNFα-producers in storing pre-formed TNFα, which is released, along with other mediators, on degranulation (Steffen et al., 1989; Gordon & Galli, 1990).

Other potential TNFα sources include astrocytes exposed to LPS or some neurotropic viruses (Robbins et al., 1987; Lieberman et al., 1989) and LPS-stimulated renal mesangial cells (Baud et al., 1989) and keratinocytes. The latter also produce TNFα in response to UV-light (Köck et al., 1990) and possibly irritants (Piguet et al., 1991). In the normal, pregnant uterus, TNFα mRNA and protein have been found in several non-macrophage cell types, including epithelial and decidual cells, as well as placental trophoblasts (Yelavarthi et al., 1991). Smooth muscle cells secrete TNFα in response to a 'non-physiological' combined stimulus of LPS with a brief exposure to protein synthesis inhibitors (Warner & Libby, 1989) and the secretory Paneth cells of normal intestine can contain TNFα transcripts (Keshav et al., 1990).

'Inappropriate' expression of TNFα has also been documented from a variety of tumour cells. Assorted EBV-transformed B-cell lines (Williamson et al., 1983), many B-cell chronic lymphocytic leukaemias (Cordingley et al., 1988) and several human epithelioid cell lines (Spriggs et al., 1988) may secrete TNFα constitutively, whilst selection of TNF-resistant murine fibrosarcoma cells led to the identification of a subset of these cells producing low levels of TNFα themselves (Rubin et al., 1986).

Finally, it has recently emerged that poorly-understood neural or neuro-endocrine pathways to TNFα production also exist. Intracerebroventricular injection of LPS induces a marked peripheral accumulation of TNFα that cannot be accounted for either by export of TNFα or leakage of LPS from the central nervous system (Ghezzi, 1992).

1.3. Induction of TNFα; gene, transcript and protein structure:-

1.3.1. Gene structure:-

Shortly after the cDNA's for human and murine TNFα were cloned (see chapter 2) the corresponding genes were also cloned and sequenced (Nedwin et al., 1985a; Semon et al., 1987), revealing that in each of these species the TNFα gene is closely linked to that of TNFβ, being

some 1100 bases 3' of the latter. This locus mapped to chromosomes 6 and 17 in the human and mouse respectively (Nedwin et al.,1985a; Nedospasov et al., 1986), the same chromosomal location as the MHC in each. Later work confirmed that the TNF locus actually resides within the MHC (Spies et al., 1986; Müller et al., 1987), though the reasons for this association are unclear.

Each TNF gene is approximately 3kb long and interrupted by three introns with the fourth exon coding for the majority of the secreted protein in each case, suggesting that the two genes arose from a duplication event. Significant homology between the TNFα and β genes only occurs in the fourth exon, though the whole TNF locus shows considerable homology between the two species.

1.3.2. Gene induction:-

The early identification, potency and importance of the LPS-stimulated macrophage as a TNFα producer have made it the most extensively analysed model of TNFα production. These studies demonstrate that TNFα production is controlled at several levels. Whilst some workers conclude that the TNFα gene is transcriptionally inactive in resting, human monocytes in vitro (Sariban et al., 1988), others have found a low level of constitutive transcription in murine peritoneal macrophages, both in vitro and in vivo (Jongeneel et al., 1989; Collart et al., 1990). All agree, however, that following appropriate stimulation the rate of TNFα gene transcription increases.

The regulatory region of this gene contains several sites which appear to be important in transcriptional control, including four with homology to recognition motifs for the NF-kappaB family of transcription factors (Sen & Baltimore, 1986) and a single MHC class II-like 'Y box' (Dorn et al., 1987). Following stimulation of macrophages with LPS, the nuclear concentration of NF-kappaB-like proteins rises (Collart et al., 1990), presumably following dissociation of these factors from I-kappaB in the cytoplasm (Baeuerle & Baltimore, 1988), and several DNA-protein complexes form in the TNFα promoter, formations which can be specifically blocked by oligo-nucleotides with the aforementioned sequences (Collart et al., 1990; Shakhov et al., 1990). Furthermore, the inclusion of two or more of the NF-kappaB motifs confers LPS-inducibility on an heterologous

promoter, and deletion analyses, using promoter/reporter gene constructs, suggest that a region containing the Y box is essential for LPS-inducibility of the TNFα promoter (Shakhov et al., 1990).

Pathways linking LPS-exposure to alterations in the binding of transcription factors to the TNFα promoter are incompletely understood. However, some features are apparent:- 1) LPS induction of TNFα appears to be mediated via interaction with a cellular receptor, CD14 (Wright et al., 1990); 2) known second messengers may be involved: inhibitors of protein kinase C can block LPS induction of TNFα (Kovacs et al., 1988), whilst raised levels of cAMP and cGMP are associated with decreased and increased TNFα production, respectively (Renz et al., 1988; Endres et al., 1991); 3) differential responses to cyclic nucleotides in these studies and the results of others, which demonstrate that cells rendered refractory to LPS with respect to TNFα production can still secrete IL-1, IL-6 and IL-8 (Takasuka et al., 1991; Fantuzzi et al., 1992), suggest that the LPS-induced pathway leading to TNFα production differs from those leading to the production of other LPS-inducible monokines; and 4) there is clearly more than one pathway to TNFα induction, since zymosan can induce TNFα from LPS-refractory cells (Takasuka et al., 1991).

Involvement of NF-kappaB proteins in TNFα transcriptional control helps to explain its inducibility by some other stimuli, particularly other cytokines (see 1.2, 1.6). However, the extent to which different pathways to TNFα induction may converge is not clear. Although activators of protein kinase C such as phorbol-esters can increase the nuclear concentration of an NF-kappaB-like protein (Griffin et al., 1989) and induce TNFα gene transcription, an area of the TNFα promoter which does not contain any NF-kappaB binding sites is sufficient for full phorbol ester-responsiveness (Economou et al., 1989b), suggesting the existence of an NF-kappaB-independent pathway to induction. Regions with homology to other known transcription factor binding motifs (such as an AP-1 site and a cAMP-responsive element) do exist in the TNFα promoter, which can also bind another transcription factor, NF-IL-6 (Hensel et al., 1989), though the importance of these findings is unclear. Nedospasov et al. (1992) have also identified another protein-binding site in the 3' untranslated region (UTR) of the TNFα gene, which may play a role in TNFα inducibility.

How the expression of the TNFα gene is restricted to certain cells is also poorly understood. Clearly, differential distribution of molecules like CD14 or NF-kappaB proteins could contribute to tissue-specific expression. However, Kruys et al. (1992) have demonstrated that components in the TNFα gene 3' UTR effectively cancel out a potentially constitutive activity of the gene promoter in some cell types, but not others.

1.3.3. TNFα transcripts:-

Following LPS stimulation of monocyte/macrophages, spliced TNFα transcripts of 1.6-1.7 kb accumulate then rapidly disappear. For example, TNFα mRNA reaches maximum levels within one hour, then becomes undetectable, by Northern blotting, within four hours of stimulating murine macrophages in vivo (Remick et al., 1989).

The short-lived nature of TNFα mRNA may be associated with the presence of a UA-rich sequence in the 3' UTR. This sequence, which is well conserved across species, contains an overlapping and repeated octomeric element (UUAUUUAU), also found in the mRNA of many other inflammatory cytokines (Caput et al., 1986), which confers instability on transcripts bearing it (Shaw & Kamen, 1986), probably mediated by a selective ribonuclease (Beutler et al., 1988).

Consideration of the quantity of TNFα secreted by the LPS-stimulated macrophage, which is difficult to account for purely by increases in mRNA levels, helped lead to suggestions of additional, post-transcriptional control of TNFα production (Beutler et al., 1986a). Han et al. (1990) subsequently showed that the same region responsible for transcript instability also bestows (with contributions from surrounding sequences) the property of translational inducibility by LPS. Though the additional LPS-stimulated pathway which leads to this induction is poorly understood, the presence of a similar sequence (derived from interferon [IFN] ß mRNA) can inhibit translation in animal, but not plant, systems (Kruys et al., 1987), suggesting the presence of a translational block, which can be overcome by an appropriate stimulus.

1.3.4. TNFα protein structure:-

The open reading frame (ORF) of an human TNFα transcript comprises 233 codons, predicting a full-length protein of 26kDa (Pennica et al., 1984), whilst secreted human TNFα is only c.17kDa. Amino-terminal sequence analysis of the purified, mature protein reveals that it derives from the carboxy-terminal 157 amino acids (aa) of the pre-protein (Aggarwal et al., 1985b).

At 76 aa the 'discarded' propeptide is significantly longer than typical signal peptides of many secreted proteins and is better conserved across species than the secreted protein (86% compared to 79% in a human-mouse comparison), suggesting that it has an important rôle. Kriegler et al. (1988) found that, in addition to the secreted form, human TNFα also exists in stimulated macrophages as an integral, transmembrane 26kDa protein with an extracellular carboxy-terminus. Thus, one function of the leader is to anchor the full-length molecule to the membrane. These authors also demonstrated that, although inactive in free solution, recombinant 26kDa TNFα can function when membrane-bound, a finding which extends to native cell-surface TNFα (Decker et al., 1987). This form of TNFα also exists in activated T-cells (Kinkhabwala et al., 1990).

Scuderi (1989) proposed that 17kDa, mature, human TNFα is finally secreted via cleavage of the 26kDa precursor at the cell-surface by a serine protease, though whether all secreted TNFα is produced in this way is unclear. In humans and mice the presence of alternative, minor cleavage sites may contribute to some heterogeneity in the size of the secreted protein (Perez et al., 1990; Cseh & Beutler, 1989) and in the latter species additional post-translational modification takes place in the form of glycosylation (Green et al., 1976), though the rôle of the carbohydrate group is unclear. Factors governing how, and to what extent, cleavage occurs are still largely unknown. However, the existence of post-translational control of TNFα secretion must be suspected, since conditions allowing the accumulation of TNFα pre-protein have been described (Zuckerman et al., 1989).

Early characterisations suggested that TNFα exists in higher molecular weight forms than the 17kDa or 26kDa monomeric entities (Matthews, 1981a; Aggarwal et al., 1985b). Whilst each 17kDa molecule has two cysteine residues, these form an intrachain disulphide bridge

(Davis et al., 1987). However, larger structures can be explained by non-covalent association of 17kDa subunits (Davis et al., 1987). Smith and Baglioni (1987) proposed that most of the activity of TNFα is associated with a homo-trimeric form, though others have postulated that significant activity can be associated with dimers of TNFα (Petersen et al., 1989). Crystallographic studies confirm TNFα's trimeric nature (Eck et al., 1988) and suggest that three 17kDa monomers, each folded into an elongated, antiparallel, β-pleated sandwich, with a 'jelly-roll' topology and some structural homology to a viral coat protein, come together to form a compact, bell-shaped structure (Eck & Sprang, 1989; Jones et al., 1989). The significance of such homology is currently unclear, as is the point at which newly-synthesised TNFα trimerises, though self-assembly in solution is possible (Davis et al., 1987).

1.4. Actions of TNFα in vitro:-

Since its early recognition as a pleiotropic molecule, the catalogue of actions attributable to TNFα has steadily grown. This list includes the following, partially-overlapping, groups of activities:- pro-inflammatory; tissue-remodelling; metabolic; cytotoxic; endocrine; and direct influences on the life-cycles of assorted pathogens.

1.4.1. Pro-inflammatory actions:-

Many of the pro-inflammatory activities attributable to TNFα can be perceived as being likely to lead to the accumulation of activated leukocytes at an appropriate site. They involve TNFα acting on cells of the immune system, as well as the vascular endothelium.

In addition to being a prime source of TNFα, cells of monocyte/macrophage lineage are also targets for its action. TNFα is chemotactic for these cells (Wang et al., 1987, 1990), can promote the maturation of promonocytic cell lines (Griffin et al., 1989; Peetre et al., 1986) and increase the survival time of monocytes in serum-free conditions (Mangan et al., 1991). Macrophages cultured in the presence of TNFα also acquire increased abilities to destroy some pathogens (Bermudez & Young, 1988), mediated, at least in part, via the induction of reactive nitrogen intermediates (Liew et al., 1990a).

TNFα's potential as a pro-inflammatory agent is also heightened by its ability to stimulate these cells and/or fibroblasts and endothelial cells, to produce other inflammatory mediators such as IL-1 (Dinarello et al., 1986), IL-6 (Kohase et al., 1986), IL-8 (Matsushima et al., 1988), GM-CSF (Munker et al.,1986), M-CSF (Oster et al., 1987), platelet activating factor (PAF)(Cammussi et al., 1987), monocyte chemotactic factor (Dixit et al., 1990), platelet-derived growth factor (Hajjar et al., 1987), PGE_2 (Dinarello et al., 1986) and itself (Philip & Epstein 1986).

Other leukocytes are similarly affected following exposure to TNFα. It can cause neutrophils to rapidly adhere to endothelial cells (Gamble et al., 1985) and is also a chemotactic factor for these cells (Wang et al., 1987). Subsequent migration away from a source of TNFα may then be inhibited (Fast et al., 1988). TNFα also primes or, provided they have migrated from suspension to be supported by a suitable matrix, actually stimulates neutrophils' functional responses (Nathan, 1987). Thus, TNFα-treatment enhances their binding to certain bacteria (Steadman et al., 1991), increases their phagocytic ability (Klebanoff et al., 1986; Van Strijp et al., 1991), promotes a respiratory burst, H_2O_2 production and degranulation (Nathan, 1987) and induces them to secrete other mediators such as PAF (Camussi et al., 1987). Eosinophils are likewise functionally activated by TNFα to show enhanced antiparasitic activity (Silberstein & David, 1986).

TNFα not only helps to activate lymphocytes but it also enhances their proliferation. Many surface markers associated with lymphocyte activation, such as TNF receptors (TNF-R's), MHC class II proteins and the Ly6A/E protein, are upregulated on T-cells, an NK cell line and LAK precursors by treatment with TNFα, as are IL-2-R's (Scheurich et al., 1987; Hackett et al., 1988; Lee J. et al., 1987; Chouaib et al., 1988; Malek et al., 1989), helping to explain several synergistic actions between TNFα and IL-2. TNFα enhances IL-2-induced secretion of immunoglobulin by B-cells (Kehrl et al., 1987) and, in combination with IL-2, induces T-cells to secrete IFN gamma (Scheurich et al., 1987). T-cells, thymocytes and B-cells all show enhanced proliferation to mitogens, or IL-2, in the presence of TNFα (Hackett et al., 1988; Ranges et al., 1988; Kerhl et al., 1987; Jelinek & Lipsky, 1987; Zucali et al., 1987), whilst Ranges et al. (1989) identified a T-cell

line for which TNFα can actually replace IL-2 as a proliferative signal. It also appears to play a rôle in the development of cytotoxic lymphocytes (Ranges et al., 1987) and augments the IL-2-induced generation of LAK cells (Espevik et al., 1988; Chouaib et al., 1988).

In addition to acting on peripheral blood elements, TNFα influences erythroid progenitors, since it inhibits colony formation by granulocyte/ macrophage-, erythroid- and multipotential- progenitor cells (Broxmeyer et al., 1986; Peetre et al., 1986).

The vascular endothelial cell responds to TNFα with morphological, secretory (see above) and cell surface changes, many of which favour the development of an inflammatory response. Following TNFα treatment, endothelial cells increase their metabolic rate, enlarge and change shape, forming intercellular gaps and thus increasing the permeability of a monolayer to macromolecules and other solutes (Stolpen et al., 1986; Cavender et al., 1989; Brett et al., 1989). At the same time, they display increased adhesiveness for leukocytes (Gamble et al., 1985; Cavender et al., 1987) (and even some bacteria [Cheung et al., 1991]), largely due to the upregulation of adhesion molecules, such as ICAM-1, ELAM-1 and VCAM-1 (Pober et al., 1986b; Bevilacqua et al., 1987; Osborn et al., 1989a). Ikuta et al. (1991) observed that such increases may lead to the binding of NK cells and a subset of memory, helper T-cells in preference to other lymphocytes. MHC class I molecules also become more abundant on these (Pober et al., 1986b) and other (Collins et al., 1986) cells.

TNFα also acts on endothelial cells to favour coagulation (Bevilacqua et al., 1986). Enhanced procoagulant-, and reduced anticoagulant-, activities have been associated with increased tissue factor expression and a decrease in endothelial cell-dependent protein C activation, respectively (Nawroth & Stern, 1986). Reduced production of thrombomodulin (Lentz et al., 1991), as well as its internalisation and degradation (Moore et al., 1989), increased levels of plasminogen activator inhibitors (Mawatari et al., 1991) and the sequestration of plasminogen activator away from the circulation (Van Hinsbergh et al., 1990) all contribute to the latter effect.

TNFα can also increase the expression of MHC class II molecules induced on these cells (Lezcynski, 1990), as well as some monocytic cell lines (Arenzana-Seisdedos et al., 1988), by IFN gamma. However,

these effects may depend on the chronology of events and the state of cellular differentiation. Leeuwenberg et al. (1988) found that TNFα added with, or before, IFN gamma actually opposed its effects on endothelial cells, whilst Watanabe and Jacob (1991) observed antagonism on a selection of mature but not immature cells.

1.4.2. Tissue-remodelling actions:-

Several actions of TNFα are involved in processes of tissue remodelling. Fibroblasts respond to TNFα with enhanced proliferation in the presence of serum (Sugarman et al., 1985; Vilcek et al., 1986), perhaps mediated by an upregulation of EGF-R's (Palombella et al., 1987). This is accompanied by a prolonged increase in collagenase production (Dayer et al., 1985) and a concurrent decrease in collagen synthesis (Solis-Herruzo et al., 1988). Under the influence of TNFα, bone and cartilage resorption are also stimulated, whilst new bone formation and the synthesis of glycosaminoglycans are suppressed (Bertolini et al., 1986; Saklatvala, 1986; Ikebe et al.,1988). TNFα also enhances the proliferation of astrocytes (Selmaj et al., 1990) and a rôle for TNFα in angiogenesis may be predicted by the observation that it induces endothelial cells to form capillary-tube-like structures when grown on collagen cells (Leibovich et al., 1987).

1.4.3. Metabolic actions:-

Early suggestions by Cerami et al. (see 1.1) that TNFα has important effects on body metabolism have since been confirmed by several studies on fat, muscle and liver cells. It downregulates the transcription of several, normally-active genes in adipocytes, as well as preventing the morphological differentiation of these cells. Specific enzymic activities reduced include LPL, fatty acid synthetase, acetyl CoA carboxylase and glycerolphosphate dehydrogenase (Pekala et al., 1983; Torti et al., 1985). TNFα also inhibits the differentiation of myocytes (Miller et al., 1988) and stimulates glycogenolysis in these cells (Lee M. et al., 1987).

Hepatoma cells produce some complement proteins and α1-chymotrypsin in response to TNFα, whilst decreasing their synthesis of albumin and transferrin (Perlmutter et al., 1986), suggesting a possible rôle for TNFα in acute phase protein synthesis.

1.4.4. Cytotoxic actions:-

Whilst TNFα generally does not kill normal cells, responses can be changed when cells become transformed or parasitised. Following Carswell et al.'s original observation (1975) that TNFα was toxic to cells derived from a murine fibrosarcoma, subsequent surveys showed that 30-50% of transformed cell lines are susceptible to the cytotoxic or sometimes cytostatic action of TNFα (Williamson et al., 1983; Sugarman et al., 1985). Wong and Goeddel (1986) found that TNFα can also kill some cells infected with certain viruses, whilst others have since shown that simply expressing some viral proteins (such as Adenovirus E1A or SV40 large T) can convert a cell to TNFα sensitivity (Chen M-J. et al., 1987; Duersken-Hughes et al., 1989; Ames et al., 1990). TNF's α & β appear to be major mediators of the antiviral activity of NK cells by such actions (Paya et al., 1988). Other intracellular pathogens, such as Mycobacteria, and their antigens, can also render cells more sensitive to TNFα cytotoxicity (Filley & Rook, 1991).

Some exceptions to the above generalisations can be found. High doses of TNFα can kill normal endothelial cells (Robaye et al., 1991), though this effect may depend on cellular sub-type, as Meyrick et al. (1991) found that TNFα could kill cells derived from the aorta, but not the pulmonary microvasculature, of the same animal. TNFα is also toxic to normal oligodendrocytes and can damage the myelin they produce (Robbins et al., 1987; Selmaj & Raine, 1988). Furthermore, it can combine with IFN gamma to kill pancreatic islet cells in culture (Pukel et al., 1988). However, the evolutionary reasons for such apparently undesirable activities are unclear. Also in contrast to its general actions, TNFα may actually be a growth factor for a few transformed cells, notably those derived from some B-cell chronic lymphocytic leukaemias (Cordingley et al., 1988; Digel et al., 1989).

1.4.5. Endocrine actions:-

TNFα forms one link between the body's immune and endocrine systems. It induces the release of ACTH, growth hormone and thyrotropin from pituitary cells (Milenkovic et al., 1989), yet inhibits ACTH-, FSH- and HCG- induced steroidogenesis in adrenal, ovarian and testicular cells respectively (Jäättelä et al., 1990;

Adashi et al., 1989; Warren et al., 1989). In contrast, the direct effects of TNFα on steroidogenesis in these cells is stimulatory (Darling et al., 1989; Roby & Terranova, 1988; Warren et al., 1989). Whilst some of these actions presumably help to mobilise body resources during illness, others may be involved in reproductive biology, growth (see 1.8.7) or have feedback implications (see 1.7).

1.4.6 Direct influences on life-cycles:-

TNFα can also affect the life-cycles of assorted pathogens in several, sometimes-surprising ways. In addition to killing some virally-infected cells, TNFα also exerts an antiviral action via the induction of 2'-5' oligoadenylate synthetase (Mestan et al., 1988; Wong & Goeddel, 1986). How much of this activity is actually mediated via the induction of interferons, with which TNFα synergises, has been a subject of dispute (Mestan et al., 1986; Kohase et al., 1986; Van Damme et al., 1987; Reis et al., 1988). However, TNFα only appears to be able to inhibit some viruses in some cell types. Schijns et al. (1991) found that of brain cells infected with Aujeszky's virus, TNFα only exerted antiviral activity in astrocytes. Furthermore, TNFα can actually enhance the proliferation of HIV in some cells (see 1.8.5).

The ability of TNFα to kill cells infected with other pathogens, or to activate macrophages to kill, has been mentioned. In addition, TNFα can simply inhibit the intracellular replication of some pathogens (de Titto et al., 1986) and may kill malarial parasites more directly (Taverne et al., 1984).

Finally, some nematodes may actually exploit TNFα to their advantage, in spite of its ability to enhance parasiticidal activity. Adult Schistosoma mansoni worms respond directly to TNFα by a 15-fold increase in egg-laying, a response the parasite may have developed to leave eggs in the protective environment of a TNF-induced granuloma (Amiri et al., 1992).

1.5. Actions of TNFα in vivo:-

Given the myriad of activities displayed by TNFα, the net effect of its administration to normal animals *in vivo* is highly dependent on the dose, route and duration of treatment. Due to the induction of other mediators, it may also, on occasion, be opposite to that expected from the results of *in vitro* studies.

Tracey *et al*. (1986) first demonstrated that the intravenous (IV) infusion of rats with high doses of TNFα reproduces most of the clinical signs of septic shock, including hypotension, metabolic acidosis, early hyperglycaemia later converting to hypoglycaemia, hyperkalaemia, bloody diarrhoea and ultimately death from respiratory arrest. Post-mortem examination of these animals revealed inflammation, haemorrhage and necrosis in many organs.

Changes seen following sub-lethal doses of IV TNFα are less severe but include some intravascular coagulation (Van der Poll *et al*., 1990), panleukopaenia then subsequent neutrophilia (Van der Poll *et al*., 1992a) and increases in energy expenditure, CO_2 production, blood cortisol and triglyceride levels, and muscle catabolism, though blood amino acid levels may actually fall (Starnes *et al*., 1988; Flores *et al*., 1989), perhaps due in part to enhanced uptake by the liver (Warren *et al*., 1987). Dinarello *et al*. (1986) also found that low doses of TNFα induce a single peak of fever, probably mediated via the direct induction of PGE_2 from hypothalamic cells. Higher doses, however, induce a biphasic response, with a second peak attributable to TNFα-induced IL-1. The importance of secondary agents in mediating TNFα actions *in vivo* is further illustrated by observations that antagonists to PAF (Sun & Hsueh, 1988), IL-1 (Everaerdt *et al*., 1992) or IL-6 (Starnes *et al*., 1990) can each confer significant protection against TNFα-induced toxicity.

In contrast to their findings following the IV administration of high doses of TNFα, Tracey *et al*. (1988) found that repeated intra-peritoneal (IP) injections of lower doses led to a different syndrome involving anaemia and weight loss, with reductions in both body protein and fat. Tissue inflammation with leukocytosis was also noted at the site of injections. Cachexia was similarly observed following the inoculation of nude mice with neoplastic cells constitutively expressing TNFα, but not with control cells (Oliff *et al*., 1987).

However, there is more than one pathway to TNFα-induced weight-loss. Whilst classical cachexia results when these cells are implanted intramuscularly, their inoculation intracerebrally induces profound anorexia (Tracey et al., 1990). Chronic infusion of TNFα can also cause some hepatic necrosis (Gaskill, 1988).

The effects of continuous, dysregulated overproduction of TNFα have been further studied using transgenic mice which constitutively express human TNFα (Keffer et al., 1991). Whilst these mice fail to put on weight normally, they also develop a chronic, inflammatory polyarthritis, a finding in keeping with a proposed rôle for TNFα in rheumatoid arthritis (see 1.8.6).

Acute, local administration of TNFα leads to inflammation, whether in the skin (Sharpe et al., 1987) or brain (Ramilo et al., 1990). This can involve oedema and an early neutrophil accumulation, particularly in the presence of a vasodilator such as PGE_2 (Rampart et al., 1989). A slower mononuclear accumulation may follow (Munro et al., 1989), whilst dendritic cells migrate to the local draining lymph node (Cumberbatch & Kimber, 1992).

Sustained infusion of TNFα in a local, skin model again leads to an early accumulation of neutrophils but after a few days the nature of the reaction changes, with epidermal thickening, fibroblastosis, increased collagen deposition and new capillary formation being evident (Piguet et al., 1990), underlining TNFα's potential rôle in tissue remodelling.

1.6. Cellular mechanisms of TNFα actions:-

Early work indicated that TNFα interacts with high-affinity cell surface receptors (Aggarwal et al., 1985a). cDNA's encoding two distinct human TNF-R's, types I and II (TNF-RI and TNF-RII) have now been cloned, sequenced and expressed (Nophar et al., 1990; Schall et al., 1990; Loetscher et al., 1990; Smith C. et al., 1990) as have their murine homologs (Barrett et al., 1991; Lewis et al., 1991). (Different authors have also called these receptors p55, p60, type B or confusingly type II receptor, and p75, p80, type A or confusingly type I receptor, respectively.)

TNF-R's have been found on most mammalian cells, except for red blood cells and platelets (Munker et al., 1987), in keeping with TNFα's widespread activities. Cells of epithelial origin seem to predominantly express TNF-RI, whilst myeloid cells may express both types (Hohmann et al., 1989, 1990a; Brockhaus et al., 1990). Whether further TNF-R's exist remains to be seen. Using a combination of high-affinity antibodies to TNF-RI and TNF-RII it is possible to totally block the binding of TNFα to some cell types but not others (Dr.B. Aggarwal, personal communication). Whilst this might point to the existence of other receptors, TNFα can bind to lipid membranes directly under some conditions, notably those of low pH (Yoshimura & Sone, 1987).

Sequence analyses suggest that each receptor is organised into extracellular, transmembrane and intracellular domains. The extracellular domains of TNF-RI and -RII share some homology in amino acid sequence, (including the conservation of several cysteine residues) with each other (28% for the human molecules) and several other proteins, including putative cytokine receptors, such as NGF-R, Ox40, Bp50, CD27, and Fas antigen (Smith C. et al., 1990; Itoh et al., 1991; Camerini et al., 1991). Expression studies have also revealed that whilst murine TNFα displays high affinity binding to both murine receptors, its affinity for TNF-RII is slightly greater than that for TNF-RI and, though murine TNF-RI shows similar affinity for human and murine TNFα, the murine TNF-RII has little affinity for the human cytokine (Lewis et al., 1991). This can perhaps be explained by the fact that the extracellular domain of TNF-RII shows less conservation across the two species than that of TNF-RI.

Following binding, TNFα-receptor complexes are internalised and the cytokine is degraded (Tsujimoto et al., 1985; Mosselmans et al., 1988). Whilst one report using microinjection techniques documents an ability of TNFα to perform some functions intracellularly (Smith M. et al., 1990), fixed, cell-surface TNFα can induce cytotoxicity (Decker et al., 1987) and one can elicit many functions associated with TNFα by using whole antibodies (but not monovalent Fab fragments, against TNF-RI at least) to each of the two receptors (Engelmann et al., 1990a; Espevik et al., 1990; Tartaglia et al., 1991), suggesting that cross-linking of receptors is sufficient to convey the correct

cellular signals for several functions. Studies with soluble receptors also suggest that an individual TNFα trimer may bind up to 3 molecules of only one type of receptor (Scheurich et al., 1992), a prerequisite condition for achieving high cross-linking efficiency.

By using agonistic, receptor-specific antibodies one can define certain TNFα actions as being mediated via TNF-RI or TNF-RII (Tartaglia et al., 1991). Functions such as cytotoxicity and the induction of the enzyme manganous superoxide dismutase (MnSOD, see below) can be elicited, in susceptible cells expressing both types of receptor, by antibodies to TNF-RI but not TNF-RII, whilst enhancement of thymocyte proliferation and induction of proliferation of CT-6 cells, previously shown to be species-specific effects (Ranges et al., 1988, 1989), may be elicited by antibodies to TNF-RII but not TNF-RI. Thus, appropriate stimulation of either receptor activates a different set of pathways.

Understanding the mechanisms by which TNFα binding to its receptors is converted into its many actions is complicated by several factors. These include:- 1) it appears to activate many, possibly inter-dependent, pathways simultaneously (not surprisingly in view of its pleiotropic nature); 2) TNFα may have different, even opposite, effects in different cell types: differential receptor distribution may contribute to such disparity, particularly since TNF-RI and TNF-RII expression can be independently regulated (Hohmann et al., 1990a), but alternative effector mechanisms could also be involved in different cells; 3) though two sets of pathways exist (see above), there seems to be some interaction between them, or between TNF-RI and TNF-RII, since one can reduce the cytotoxicity of TNFα for certain cells by blocking its binding to TNF-RII (Shalaby et al., 1990); 4) more than one pathway may lead to the same end result (e.g. cell death, see below); and 5) the intracellular domains of TNF-RI and TNF-RII share little or no homology with each other, or those of other known receptors, giving few clues as to signalling methods, though studies with deletion mutants do show that the intracellular domain of human TNF-RI is essential (Tartaglia & Goeddel, 1992).

It has been found that G-proteins, known couplers of receptors to intracellular enzyme systems, can be activated by TNFα (Imamura et al., 1988) and blocking studies suggest that these may be involved in

some, but not all, of TNFα's actions on endothelial cells (Brett et al., 1989). Furthermore, in some cell types TNFα activates phospholipase A2 (PLA_2) (Neale et al., 1988; Hollenbach et al., 1992) (an enzyme which can be influenced by G-proteins [Jelsema & Axelrod, 1987]), with the release of arachidonic acid. TNFα also increases the intracellular levels of cAMP in fibroblasts (Zhang et al., 1988), though whether this is the result of increased activity of G-protein-associated adenylate cyclase is unclear. A phosphatidylcholine-specific phospholipase C and a phospholipase D may also be activated by TNFα, leading to the formation of another second messenger, diacylglycerol (DAG) (Schütze et al., 1991; Billah et al., 1989; Bauldry et al., 1991).

It is apparent that within minutes of binding to many cell types TNFα induces the phosphorylation of several cytoplasmic and nuclear proteins (Zhang et al., 1988; Schütze et al., 1989; Shiroo & Matsushima, 1990; Guy et al., 1992), including an mRNA cap-binding protein (Marino et al., 1989), though the method(s) remains a matter of debate. Different studies have documented increased activities, in response to TNFα, of protein kinases C (Brenner et al., 1989) and A (Zhang et al., 1988) and the tyrosine kinase located within the EGF-R (Donato et al., 1989), whilst Shiroo and Matsushima (1990) proposed that a novel kinase was responsible for TNFα-induced phosphorylation of two proteins in PBM's. More recently, however, Guy et al. (1992) found that of 116 fibroblast cell proteins whose phosphorylation status was altered by TNFα, 95% were similarly affected by okadaic acid, a phosphatase inhibitor, in contrast to far less faithful mimicry shown by assorted protein kinase agonists.

One end result of TNFα action is clearly the altered expression of numerous proteins. Whilst an effect on mRNA cap-binding (see above), and hence transcript translation, may be one method of protein regulation, TNFα also induces the expression of several 'primary response genes' (Dixit et al., 1990). Genes known to be induced by TNFα include those of transcription factors such as c-fos and c-jun (AP-1) (Lin & Vilcek, 1987; Brenner et al., 1989; Dixit et al., 1989), which may then mediate the induction of 'secondary genes'. A growing list of other transcription factors either induced or initially activated by TNFα, has been compiled. These include:- NF-kappaB

(Griffin et al., 1989) (helping to account for the ability of TNFα to induce itself); NF-GMa (Shannon et al., 1990); and IRF-1 (Fujita et al., 1989). TNFα may also decrease the DNA-binding activity of others such as C/EBP (Ron et al., 1990). Whilst pathways linking TNFα exposure to altered nucleic acid-protein bindings are incompletely understood, it does appear that the enhanced binding of NF-kappaB is dependent on DAG-mediated activation of a sphingomyelinase, to produce another second messenger, ceramide (Schütze et al., 1992).

How TNFα induces the killing of some cell types and not others has also been a matter of interest. Matthews et al. (1987) found that the cytotoxicity of TNFα was markedly reduced under anaerobic conditions, implicating oxidative damage as a cause of death. Oxygen radicals can originate from metabolism of arachidonic acid, amongst other sources, and inhibitors of PLA_2 or arachidonate metabolism may inhibit TNFα-induced cytotoxicity (Matthews et al., 1987; Neale et al., 1988). Agarwal et al. (1988), however, found that TNFα induces ADP-ribosylation (also known to be a potential route to cytotoxicity [Skidmore et al., 1979]) in susceptible cells and that inhibitors of this process were protective. It seems probable, therefore, that more than one pathway may be involved in TNFα-mediated cytotoxicity, as one might expect from the observation that TNFα can cause both apoptotic and necrotic forms of cell death (Laster et al., 1988).

Early receptor studies (Kull et al., 1985) failed to implicate the absolute presence or absence of TNFα binding as a means of determining TNFα-susceptibility or -resistance. The facts that an inhibitor of transcription such as actinomycin D can render cells more susceptible to TNFα (Ostrove & Gifford, 1979) and that TNFα can induce tolerance to subsequent, combined treatment (Hahn et al., 1985) have been taken to suggest that TNFα may also induce protective factors. At least three such proteins have been found:- MnSOD (Wong & Goeddel, 1988), a scavenger of free radicals, plasminogen activator inhibitor type-2 (Kumar & Baglioni, 1991) and A20 (Opipari et al., 1992), a novel protein whose function is less well understood. Wong and Goeddel (1988) also suggested that, since some TNFα-resistant cells were found to express MnSOD constitutively, susceptibility to TNFα may depend on the levels of this enzyme. Hollenbach et al. (1992), however, found that TNFα could not activate PLA_2 in some TNFα-resistant cell lines

unless actinomycin D was present, when these cells became susceptible. Thus, not surprisingly, there may be more than one way of achieving TNFα resistance.

1.7. Controlling TNFα activity:-

To derive benefit from TNFα, whilst avoiding its harmful effects, its activity must clearly be tightly regulated, yet capable of adjustment to suit changing needs. Several factors affecting the production and availability of, as well as cellular responsiveness to, TNFα have been identified, though the extent to which each operates in individual situations is poorly understood.

Since TNFα is not produced without appropriate stimulation, the successful removal of a pathogen by the inflammatory reactions it provokes and the short-lived nature of TNFα transcripts (see 1.3.3) would appear to be two methods by which TNFα production may be limited, though the potential for spatial amplification of TNFα production, mediated by LPS/TNFα-induced cytokines, such as IL-1, TNFα and GM-CSF, clearly exists. Though precise details of the downregulation of TNFα transcript production and translation are poorly understood, many mediators are known to reduce or prevent TNFα production, including:- PGE_2 (Renz et al., 1988); corticosteroids (Beutler et al., 1986a); TGFβ (Espevik et al., 1987; Chantry et al., 1989); IL-4 (te Velde et al., 1990); IL-6 (Aderka et al., 1989); and IL-10 (de Waal et al., 1991). Since several of these agents can be induced by LPS and/or TNFα (see 1.4, 1.5), they may constitute the effectors of feedback mechanisms by which TNFα production can be switched off and/or limited. In the case of failure to clear some offending pathogens, production may also be restricted by the development of tolerance to the inducing stimulus, as occurs with LPS (Takasuka et al., 1991). The potential to boost TNFα production under certain circumstances also exists. Exposure to IFN gamma (Beutler et al., 1986b), or the stimulation of α-adrenergic receptors (Spengler et al., 1990), markedly increase the expression of LPS-induced TNFα. IFN gamma can also enhance the induction of TNFα by other cytokines (Hart et al., 1988; Nedwin et al., 1985b) and overcome the post-transcriptional blockade of TNFα production imposed by corticosteroids (Luedke & Cerami, 1990).

TNFα's availability can be affected by its distribution, clearance from the body and the presence of specific inhibitors. Putative controls over the release of mature TNFα from its cell-surface form (see 1.3.4) may serve to regulate the proportions of TNFα available to act locally or more distantly. Furthermore, TNFα can bind reversibly to heparin (Lantz et al., 1991) and it is possible, therefore, that sub-saturating amounts of tissue-derived TNFα may be retained close to the site of production, by heparan sulphate in connective tissue. Similarly, TNFα secreted into certain body compartments such as the sub-arachnoid space or pulmonary alveoli may be 'trapped' by structural barriers (Waage et al., 1989; Nelson et al., 1989). Any TNFα reaching, or produced within, the circulation, may still have a limited distribution, since it can bind to α-2-macroglobulin (Wollenberg et al., 1991) and thus perhaps be targeted to certain tissues via receptors for this carrier.

Intravenously administered TNFα is rapidly cleared from the circulation with a half-life of only 6-7 minutes (Beutler et al., 1985d). Much of its removal can be accounted for by binding to TNF-R's (and its subsequent internalisation and degredation) within well-vascularised tissues. However, TNFα can also bind to the renal protein uromodulin (Hession et al., 1987), hence allowing some additional clearance via the kidney.

Two other TNFα-binding proteins, sharing immunological identity with TNF-RI and TNF-RII, and able to inhibit the cytotoxicity of TNFα by competing with cell receptors for its binding, have been found in urine, purified and partially sequenced (Seckinger et al., 1988, 1989, 1990; Engelmann et al., 1989, 1990b). Sequence analysis suggests that they derive from cleavage of the extracellular domains of the two receptors (indeed sequence derived from one of these proteins was used to help clone the cDNA for human TNF-RI [Nophar et al., 1990]). Furthermore, there is a large circulating pool of these proteins in the plasma of normal individuals (Aderka et al., 1991), a pool which increases following exposure to LPS or TNFα (Van der Poll et al., 1992b). They are thus likely to be an important physiological method of inactivating circulating TNFα. Curiously, they may also actually help enhance TNFα activity in situations where TNFα enjoys a longer half-life (such as body compartments, see above) by stabilising its

trimeric structure (Aderka et al., 1992). An 'activity-prolonging' function has also been proposed (Bendtzen et al., 1990) for the high levels of non-neutralising auto-antibodies to TNFα seen in the plasma of some individuals (Fomsgaard et al., 1989), though their significance is poorly understood.

Responsiveness to TNFα may be adjusted intracellularly (e.g. by the induction of protective proteins and hence tolerance [see 1.6]) or at the level of receptor expression. On activation, the initially low numbers of TNF-R's on most resting lymphocyte subsets rises (Kerhl et al., 1987; Munker et al., 1987; Scheurich et al., 1987; Owen-Schaub et al., 1989), largely via an increase in TNF-RII (Erikstein et al., 1991; Ware et al., 1991), though NK and LAK cells may also increase levels of TNF-RI (Naume et al., 1991; Dett et al., 1991). In contrast, stimulation of activated T-cells via the TCR leads to an early downregulation of TNF-R's, before TNFα is produced itself (Ware et al., 1991), thus limiting a possible autocrine stimulatory loop. Exposure of macrophages, but not endothelial cells, to LPS leads to a rapid, and total, internalisation of their TNF-R's (Ding et al., 1989), again preventing the action of TNFα on its source cell. Furthermore, neutrophils shed their receptors on exposure to formyl-Met-Leu-Phe peptide, C5a and GM-CSF (Porteu & Nathan, 1990). Since these might be found, or induced, in the vicinity of bacteria, one may envisage that having been successfully summoned to an appropriate site, neutrophils can help inhibit further TNFα activity by raising the local (and possibly systemic) concentration of soluble receptors.

Not surprisingly, many of TNFα's actions may be modified by the presence of other mediators. TNFα is more toxic to normal cells and tissues in the presence of some bacterial toxins, including LPS (Rothstein & Schreiber, 1988; Louise & Obrig, 1991), though how much of this synergy actually involves endogenous mediators is unclear. IFN gamma is also known to enhance many of TNFα's actions (and vice versa - see 1.4). These include:- its cytotoxicity to many cell lines (Williamson et al., 1983; Sugarman et al., 1985); the activation of neutrophils (Shalaby et al., 1985; Djeu et al., 1986), endothelial cells (Doukas & Pober, 1990) and keratinocytes (Barker et al., 1990); and the suppression of haematopoiesis (Broxmeyer et al., 1986). Furthermore, some activities, such as the activation of macrophages to

kill certain cells and parasites (Esparza et al., 1987; Liew et al., 1990a) and the induction of class II molecules on pancreatic islet cells (Pujol-Borrell et al., 1987), may only be seen when both of these cytokines are present. Part of this synergy may be explained by the potential of IFN gamma to upregulate TNF-R's on some cells (Aggarwal et al., 1985a), an ability sometimes exhibited by other IFN's (Billard et al., 1990). However, other pathways are also involved as Johnson and Pober (1990) found that IFN gamma could not upregulate TNF-R's on the same endothelial cells where it synergised with TNFα in the induction of class I MHC molecules.

Additive or synergistic effects may also be seen with IL-1 (e.g. Seelentag et al., 1987; Ranges et al., 1988), a cytokine with which TNFα shares many pro-inflammatory and other activities (Pober et al., 1986a,b; Le & Vilcek, 1987). This overlapping pattern of activity includes phosphorylation of many of the same proteins (Guy et al., 1991) and induction of the same transcription factors (Osborn et al., 1989b) and proteins (Beresini et al., 1988). Why should there be such redundancy of function between these frequently co-induced molecules? Observations that Epstein-Barr virus can inhibit TNFα gene transcription (Gosselin et al., 1991) and that, by its homology to the TNF-R's, the T2 protein of Shope fibroma virus is actually a form of soluble TNF-R (Smith et al., 1991) suggest one possibility. Since pathogens can apparently acquire the ability to subvert one cytokine's activities, then having others with overlapping abilities could be an evolutionary advantage.

TNFβ is another such mediator. Like TNFα, TNFβ molecules form homo-trimers with a similar 3D structure (Eck et al., 1992) and a comparable ability to TNFα to bind to TNF-RI (Schall et al., 1990). Thus, in sub-saturating amounts, the two often display additive activities. There are significant differences between them, however. Though TNFβ can also bind to TNF-RII, it shows slightly less affinity for this molecule and its soluble form than does its 'twin' (Smith C. et al., 1990; Engelmann et al., 1990b). Somewhat surprisingly, it also shows considerably less affinity than TNFα for the soluble form of TNF-RI (Pennica et al., 1992). It is produced by a more limited cell range (activated lymphocytes) and in these cells TNFα and TNFβ induction is independently regulated (Cuturi et al., 1987; Sung et

al., 1988a). When coinduced, TNFß is also produced more slowly than TNFα (Nedwin et al., 1985b). Furthermore, TNFß is markedly less potent than TNFα in many activities, such as inducing endothelial cells to secrete IL-1 (Locksley et al., 1987) and to adhere to neutrophils (Desch et al., 1990). Broudy et al. (1987) also found that, in contrast to TNFα, TNFß was totally unable to induce haematopoietic growth factors from endothelial cells at any concentration tested, whilst Oster et al. (1987) even found that TNFß could block a similar action of TNFα on monocytes. This would suggest either that TNFß is incapable of adequately cross-linking one of the TNF-R's or that not all of TNFα's actions are signalled simply by cross-linking TNF-R's (see also chapter 3). Whether the existence of a second TNF may also be due to previous outmanoeuvreing of a pathogen's subversive activities and/or perhaps to a need to respond slightly differently in different circumstances remains to be seen.

Finally, the activity of TNFα may be affected by the presence of antagonistic agents. These include other LPS-inducible monokines such as TGFß, which may be described as generally immunosuppressive (Kehrl et al., 1986; Ristow, 1986; Rook et al., 1986) and which also induces directly opposing effects to TNFα on collagen and proteoglycan synthesis (Ignotz & Massagué, 1986; Chen J. et al., 1987) and curiously, IL-1, since it may actually inhibit some aspects of endothelial cell activation caused by TNFα (Cavender & Edelbaum, 1988; Leszcynski, 1990). Furthermore, IL-1 can cause a temporary reduction in TNF-R numbers on some cells, as well as inducing longer-lasting tolerance to TNFα cytotoxicity (Holtmann & Wallach, 1987). Consequently, quantitative, temporal and spatial differences in the production and distribution of many other cytokines are also of importance in ultimately determining TNFα activity, adding to the complexity of its control.

1.8. TNFα in disease and development:-

Prominent rôles have been proposed for TNFα in many diseases, often following demonstrations of its altered production and/or ability to modify the disease outcome, or to reproduce some of its features. However, since the induction of TNFα does not necessarily mean that the same range of activities associated with giving a similar quantity of TNFα will follow (see 1.7), studies measuring alterations to disease progression when endogenous TNFα activity is blocked are invaluable in confirming its net effect. On the basis of such findings, modification of TNFα activity has been suggested as a possible therapeutic approach for some illnesses. Such treatments merit caution, however. Whilst controlled TNFα production can contribute to the elimination of a pathogen, acute overproduction in the face of an overwhelming infection, or more chronic dysregulations of its activity, which may occur, for example, during persistance of some stimuli, can clearly be deleterious. Consequently, an underlying theme in many diseases is that TNFα can be simultaneously associated with both beneficial and harmful actions. Furthermore, TNFα is but one of a network of mediators and cannot be considered in isolation.

1.8.1. Acute bacterial infection:-

The finding that antagonising TNFα activity could protect mice from the lethal effects of LPS (see 1.1) has since been extended to an ability of anti-TNFα antibodies to protect baboons from an experimentally-induced lethal bacteraemia (Tracey et al., 1987). Such treatments were only effective if given prior to, or within a very short space of time after, the lethal infusion, a finding consistent with TNFα inducing a cascade of other mediators (see 1.4, 1.5). Elevated levels of TNFα have been detected in the sera of patients suffering from septic shock and show some correlation with the outcome (Calandra et al., 1990). TNFα can also be found in the sera and other body fluids of patients with localised infections such as pneumonitis and meningitis, though these levels do not always correlate with clinical severity (Waage et al., 1989; Moller et al., 1991; Millar et al., 1989; Nohynek et al., 1991). This fits with findings that in more slowly-progressive models of septic disease TNFα may exert a predominantly protective effect. For example, anti-TNFα antibodies

capable of protecting against the lethal effects of IV LPS could not protect against lethality in one model of peritonitis (Bagby et al., 1991) and even increased mortality in another (Echtenacher et al., 1990). Predominantly protective roles for TNFα have also been proposed in Listeriosis, where anti-TNFα treatment can convert a mild infection into a lethal one (Havell, 1987), and in Salmonellosis where similar treatment also exacerbates the infection (Mastroeni et al., 1991; Tite et al., 1991), partly by abolishing TNFα-induced enhancement of immunity. An IP injection of TNFα may even enhance survival when given prior to an inoculation of Salmonella (Nakano et al., 1990).

1.8.2. Protozoal infection:-

TNFα has not only been incriminated in the cachexia of trypanosomiasis (see 1.1) but raised serum levels of TNFα have been observed in other parasitic diseases such as Leishmaniasis and Malaria (Scuderi et al., 1986). Higher TNFα concentrations can be associated with several manifestations of severe malaria (Shaffer et al., 1991) and antibodies to TNFα can be protective against one such syndrome, cerebral malaria, in a murine model (Grau et al., 1987), though these mice still eventually die from other manifestations of the disease. In this context, TNFα is likely to be simultaneously playing a protective rôle since it may be involved in killing malarial parasites (Taverne et al., 1984), especially in combination with IFN gamma and other serum factors (Naotunne et al., 1991). A predominantly protective role has been proposed for TNFα in Leishmaniasis, where anti-TNFα treatments worsen the condition and injections of TNFα lead to clinical improvement (Titus et al., 1989; Liew et al., 1990b). A major involvement of IFN gamma is also likely here since the two synergise in mediating the killing of these organisms (Liew et al., 1990a) and the latter primes monocytes for TNFα production in response to Leishmania donovani (Reiner et al., 1990).

1.8.3. Granuloma formation:-

Prominent rôles for TNFα have also been found in more chronic granulomatous and fibrotic types of disease. Elevated levels of probably locally-produced TNFα have been found in the pleural fluid of patients with tuberculous pleurisy (Barnes et al., 1990) and in the

serum of patients with other Mycobacterial diseases such as leprosy (Sarno et al., 1991), in keeping with findings that Mycobacteria can directly induce TNFα production (see 1.2). Macrophages from tuberculosis (TB) patients also seem to be primed to secrete more TNFα in response to LPS (Ogawa et al., 1991). Whilst it is possible that chronic overproduction of TNFα contributes to the cachexia seen in TB, it may play a predominantly beneficial rôle in this disease. Not only can TNFα kill some infected cells and help macrophages to kill Mycobacteria (see 1.4) but anti-TNFα treatment prevents or disperses granuloma formation in BCG-infected mice, allowing fatal dissemination of the organism (Kindler et al., 1989). TNFα also appears to be responsible for the formation of other granulomata. A single injection of TNFα bestows on SCID-mice a previously absent ability to form granulomata in response to Schistosoma mansoni eggs (Amiri et al., 1992). A major part for TNFα in some fibrotic diseases also seems likely, since anti-TNFα treatment inhibits the pulmonary fibrosis seen after an injection of bleomycin (Piguet et al., 1989).

1.8.4. Cancer:-

Elevated serum levels of TNFα may be present in many people with active neoplasia (Balkwill et al., 1987) and although raised concentrations of soluble TNF-R's are also found in a high percentage (Aderka et al., 1991), a rôle for TNFα in the cachexia suffered by these patients is suggested by demonstrations that anti-TNFα treatment can attenuate weight loss in some cancer models (Sherry et al., 1989; Yoneda et al., 1991). Whilst the source of this TNFα may occasionally be the tumour cells themselves (see 1.2), tumour-infiltrating macrophages and lymphocytes produce TNFα (Beissert et al., 1989; Barth et al., 1991), presumably in response to components of the transformed cells (see 1.2). Although TNFα can be directly toxic to many transformed cells, experiments using some TNFα- producing (and hence -resistant) tumour cells have shown that their growth may actually be prevented by TNFα-mediated recruitment of lymphocytes (Asher et al., 1991). The spontaneous regression of some tumours following acute administration of TNFα also appears not to be mediated via direct cytotoxicity. TNFα destroys the center of an SA1 sarcoma in mice via a haemorrhagic reaction and whilst the peripheral rim of tumour tissue

survives in some mice, in others this too may regress following TNFα treatment, but only in the presence of T-cells (Havell et al., 1988). Similarly, Palladino et al. (1987) demonstrated that TNFα can induce the haemorrhagic necrosis of a MethA sarcoma when grown in a vascular, subcutaneous site but not intraperitoneally. The action of LPS on these tumours also involves central haemorrhagic necrosis (Algire et al., 1947), though anti-TNFα antibodies inhibit this reaction by only 50%, suggesting that other mediators are also involved (North & Havell, 1988). Why should TNFα act selectively on the vascular bed of tumours? The answer may partly involve 'Vascular Permeability Factor', a tumour-derived protein which can synergise with TNFα in the induction of procoagulant activity on local endothelial cells (Clauss et al., 1990).

1.8.5. Lentiviral disease:-

Elevated levels of circulating TNFα, correlating to some extent with disease progression, are also seen in both adults and children with AIDS (Lähdevirta et al., 1988; Rautonen et al., 1991). Consequently, chronic overproduction of TNFα could mediate some of the cachexia and other pathologies, such as inflammatory cell infiltration, lymphoproliferation and demyelination, seen in this disease. A possible rôle for TNFα in the generation of AIDS-associated Kaposi's sarcomata has also been postulated following demonstration of its accumulation at these sites (Oxholm et al., 1989).

The mechanism of TNFα production in AIDS patients has been a matter of debate. Whilst infection with agents known to be common secondary pathogens in AIDS, such as cytomegalovirus, can be associated with a prolonged increase in TNFα levels (Tilg et al., 1991), some workers have observed spontaneous secretion of TNFα by monocytes or macrophages either derived from AIDS patients (with or without secondary infections) or infected with HIV in vitro (Wright et al., 1988; Roux-Lombard et al., 1989; D'Addario et al., 1990; Krishnan et al., 1990; Vyakarnam et al., 1990; Israel-Biet et al., 1991; Mabondzo et al., 1991). Merrill et al. (1989) found that the act of gp120, the envelope glycoprotein of HIV, binding to its cellular receptor CD4, was capable of inducing TNFα and this has been confirmed by Clouse et al. (1991). TNFα is also secreted during the fusion of SIV-infected

macrophages with $CD4^+$ lymphocytes, which may involve similar binding (McEntee et al., 1992). HIV or gp120 can even induce TNFα from rat brain cells (Merrill et al., 1992). However, it is possible that other mechanisms might also be involved in the induction of TNFα by HIV (see chapter 5, part i). Others have documented an increased ability of cells from AIDS patients, or infected cell-lines, to respond to LPS, Sendai virus or other stimuli (in the case of T-cells) by TNFα production, both in terms of their sensitivity and levels produced (Hober et al., 1989; Roux-Lombard et al., 1989; D'Addario et al., 1990; Vyakarnam et al., 1991). This enhancement may involve an increased stability of TNFα transcripts (Voth et al., 1990) and/or priming of macrophages by the high levels of a form of IFNα seen in many AIDS patients (Lau & Livesey, 1989). Enhanced sensitivity to TNFα may also be induced by this interferon via an upregulation of TNF-R's (Lau et al., 1991). Interestingly, B-cells from AIDS patients may spontaneously secrete TNFα, a secretion which is enhanced in the presence of gp120 (Rieckmann et al., 1991). Synthetic peptides derived from both gp120 and the transmembrane viral protein gp41 have also been seen to enhance the secretion of TNFα by normal PBM's in response to LPS (Tyring et al., 1991).

Observations of increased TNFα production in the presence of HIV have not been universal, however. Many workers have failed to observe either constitutive secretion of TNFα by chronically-infected cell lines, or the release of TNFα by monocytes/ macrophages on infection with HIV or SIV, nor any alteration in their response to LPS (Molina et al., 1989, 1990a,b; Kornbluth et al., 1990; Munis et al., 1990; Horvath et al., 1991; Peters et al., 1991) (though Molina et al. [1989] did find that a monocytic cell line could respond with enhanced TNFα production in response to LPS at the time of acute infection). Others have even documented defective TNFα production by PBM's from AIDS patients (Ammann et al., 1987; Cox et al., 1990) and a loss of the ability of macrophages from simian AIDS cases to respond to LPS on culture (Horvath et al., 1991), though the reasons for such contrasting findings are presently unclear.

Clouse et al. (1989) observed that the supernatants of LPS-stimulated macrophages could enhance the replication of HIV in a chronically-infected T-cell clone and that much of this phenomenon was

mediated by TNFα. This effect has been confirmed in some T-cells (Folks et al., 1989), primary monocytes (Michihiko et al., 1989; Mellors et al., 1991) and glial cells (Tornatore et al., 1991), and extends to enhancement of SIV replication in macrophages (Walsh et al., 1991), though not all models of HIV infection show this response (Folks et al., 1987; Ito et al., 1989; Hazan et al., 1990; Lacoste et al., 1990). Novak et al. (1990) found that TNFα even caused resistant cells from some donors to become permissive for HIV infection and Kobayashi et al. (1990a) demonstrated that spontaneous secretion of both TNF's by HTLV-1-infected T-cell lines helps to mediate their high susceptibility to infection. Much of this enhancing effect is attributable to TNFα inducing the binding of NF-kappaB proteins to the long terminal repeat (LTR) of the HIV DNA provirus and increasing transcription from it (Duh et al., 1989; Israel et al., 1989; Osborn et al., 1989b). TNFα can also synergise with IL-6 in enhancing HIV replication (Poli et al., 1990a).

Induction of TNFα by HIV and vice versa suggests the possibility of an autocrine stimulatory loop and indeed antibodies to TNFα can suppress the constitutive production of HIV in a chronically-infected promonocytic line (Poli et al., 1990b; Locardi et al., 1990). Interestingly, the cell surface form of TNFα may be responsible for much of this constitutive induction (Tadmori et al., 1991). However, it is difficult to reconcile such a positive feedback process in vitro with a disease which is slowly progressive in vivo, without the existence of negative feedback processes. Not surprisingly, TNFα also appears to play a part in more than one such process. Matsuyama et al. (1989a) observed that in addition to being able to enhance the transcription of HIV in chronically-infected T-cell lines, TNFα could also kill these cells, whilst Wong et al. (1988) found that TNFα, in combination with IFN gamma, could also decrease the susceptibility of some T- and B- cell lines to HIV infection. The latter authors have subsequently found that some infected cells may be rendered susceptible to the cytotoxic action of TNFα via an inability to produce MnSOD in response to TNFα (Wong et al., 1991). TNFα may also inhibit the stimulatory effect that HIV can exert on B-cell function (Delfraissy et al., 1991).

1.8.6. Immunologically-mediated disease:-

TNFα appears to be an important mediator of many 'immunological' diseases. Not only is it induced at the site of delayed type hypersensitivity (and irritant) reactions, but anti-TNFα antibodies can also prevent their development (Piguet et al., 1991). Similarly anti-TNFα treatment alleviates much of the pathology associated with immune-complex deposition in the lung (Warren et al., 1991) and an antibody which neutralises both TNF's can be protective in one model of experimental allergic encephalomyelitis (EAE) (Ruddle et al., 1990). Interestingly, astrocytes from strains of rats which are susceptible to this disease can be primed for TNFα production by IFN gamma, whilst those from resistant strains cannot (Chung et al., 1991). Both TNF's have also been found at active sites of multiple sclerosis (MS), which shares many morphological features with EAE (Hofman et al., 1989; Selmaj et al., 1991). T-cell clones derived from the CSF of MS patients have been found to secrete high levels of this cytokine (Benvenuto et al., 1991).

TNFα also seems to be involved in autoimmune disease. Not only can it be found in the synovial fluid of most rheumatoid arthritis joints (Hopkins & Meager, 1988) but anti-TNFα antibodies can attenuate pathology in the DBA/1 collagen type II-induced model of autoimmune arthritis (Feldmann et al., 1992). Conversely, therapy with TNFα can delay the onset of glomerulo-nephritis seen in murine autoimmune lupus nephritis (Jacob & McDevitt, 1988) (though others have found that it can aggravate the problem [Brennan et al., 1989]). Furthermore, susceptible mice have a reduced ability to secrete TNFα and this correlates with an RFLP in the TNFα gene (Jacob & McDevitt, 1988).

TNFα activity may also be prominent in transplant-related problems. It is induced during cardiac transplant rejection (Arbustini et al., 1991) and increased serum levels of TNFα are seen both during rejection of kidney transplants (Maury & Teppo, 1987) and prior to complications of bone-marrow transplants (Holler et al., 1990), in keeping with demonstrations of the induction of high levels of TNFα during a mixed lymphocyte response (Shalaby et al., 1988). This induced TNFα seems to be involved in causing some of the

manifestations of graft-versus-host disease, as anti-TNFα antibodies can prevent the skin and gut lesions seen in the latter (Piguet et al., 1987).

1.8.7. Development:-

Finally, TNFα appears to play a rôle in normal development. Some TNFα is found in the normal pregnant uterus (see 1.2). It is also expressed constitutively in the thymus (Giroir et al., 1992) and blocking its activity in the foetus or neonate leads to a marked suppression in early growth (de Kossodo et al., 1992).

1.9. The potential rôle of TNFα in ovine disease:-

A standard text of sheep medicine (Blood et al., 1979) reveals several ovine diseases where one could speculate that TNFα might play a prominent rôle, either because of their clinical signs and/or their similarity to diseases of other species where TNFα is known to be important.

Sheep, particularly neonatal lambs, are susceptible to septicaemias caused by a wide range of both gram -negative and -positive organisms. Such problems include colibacillosis, listeriosis, salmonellosis, leptospirosis, anthrax, erysipelas and, occasionally, pasteurellosis, as well as streptococcal and staphylococcal ('tick pyaemia') septicaemias. Though several of these can also cause more slowly progressive and/or localised septic disease, each can feature acute mortality with many symptoms that are highly reminiscent of excessive TNFα activity, such as fever, widespread haemorrhages and/or oedema, organ necrosis, respiratory distress and diarrhoea or dysentery.

Similar signs may also be seen:- in enterotoxigenic forms of colibacillosis and salmonellosis, where endotoxin may be absorbed from the gastro-intestinal tract; following acute infections of the mammary gland with Pasteurellae or Staphylococci; and in some viral diseases, such as 'blue tongue' and 'sheep pox'. It is also conceivable that TNFα activity could contribute to the haemorrhages, oedema and mortality seen in the many different forms of clostridial disease to which this species is prone.

A number of severe protozoal diseases also afflict the sheep. As in other species, toxoplasmosis can induce an encephalitis or pneumonia, though it is more commonly associated with abortion and neonatal death, whilst babesiosis involves parasitaemia, fever, intravascular clotting and haemorrhages. An involvement of TNFα in modulating the life-cycles of, or mediating the pathologies caused by, either of these organisms would seem to be a possibility.

Judging from their respective pathologies, TNFα could also play significant parts in 'louping Ill', an acute, febrile, viral encephalomyelitis, which involves perivascular accumulations of inflammatory cells in the meninges and CNS, and 'border disease', another virally-induced disease featuring defective myelination of the CNS of new born lambs.

Sheep are also prone to many chronic diseases involving cachexia and where TNFα may therefore be important. Like other species they can succumb to TB, as well as Johne's disease, a debilitating, enteric illness caused by another mycobacterium. Actinobacillosis is a not uncommon ovine disease, involving fibrosis and a pyo-granulomatous response, which could be mediated, at least in part, by TNFα, whilst maedi-visna disease, a lentivirally-induced illness, involving multi-organ inflammatory cell infiltration, lymphoproliferation and cachexia, has several features in common with AIDS (see chapter 5, part i). Scrapie is another slowly-progressive disease involving emaciation and neuronal degeneration. Finally, whilst neoplasia is not particularly common in the sheep, some flocks may have a high incidence of adenocarcinoma of the small intestine and the debilitating illness 'jaagsiekte' or 'driving sickness' results from a retrovirally-induced tumour of the lung.

1.10. TNFα in ungulates:-

The sheep is an animal of undoubted agricultural importance. Consequently, the understanding and prevention of ovine disease is of tremendous economic concern world-wide. In 1989, however, when the work described in the following chapters commenced, apart from a documented ability of human TNFα to mimic many endotoxin-like effects in live sheep (Horvath et al., 1988), very little was known about TNFα in this species. Such studies have since been confirmed and extended

by others (Johnson et al., 1989; Wheeler et al., 1990; Redl et al., 1990) and more recently, Winstanley (1992) demonstrated that recombinant human TNFα (rhTNFα) can bind to ovine leukocytes.

A little more was known about TNFα in other ungulates. The cDNA encoding bovine TNFα had been cloned and sequenced, though only the deduced amino acid sequence had been presented (Goeddel et al., 1986). Recombinant bovine TNFα (rboTNFα) had also been expressed and a few papers were beginning to document that it had similar activities to TNFs'α from other species, such as the induction of cachexia when repeatedly administered to cattle (Ohmann et al., 1989b) and an antiviral activity (Campos et al., 1987) mediated via an ability to induce 2'-5' oligo-adenylate synthetase in some cells (Campos et al., 1988) but not others (Ohmann & Babiuk, 1988; Ohmann et al., 1989a). RboTNFα has also found to be effective in inducing lymphocyte localisation in sheep skin (Kalaaji et al., 1990). More recently, Chiang et al. (1991) demonstrated that it could enhance some functions of bovine neutrophils. It has also now been shown that, as in other species, the TNF locus is linked to the MHC locus in goats (Cameron et al., 1990).

A few studies have been dedicated to the detection of ungulate TNF'sα. Kenison et al. (1988) developed a radioimmunoassay (RIA) for bovine TNFα, though, since this is based on a polyclonal antiserum, it may be subject to interference by TNFα-binding proteins (see chapter 4). Using this assay, Peel et al. (1990) could detect circulating bovine TNFα following the administration of LPS to calves, but not in calves with Salmonellosis. Adams and Czuprynski (1990) demonstrated that TNFα could be induced from bovine macrophages by LPS and detected using TNF-sensitive murine cells. They also found that the molecular weight of bovine TNFα was consistent with a multimeric form. Similar findings have also been made in horses (Seethanathan et al., 1990; Mackay et al., 1991a), where TNF-like activity can be detected in serum after giving LPS (Morris et al., 1990; Mackay et al., 1991b) and in some cases of neonatal septicaemia (Morris & Moore, 1991). As in other species, dexamethasone appears to inhibit TNF production in the horse (Morris et al., 1991). Baarsch et al. (1991) have also been able

to detect porcine TNFα using cytotoxicity assays and the cDNA encoding TNFα in this species has now been sequenced (Pauli et al., 1989; Drews et al., 1990).

1.11. Aims of the project:-

Whilst some studies have now documented that other ovine cytokines such as IL-1, IL-2 and IFN gamma show significant homology in structure and/or function to their human and murine counterparts (Fiskerstrand & Sargan, 1990; Fiskerstrand et al., 1992, Andrews et al., 1991; Seow et al., 1990; McInnes et al., 1990), some features of the ovine immune system show marked differences to other species. For example, there seems to exist a large population of functionally-important pulmonary intravascular macrophages in sheep, whereas these are not found in humans (Warner et al., 1986). Consequently one cannot assume that aspects of the immune system, including TNFα biology, will necessarily be the same in the two species. The importance of this point was made all the more clear when preliminary experiments, described in chapter 4, failed to detect cytotoxic activity in the supernatants of LPS-stimulated ovine macrophages using murine cell lines known to be sensitive to TNF's from many species.

In the absence of any knowledge about ovine TNFα, it was essential to establish some basic information about this molecule (including proof of its existence) before being able to investigate its rôle in specific diseases. The following chapters therefore describe how the cDNA encoding ovine TNFα was cloned and sequenced (chapter 2), before recombinant ovine TNFα (rovTNFα) was expressed and characterised (chapter 3). Chapter 4 discusses how antibodies raised to the recombinant protein were used in the immunological detection of ovine TNFα and presents reasons for the apparent failure of TNF-sensitive murine cells to respond to ovine TNFα. Finally, these reagents were then applied in a preliminary study of the rôle of ovine TNFα in one particular problem, maedi-visna disease (as described in chapter 5, part i).

CHAPTER 2:- CLONING AND SEQUENCING THE cDNA ENCODING OVINE TNFα.

2.1. Introduction:-

A preliminary objective in studying ovine TNFα was to clone and sequence the cDNA encoding it, in order to:- 1) demonstrate the presence in sheep of a mRNA species which might encode a protein homologous to TNF'sα from other species; 2) derive predictive information on the structures of such an ovine TNFα and some of the elements which might (by analogy to other species) be involved in regulating its synthesis; and 3) obtain DNA probes suitable for use in studies of the induction of ovine TNFα mRNA.

Prior to commencing this work, the sequences of cDNA's encoding TNFα in humans (Pennica et al., 1984; Marmenout et al., 1985; Wang et al., 1985), mice (Pennica et al., 1985; Fransen et al., 1985) and rabbits (Ito et al., 1986) had been published. The strategies used in these studies were broadly similar. Initially, TNFα cDNA clones were identified by screening cDNA libraries with degenerate oligo-nucleotide probes, which had been constructed based on knowledge of partial amino acid sequences of purified TNFα. Expression of the cloned cDNA's, to produce proteins with appropriate characteristics, was used to confirm that these were indeed TNFα encoding cDNA's. Clones thus derived from one species were then used to probe libraries from another species and a sufficient degree of homology of TNFα sequences between species enabled this approach to succeed.

Against this background, one possible method for cloning ovine TNFα cDNA would have been to screen a suitable, ovine, cDNA library with a DNA probe from one of these species. Indeed this was an approach adopted by other workers (see discussion [2.3]), who, it later transpired, were concurrently working towards the same objective.

In comparison with the laborious techniques involved in screening libraries, cloning techniques based on the use of the polymerase chain reaction (PCR) (Mullis & Faloona, 1987), to generate large amounts of DNA of specific, primer-directed sequence, offer a potentially quicker route to cloning a gene from one species, when the equivalent genes in other species have already been sequenced. These techniques require identification of regions of the gene where sequence is extremely well conserved across species, enabling the construction of 'inwardly-

facing' primer pairs, which should be capable of annealing to the cDNA for the equivalent gene in most species. These primer pairs may then be used to direct the amplification of an internal fragment of the cDNA for that gene of the species under study (Ohara et al., 1989). This approach has been used successfully in cloning a number of genes, including those of other ovine cytokines, such as IL-1ß (Fiskerstrand & Sargan, 1990), IL-2 (Seow et al., 1990) and IFN gamma (McInnes et al., 1990).

There are shortcomings to this approach, however, when trying to obtain an accurate and complete gene sequence. One problem is caused by the relatively poor fidelity of sequence reproduction by Taq polymerase, the thermostable enzyme preferred for PCR, when compared to other DNA polymerases (Saiki et al., 1988). This can be overcome by obtaining a consensus sequence from several PCR-derived clones, or by directly sequencing the PCR product. A second problem is that of obtaining complete sequences, including the 5' and 3' regions flanking the internal fragment amplified by the initial 'direct' PCR. At least two approaches to this problem have been described. One, 'anchored' PCR, involves ligating an oligonucleotide, of known sequence, to a terminus and performing a PCR on this template using one outwardly-facing primer specific for the cDNA and one oligonucleotide-specific primer (Ohara et al., 1989). This obviously requires a PCR and cloning/sequencing procedure for each end of the transcript. A second approach, 'inverse' PCR, in which the molecule concerned is circularised and used as the template in a PCR using a pair of specific, outwardly-facing primers (Ochman et al., 1988; Triglia et al., 1988), offers the potential to amplify both 5' and 3' ends with a single PCR.

The strategy I chose to follow, therefore, was to generate, sequence and clone an internal fragment of ovine TNFα cDNA via direct PCR, using primers complementary to regions which are highly conserved between human, mouse and rabbit TNFα cDNAs. This was to be followed by an inverse PCR, using primers specific to the ovine sequence, to amplify the 5' and 3' termini, prior to their cloning and sequencing.

The ability to obtain large numbers of alveolar macrophages by lung lavage and the known inducibility of TNFα mRNA in cells of monocyte/macrophage lineage by LPS (Beutler et al., 1986a) suggested a source of mRNA from which to prepare a suitable cDNA template.

The cloning vectors used in this work were the multifunctional 'phagemids pTZ18R and pTZ19R (Mead et al., 1986). Whilst these were primarily selected because of their ready availability, other features of these vectors which contributed to their suitability for this work include:- 1) an ampicillin resistance gene, allowing identification of transformed bacteria (from an ampicillin-sensitive host strain) on ampicillin-containing solid media; 2) a multiple cloning site, conferring, on appropriately digested vectors, the ability to accept inserts with a wide range of termini; 3) flanking the multiple cloning site, a lac Z gene, whose disruption by the presence of inserted DNA allows the identification of recombinants by colony colour when a suitable lac Z substrate and chromogen are included in media; 4) an F1 origin of replication, allowing synthesis of large amounts of single-stranded DNA, suitable for sequencing, when transformed bacteria of appropriate phenotype are superinfected by the helper 'phage M13KO7; 5) high transformation efficiency due to their small size (<3kb); and lastly 6) the orientation of the multiple cloning site with respect to the F1 origin of replication is reversed between the 2 vectors, allowing single-stranded DNA to be produced from both strands of an insert when it is excised from one 'phagemid, by a pair of restriction endonucleases which leave different termini, and inserted into the other vector (prepared by digestion with the same enzymes). This latter feature facilitates the sequencing of larger inserts. 'Reverse' sequencing primer, which anneals to a region of 'phage DNA adjacent to the multiple cloning site and directs DNA polymerisation across it, is a suitable primer for use in the initial sequencing of DNA inserted in these vectors.

2.2.Results:-

2.2.1.Preparation of first strand ovine cDNA:-

Lavaging the lungs of a freshly-slaughtered, healthy, Scottish Blackface ewe yielded a total of 10^8 cells. Selection of the adherent fraction 24 hours after they were plated out (at 5 x 10^5/ml in 2 x 225cm^2 tissue culture flasks) left a population comprising approximately 90% macrophages, as assessed by morphology under the light microscope. An estimated 250µg of total RNA was collected and purified from these cells 2 hours after they were then stimulated with 100ng/ml of LPS.

Several distinct bands, including putative 28S and 18S RNA bands, were clearly visible when an aliquot of this preparation was assessed by agarose gel electrophoresis (fig.2.1a), suggesting that there had been no wholesale deterioration of the RNA during preparation.

An estimated 300ng of cDNA was then produced when 12µg of this RNA was used as the template in a 'first strand only' cDNA synthesis reaction. Analysis of the labelled fraction of this reaction indicated that a wide range of sizes of cDNA had been formed (fig.2.1b).

2.2.2.Initial PCR's:-

Primers A and B (fig.2.2) were selected, following a comparison of published TNFα cDNA sequences, for use in direct PCR. A single base difference exists between the species' sequences within the regions from which each of these primers were derived, hence inositol, or a choice of nucleotides, was used in the corresponding primer positions to allow for possible mismatches. Use of these primers in a PCR on suitable human cDNA should amplify a 590bp fragment of DNA.

Initial PCR's, performed at annealing temperatures of 48°C or 45°C using primers A and B at 0.1 or 1µM each, on 5 or 25ng of cDNA template, failed to produce any DNA (as assessed by UV trans-illumination of ethidium bromide-containing gels, following electrophoresis of an aliquot of reaction mixes) with the exception of possible primer-dimers (fig.2.1c).

Figure 2.1:-

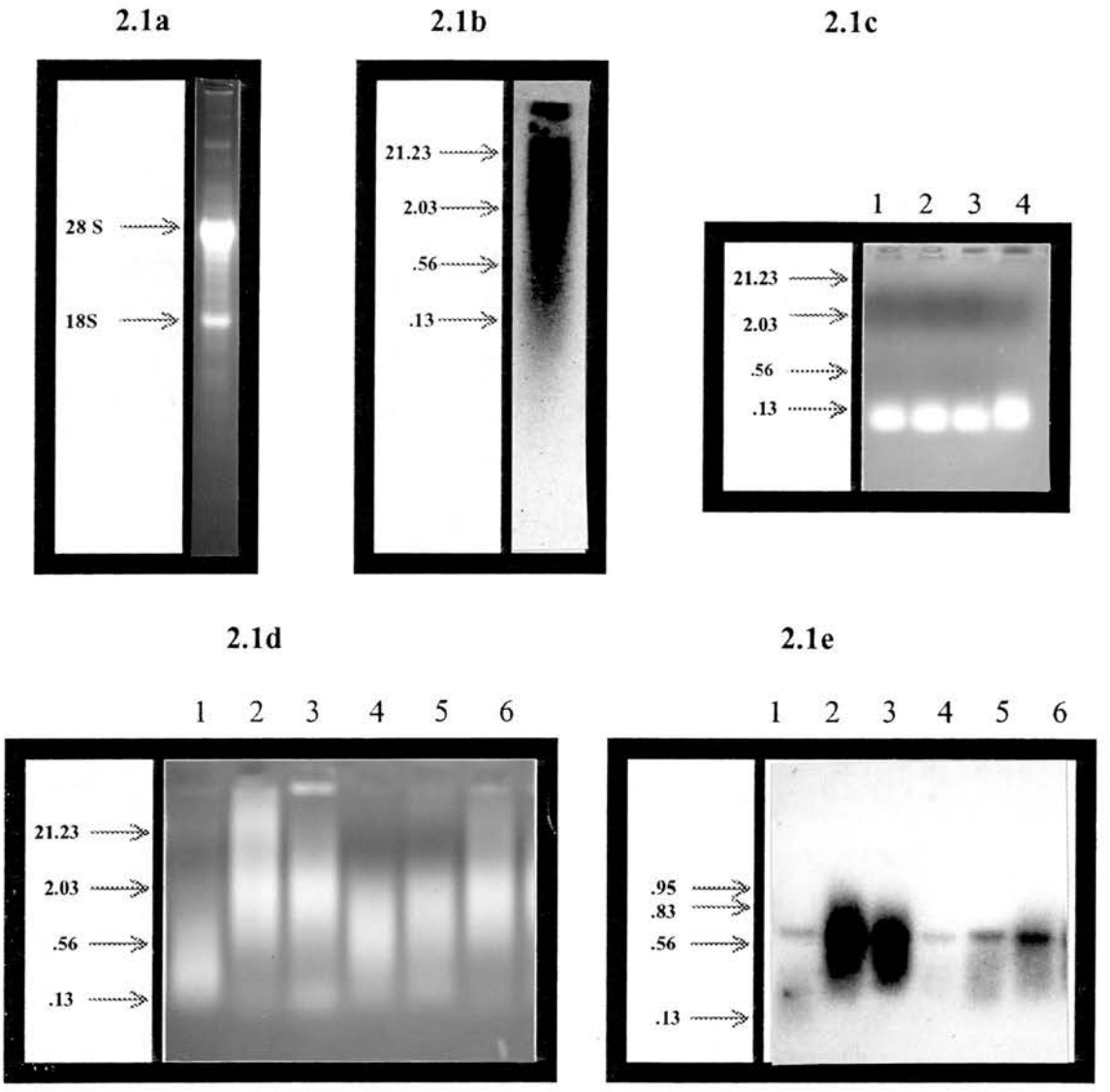
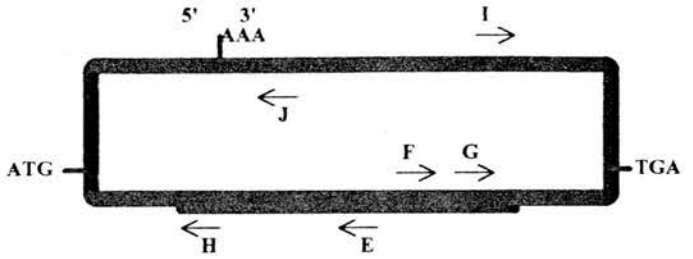


Figure 2.1:- photographs of transilluminated, RNA (2.1a) & DNA (2.1c,d) gels, an autoradiograph of a DNA gel (2.1b) & a Southern blot of the gel shown in 2.1d (with corresponding lane no.s), following hybridisation to an human TNFα cDNA probe (2.1e). DNA gels were 1.2% agarose. The positions of putative 28S & 18S RNA bands or molecular size markers (in kb) are indicated. Samples submitted to electrophoresis comprised:- a 10μg aliquot of the RNA preparation used for 1st strand cDNA synthesis (2.1a), 5μl of a labelled, 1st strand cDNA synthesis reaction (2.1b) & 10% aliquots of PCR's performed at annealing temperatures of 45^{0}C (2.1c) or 40^{0}C (2.1d). Primers A & B (fig.2.2) were used in these PCR's at 1μM (2.1c, lanes 2 &4; 2.1d, lanes 1 & 2), 0.1μM (2.1c, lanes 1& 3; 2.1d, lanes 3& 4) or 0.01μM (2.1d, lanes 5 & 6) on templates of 25ng (2.1c, lanes 1& 2; 2.1d, lanes 2,3 & 6) or 5ng (2.1c, lanes 3 & 4; 2.1d, lanes 1,4 & 5) of 1st strand cDNA.

Figure 2.2:-

2.2a

2.2b



2.2c

Primer	5' Sequence 3'	Position
A	CTC AGC CTC TTC TCI TTC CTG	249-269
B	CCA AAG TAG ACC TGC CCR GAC TC	838-816
C	CAC CAC GCT CTT CTG CC	284-300
D	GGT CAC CCT TCT CCA ICT GG	771-752
E	GCA GAG AGG ATG TTG ACC	674-657
F	ATC AAG AGC CCT TGC CAC AGG	676-696
G	AAG CCC TGG TAC GAA CCC	721-738
H	ACC AGA GGC CTG TTG AAG G	380-362
I	AAC ACA TCT GAG CCA AGG C	1430-1412
J	AGT CAT GCC TGT AAC CGC	1600-1617

Figures 2.2a & b:- schematic diagrams (not to scale) of TNFα cDNA molecules, derived from the human & sheep (after circularisation), respectively. The annealing positions of primers used in the work described in this chapter are indicated, in addition to the first (ATG) & last (TGA) codons of the coding region & the polyadenylated tail (AAA). The bold segment of the ovine molecule represents the portion amplified & cloned following direct PCR.
Figure 2.2c:- the actual sequences of individual primers. I represents an inositol residue & R . a 50.50 mix of A & G residues. Position no.s from which these sequences derive are according to Pennica et al. (1984) for primers A-D, or fig.2.8 for all other primers. Descending position no.s indicate antisense primers.

Analyses of subsequent PCR's, where similar primer and template concentrations were used, revealed that a range of sizes of DNAs were produced, however, when the annealing temperature was lowered to 40°C (fig. 2.1d).

Southern blotting, followed by hybridisation to an human TNFα cDNA probe with washing to only a low final stringency (1xSSC at 50°C), then revealed the presence of a band of DNA (c.600bp), which hybridised more strongly than the rest of the smear (fig.2.1e), suggesting that some desired amplification had taken place, even though it had been insufficiently specific.

2.2.3.Subsequent nested PCR:-

Primers C and D (fig.2.2) were then selected, by the same criteria used in the selection of primers A and B, for use in a 'nested' PCR to specifically amplify a fragment within the c.600bp band. Using these primers in a PCR on a human cDNA template should amplify a DNA fragment of 488bp.

Their use, over a range of concentrations, in PCR's performed at an annealing temperature of 48°C using 1µl of a previous reaction (fig.2.1d, lane 4) as template, led to the amplification of a c.500bp fragment (fig. 2.3a), which hybridised to the same human probe when used at the above stringency (fig.2.3b).

2.2.4.Cloning and sequencing of the c.500bp PCR product:-

When the remainder of these reaction mixes were pooled, an estimated 1µg of the c.500bp product was available, after appropriate preparation (terminal phosphorylation, end filling and preparative electrophoresis), for 'blunt-ended' ligation into pTZ19R. 4 similar, repeat PCR's (conditions of fig. 2.3a, lane 1) generated an estimated 2µg of c.500bp product, after preparation, for use in direct sequencing (and, later, as a probe). Gene cleaning (Bio 101), which was used for final purification in each case (after preparative electrophoresis and band excision), led to satisfactory recovery of the c.500bp product (fig.2.3c,d).

Direct sequencing of this product using primer C yielded some short stretches of readable sequence which showed homology with sequence contained in human TNFα cDNA, whilst analysis of a direct sequencing

Figure 2.3:-

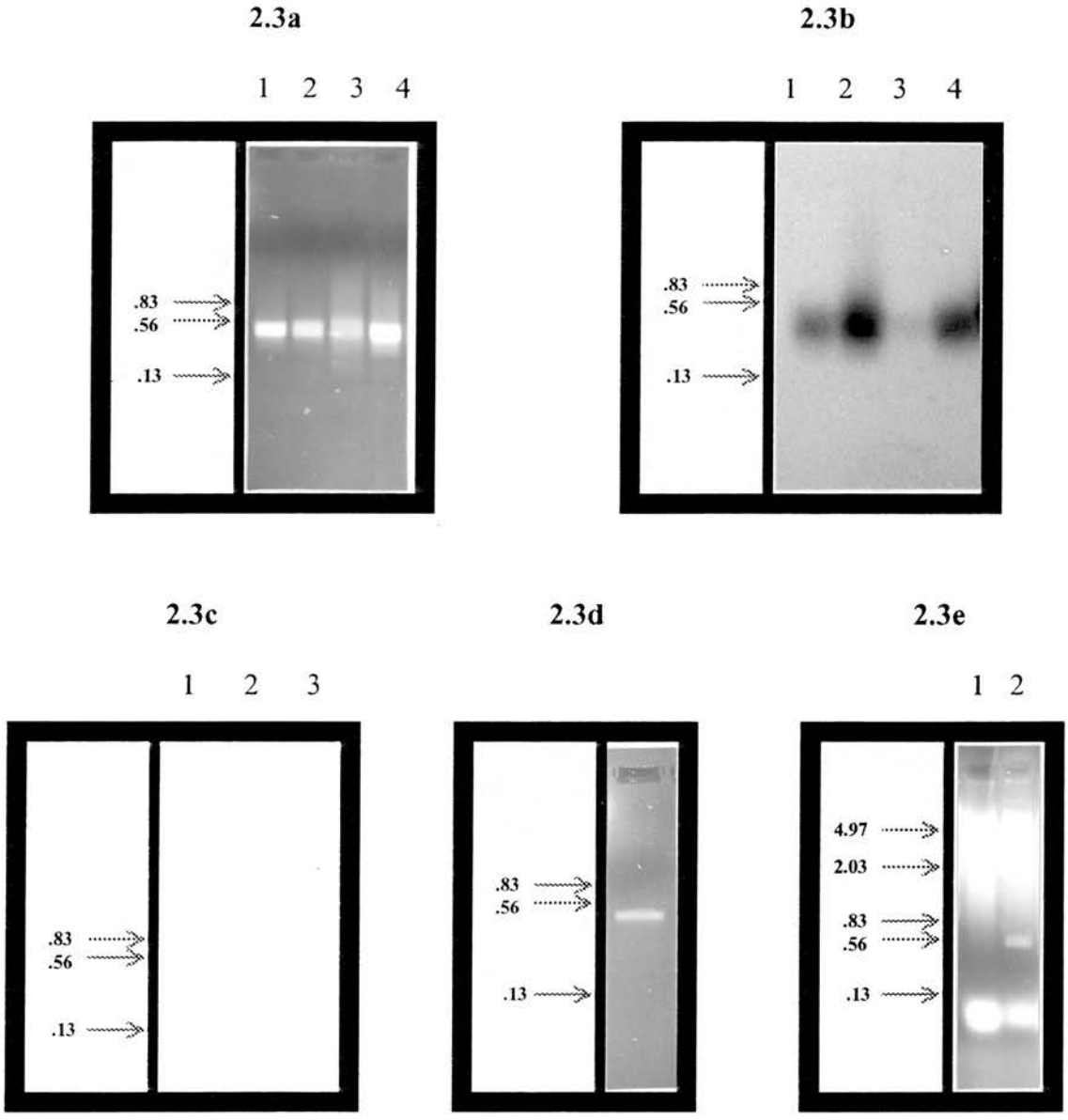


Figure 2.3:- photographs of transilluminated, 1.2% agarose DNA gels (2.3a,c,d & e) & a Southern blot of the gel shown in 2.3a (with corresponding lane no.s), following hybridisation to an human TNFα cDNA probe (2.3b). The positions of molecular size markers (in kb) are shown for each. Samples submitted to electrophoresis comprised:- 10% aliquots of PCR's (2.3a, lanes 1-4), the products of four repeat PCR's (conditions as for fig.2.3a, lanes 1 or 4), after pooling, terminal phosphorylation, end-filling, purification & division between 3 lanes (2.3c), 5% of the final yield after the c.500bp product was excised from the gel in 2.3c & purified by gene clean (2.3d) & miniplasmid preparations (following digestion with HindIII, then EcoRI) from a pTZ19R-transformed E.coli colony or povTNF1 (2.3e, lanes 1 & 2, respectively). PCR's used primers C & D (fig.2.2) at 0.1, 1, 0.01 or 0.1 μM (2.3a, lanes1-4, respectively) on a template of 1μl of a previous PCR (see fig.2.1d, lane 4).

reaction using primer D produced a long stretch (c.300b) of readable sequence, which, when converted into sense form, also showed a high degree of homology to a region of human TNFα cDNA contained between the sequences of primers C and D.

Knowledge of this ovine sequence then allowed the selection of primers E and F (fig.2.2), primarily for subsequent use in an inverse PCR. However, the use of primer E in a 'nested' direct sequencing reaction extended the known ovine sequence by a further c.100b and confirmed, in the region where there was overlap, the sequence obtained using primer D. (The regions for which sequence was derived from any of the sequencing reactions described above or below are shown schematically in fig.2.7.)

A single recombinant clone, designated povTNF1, was obtained following transformation of bacteria with the products of a ligation reaction involving 50ng of c.500bp product and 50ng of SmaI-digested pTZ19R. Earlier reactions, involving 25, 50 or 100ng of PCR product, and simultaneous reactions, involving 25 or 100ng of PCR product, failed to produce any recombinant colonies when their products were used for transformation. In each case large numbers (>1000) of colonies were obtained in control transformations involving undigested plasmid and no colonies were seen on plates coated with untransformed bacteria. More colonies were obtained on control plates derived from reactions involving ligase and digested vector than on those derived from reactions with digested vector but no ligase (60 vs 20 and 83 vs 12). Numbers of non-recombinant colonies growing on plates derived from reactions with PCR product were similar to those on plates derived from control reactions with vector and ligase but no added DNA.

When plasmids were recovered from povTNF1 and digested with HindIII then EcoRI (HindIII and EcoRI sites flank the SmaI site of pTZ18R and 19R) a c.500bp fragment was excised (fig.2.3e). A preparation of single-stranded (ss) DNA from this clone produced an estimated 10µg of DNA. The analyses of sequencing reactions involving this ssDNA and initially reverse sequencing primer, then, once the orientation of the insert had been established, primers C then F, showed that the DNA which had been ligated into pTZ19R was 491bp long and contained the sequence of primers C and D at either end. The intervening region was

highly homologous to that region of human TNFα cDNA between the sequences from which primers C and D were derived and, where it overlapped, was in perfect agreement with the sequence obtained by direct sequencing of the c.500bp product.

2.2.5.Northern blot analysis of the induction of ovine TNFα mRNA:-

Lavaging the lungs of a healthy Scottish Blackface ewe yielded 1.4 x 10^8 cells. This population, which was immediately divided between 7 x 75cm^2 tissue culture flasks containing 25mls of medium with or without 100ng/ml LPS, produced approximately 30µg of RNA per flask when the total RNA was collected and purified at assorted time points thereafter.

Fig.2.4 shows the result when an estimated 10µg of RNA from each time point were submitted to electrophoresis then Northern blotting and the blot washed, after hybridisation to an ovine TNFα cDNA probe (povTNF1 insert, excised from a large-scale plasmid preparation and purified by electrophoresis and gene-cleaning, then radio-labelled), to a final stringency of 0.3xSSC at 52°C.

The probe appeared to hybridise to a single species of RNA. This RNA had a slightly slower mobility through the gel than 18S RNA and was inducible, reaching a maximum density between 90 and 180 minutes after plating out in the presence of LPS. However, it was also noted that a similar density was achieved in the absence of added LPS.

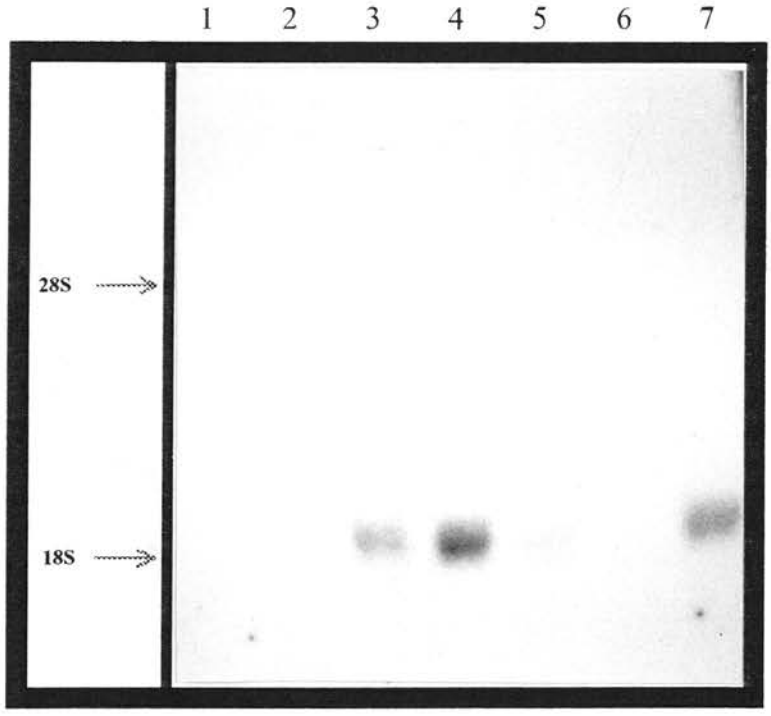
A previous Northern blotting experiment, which was performed, using cells from another sheep, with a slight change in protocol (the addition of LPS was delayed for 24 hours, by which time the adherent fraction had been selected, and RNA collected thereafter), failed to demonstrate any hybridisation to RNA, even though 10µg of the RNA preparation used for first strand cDNA synthesis were loaded on the same gel and prolonged exposure times were used for autoradiography.

2.2.6.Synthesis of double-stranded cDNA for use in inverse PCR:-

The Northern blotting experiments described above suggested that the RNA preparation previously used for production of first strand cDNA may not have been particularly rich in TNFα transcripts. Double-

Figure 2.4:-

2.4a



2.4b

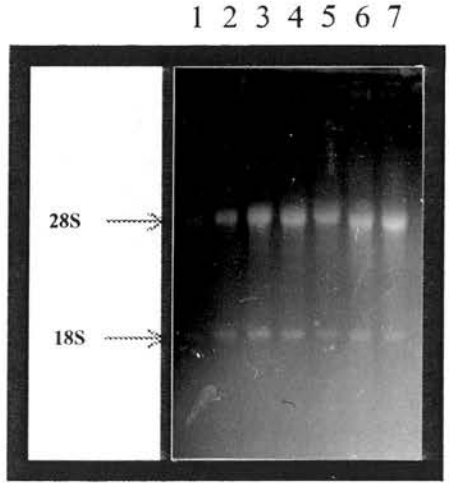


Figure 2.4:- a Northern blot (2.4a) following hybridisation to an ovine TNFα cDNA probe (povTNF1 insert) & the transilluminated gel (with corresponding lane no.s) from which the blot was taken (2.4b). The positions of putative 28S & 18S RNA are indicated.. Samples submitted to electrophoresis comprised RNA collected from ovine lung-cells 0, 0.75, 1.5, 2.25, 3 or 24 hours (lanes 1, 2, 3, 4 & 7, 5, 6, respectively) after plating out in the presence (lanes 1-6), or absence (lane 7) of 100ng LPS/ ml .

stranded (ds) cDNA was therefore synthesised using a template of RNA demonstrated to be relatively rich in ovine TNFα mRNA (RNA collected 2.25 hours after plating out with LPS [see fig.2.4 lane 4]).

When 12µg of this RNA preparation were used in the synthesis of ds cDNA, 240ng of first strand cDNA were synthesised and an estimated 88% of this was converted into ds cDNA. Analysis of an aliquot of labelled second strand synthesis reaction, by electrophoresis and auto-radiography, suggested that a wide range of sizes of ds cDNA had been formed. A pellet of 'circularised' cDNA, ready for use as the template in an inverse PCR, was obtained after purification of a ligation reaction involving 100ng of ds cDNA in dilute solution.

2.2.7.Initial inverse PCR:-

The positions of the sequences from which primers E and F were derived were such that it was anticipated that the use of these primers in an inverse PCR on an appropriate template would amplify a DNA fragment almost as long as the full length of ovine TNFα cDNA, hence having considerable overlap with the region already cloned. Whilst the exact length of ovine TNFα cDNA was unknown, the lengths of human, murine and rabbit TNFα cDNA's are all between 1600 and 1700 bp's. The results of Northern blotting, however, suggested that the ovine sequence could be slightly longer (see discussion [2.3]).

An inverse PCR performed on 25ng of this 'circularised' template using primers E and F and an annealing temperature of 45°C led to the formation of a major product of only c.1000bp, as well as minor products of larger and smaller size (fig.2.5a). This major product appeared to remain hybridised to the central region probe (povTNF1 insert) after washing to a high stringency (0.3xSSC, 65°C)(fig.2.5b).

2.2.8.Investigation of the c.1000bp product:-

Consideration of the ways in which a smaller than anticipated product could have arisen led to several different theories. These included the following:- 1) during first strand cDNA synthesis, DNA polymerisation may have terminated prior to reaching the 5' terminus, or the oligo dT primer might have annealed to an A-rich region other than the 3' terminus, leading in either case to the production of a 'shortened' cDNA; or 2) during template preparation, insufficient

Figure 2.5:-

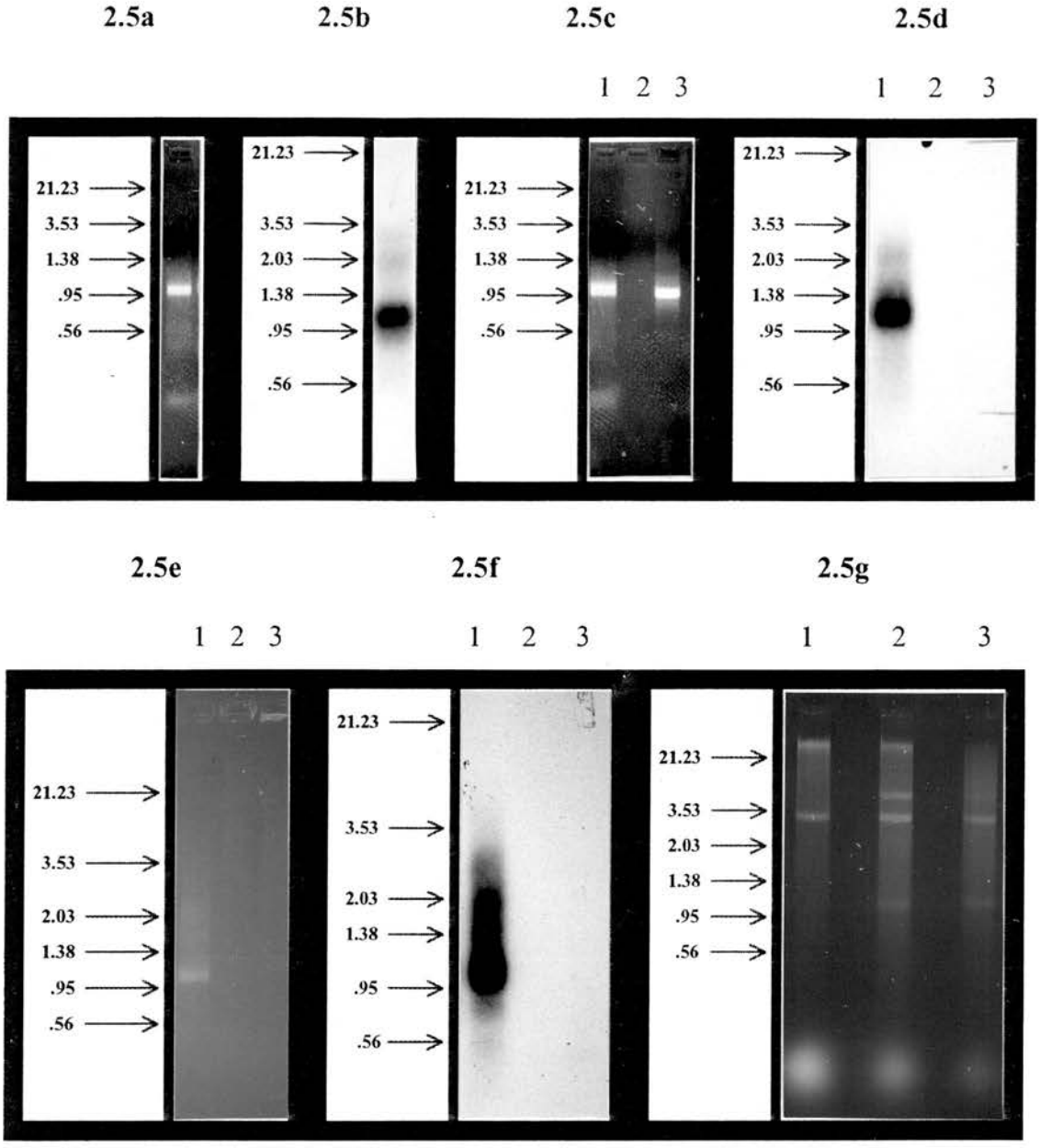


Figure 2.5:- photographs of transilluminated, 1.2% agarose gels (2.5a, c, e & g) & Southern blots of the gels shown in 2.5a, c & e (with corresponding lane no.s), following hybridisation to an ovine TNFα cDNA probe (povTNF1 insert) (2.5b, d & f, respectively). The positions of molecular size markers (in kb) are shown for each. Samples submitted to electrophoresis comprised 10% aliquots of PCR's (2.5a, c & e) & miniplasmid preparations , following digestion with HindIII then EcoRI, taken from a pTZ19R-transformed E.coli colony, povTNF2 & povTNF3 (2.5g, lanes 1-3, respectively). Primers (fig.2.2) used in PCR's were:- E & F (2.5a; 2.5c, lane 1); E only (2.5c, lane 2; 2.5e, lane 2); F only (2.5c, lane 3); E & G (2.5e, lane 1); or G only (2.5e, lane 3). PCR templates comprised circularised cDNA (2.5a) or 1μl of the product shown in 2.5a (2.5c, lanes 1-3; 2.5e, lanes 1-3).

dilution of the cDNA might have occurred for the ligation reaction to result in circularisation. This latter possibility could have resulted in the head-to-head, or tail-to-tail, ligation of some abundant molecules and the subsequent amplification of an internal portion by either primer alone.

To test the second hypothesis, PCR's were performed using primers E and F, or either primer alone, at an annealing temperature of 45°C with 1µl of the original inverse PCR product as template. A band c.1000bp was produced using primer F alone, initially suggesting that some form of tail-to-tail ligation might have occurred (fig.2.5c). However, Southern blotting analysis, using the probe and stringencies described above, subsequently demonstrated that although a component of the c.1000bp product generated in the presence of both primers hybridised to the ovine TNFα probe, the c.1000bp band generated by primer F alone did not (fig.2.5d).

2.2.9.Semi-nested inverse PCR:-

Whilst some amplification of ovine TNFα cDNA had clearly occurred during the original inverse PCR, the above results suggested that there was likely to be considerable contamination caused by non-specific amplification of other molecules by primer F. Primer G (fig.2.2) was therefore selected in order to anneal to a site just 3' to the complementary region of primer F in a 'semi-nested' PCR.

Whilst no significant product was formed when either primer alone was used, a PCR, performed on 1µl of the original PCR product using primers E and G with an annealing temperature of 44°C, again produced a major product c.1000bp (fig.2.5e), which clearly hybridised to the central region clone (p.ovTNF1 insert, final washing stringency as above) (fig.2.5f).

2.2.10.Cloning and analysis of the c.1000bp product of semi-nested PCR:-

Preparative electrophoresis of the remainder of this and 3 identical PCR's, with recovery of the c.1000bp band by excision and gene cleaning, yielded 1.5µg of DNA for direct sequencing. However, attempts to directly sequence this product using primers E or G failed to produce any substantial lengths of readable sequence.

3 further identical PCR's produced 750ng of this product in LGT agar, after preparation, for use in ligation. No colonies with recombinant plasmids resulted from initial ligation reactions involving 25, 50 or 100ng of PCR product. Control plates gave similar results to previous cloning experiments. Following subsequent identical ligation reactions and transformations, however, approximately 100 recombinant colonies were derived from each reaction. Use of an ovine TNFα cDNA probe (c.500bp product of direct PCR) in colony hybridisations identified only 2 which were positive for the presence of homologous sequence, when washed to a final stringency of 0.2xSSC at 60°C. A band of appropriate size could be excised from a miniplasmid preparation of each of these two colonies, which were designated povTNF2 and povTNF3 (fig.2.5g).

11µg of ssDNA were obtained for sequencing when preparations were made from each colony. The analyses of initial sequencing reactions using reverse sequencing primer produced c.350b of readable sequence from each and revealed that their plasmids contained similar DNA fragments inserted in the same orientation. The sequences of these inserts began with that of primer G and continued with sequence totally homologous to the short region of povTNF1 insert 3' to that from which primer G was derived, before continuing with sequence showing a high degree of homology to the equivalent region of the human molecule. Further sequencing reactions using primer G as a sequencing primer extended knowledge of the inserts' sequences by a further c.80b and confirmed the sequence already obtained in the region of overlap.

50ng of EcoRI, HindIII-excised insert was obtained in LGT from a miniplasmid preparation of each colony and ligated to 50ng of EcoRI, HindIII-cut pTZ18R. >200 recombinant colonies resulted, most of which hybridised to an ovine TNFα cDNA probe (c.500bp product of direct PCR, using stringencies as before). After preparing plasmids from 8 representative colonies derived from each original clone, EcoRI and HindIII digestion released a c.1000bp insert from all of them.

10µg of ssDNA were then obtained from 4 of these clones:- povTNF4 and povTNF5 (derived from ligation of the insert of povTNF2) and povTNF6 and povTNF7 (derived from ligation of the insert of povTNF3). Subsequent sequencing reactions using reverse sequencing primer

produced c.350b of readable sequence. When converted to sense form, these sequences comprised the sequence of primer E, then 23 or 76 bp (for povTNF4,5 and povTNF6,7 respectively) of sequence showing total homology with the c.500bp product of direct PCR 5' to the region from which primer E was derived, a short poly-A tract (11 or 5 bases) and finally, sequence with homology to the extreme 3' end of the human molecule. Knowledge of this sequence was confirmed, where there was overlap, and extended (by c.80b) using primer E as a sequencing primer in further reactions.

Primer I (fig.2.2) was then selected to assist in sequencing the remainder of these inserts and its use in subsequent sequencing reactions produced a further c.400b of sequence. The distal end of this new sequence contained several bases of totally homologous overlap (when converted to sense form) with the sequences of povTNF2 and povTNF3 derived using primer G. Thus, these clones contained the entire sequence of ovine TNFα cDNA 3' to the complementary region for primer G, though they clearly lacked several hundred bases of 5' sequence.

These sequencing reactions also showed that the sequences of povTNF4 and povTNF5 were identical, as were the sequences of povTNF6 and povTNF7. However, a handful of minor differences did exist between the sequences of povTNF2 and povTNF3 and between povTNF4,5 and povTNF6,7 (see discussion [2.3]).

2.2.11.Attempts to amplify the 5' terminus of the molecule:-

The above sequences demonstrated that the c.1000bp major product of semi-nested inverse PCR derived from amplification of incomplete cDNA molecules. The use of two new ds cDNA preparations as the template, after circularisation, in inverse PCR's with primers E and G (using the same annealing temperatures as before) failed to produce a larger major product (fig.2.6a) even though the RNA preparation was submitted to a further round of phenol/chloroform purification and longer incubation times (2.5 hours) were used for first strand synthesis.

As well as the major product, it was evident that there were larger molecules in both the original and semi-nested inverse PCR's which hybridised to the ovine TNFα probe, including a concentration of molecules c.2000bp (fig.2.5b,d,f). Preparative electrophoresis in LGT

agarose of 10µl of the original inverse PCR allowed separation of this c.2000bp band (fig.2.6b) and its subsequent excision. However, when this DNA was used as the template in subsequent PCR's with primers E and G (1µl LGT agarose/reaction, annealing temperature 44°C) the major product was once again c.1000bp (fig.2.6c), suggesting that the c.2000bp band was a concatemer of the c.1000bp band, produced as a result of Taq polymerase working in a 'rolling circle' fashion on the original circular template (see discussion [2.3]).

To ascertain whether amplification of more complete cDNA molecules had occurred during the initial inverse PCR, a test PCR was performed, using an annealing temperature of 44°C, on 1µl of the original inverse PCR product with primers E and C (since the c.1000bp band was clearly derived from amplification of cDNA molecules lacking the 5' terminus, including the region to which primer C should anneal). A product of appropriate size (c.387bp) was generated (fig. 2.6d), indicating that more complete molecules were present.

A further primer, primer H (fig.2.2) was therefore selected to anneal close to the 5' extremity of the known sequence and used in a fully-nested PCR on 1µl of the original inverse PCR product, with primer G, at an annealing temperature of 45°C. The use of these primers in PCR on a complete, circularised cDNA template should amplify a fragment lacking 340bp of the central region. Consequently such a product would share only a limited stretch of sequence with povTNF1 but several hundred bp of sequence with povTNF2-7.

The major product of this PCR was once again c.1000bp. However, a c.1300bp product was also prominent (fig.2.6e). Both bands appeared to hybridise to an ovine TNFα probe (povTNF2 insert, final purification by electroelution) when washed to a final stringency of 0.3xSSC at 65°C (fig.2.6f).

2.2.12.Analysis and cloning of the c.1300 bp product:-

Since it was a more appropriate size, subsequent analysis concentrated on the c.1300bp product. 2 and 1.5 µg of this product were obtained, after preparation, for direct sequencing and cloning respectively, from the remains of this and 6 identical PCR's. (Final purification of this band after preparative electrophoresis was by electroelution.)

Figure 2.6:-

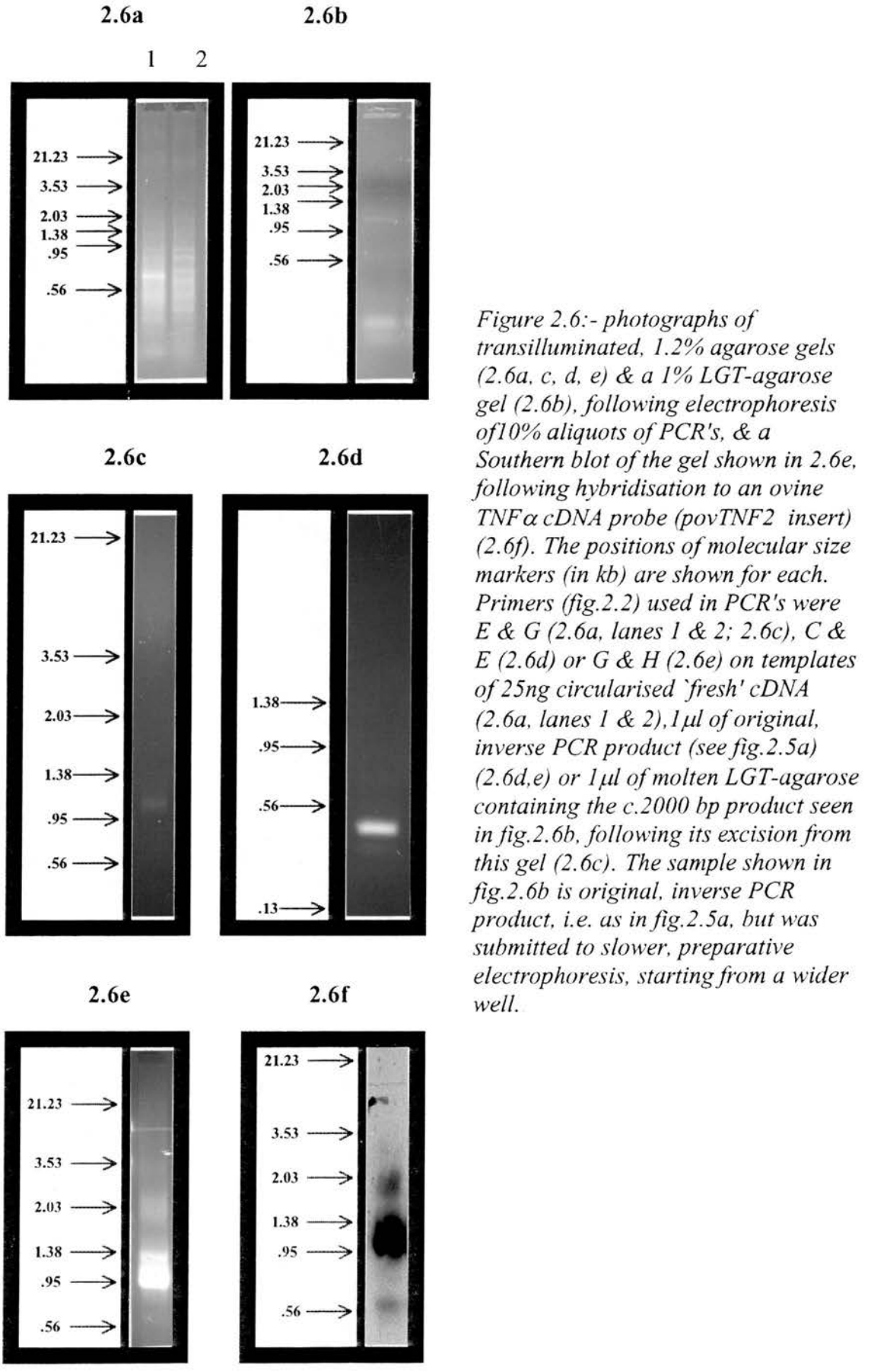


Figure 2.6:- photographs of transilluminated, 1.2% agarose gels (2.6a, c, d, e) & a 1% LGT-agarose gel (2.6b), following electrophoresis of10% aliquots of PCR's, & a Southern blot of the gel shown in 2.6e, following hybridisation to an ovine TNFα cDNA probe (povTNF2 insert) (2.6f). The positions of molecular size markers (in kb) are shown for each. Primers (fig.2.2) used in PCR's were E & G (2.6a, lanes 1 & 2; 2.6c), C & E (2.6d) or G & H (2.6e) on templates of 25ng circularised `fresh' cDNA (2.6a, lanes 1 & 2),1μl of original, inverse PCR product (see fig.2.5a) (2.6d,e) or 1μl of molten LGT-agarose containing the c.2000 bp product seen in fig.2.6b, following its excision from this gel (2.6c). The sample shown in fig.2.6b is original, inverse PCR product, i.e. as in fig.2.5a, but was submitted to slower, preparative electrophoresis, starting from a wider well.

Analysis of a direct sequencing reaction using primer H produced c.300b of readable sequence, which (when converted into sense form) showed homology with the 5' region of human TNFα cDNA and, in the short region where there was overlap, total homology with the insert of povTNF1.

10, 8 and 12 recombinant colonies resulted from transformations with the products of ligation reactions involving 25, 50 or 100 ng of PCR product respectively. However, only 1 of these showed positive hybridisation to an ovine TNFα probe (c.1000bp product of semi-nested inverse PCR) when washed to a final stringency of 0.2xSSC at 60°C. Although no insert could be freed from plasmids prepared from this clone, which was designated povTNF8, ssDNA was prepared from it and submitted to sequencing reactions, initially with reverse sequencing primer. The readable sequence generated by this reaction corresponded to that of primer G and some 340b with >99% identity to the sequences of povTNF2 and povTNF3 immediately 3' to the annealing site of primer G.

Since the insert of povTNF8 could not be freed and inserted into pTZ18R, primer J (fig.2.2) was selected in order to sequence its other end. Sequencing reactions using this primer produced c.400b of readable sequence. These comprised the terminal (3') few bases of the ovine TNFα cDNA sequence (according to povTNF4-7), including a short (11b) poly-A tract, then a stretch with homology to the extreme 5' terminus of the human sequence extending to the region from which primer H was derived, and finally, the multiple cloning site of pTZ19R, where it could be seen that a mutation (likely to be responsible for the inability to free the insert) had occurred. After the first 10 bases of the ' TNFα 5' ' stretch this sequence reached the extent of the sequence derived by direct sequencing of the c.1300bp PCR product and in the subsequent region of overlap showed total homology with it.

2.2.13.Final sequence:-

The complete sequence of ovine TNFα cDNA is presented in fig.2.8. This represents a composite, consensus sequence of all the overlapping stretches of sequence represented in fig.2.7.

Figure 2.7:-

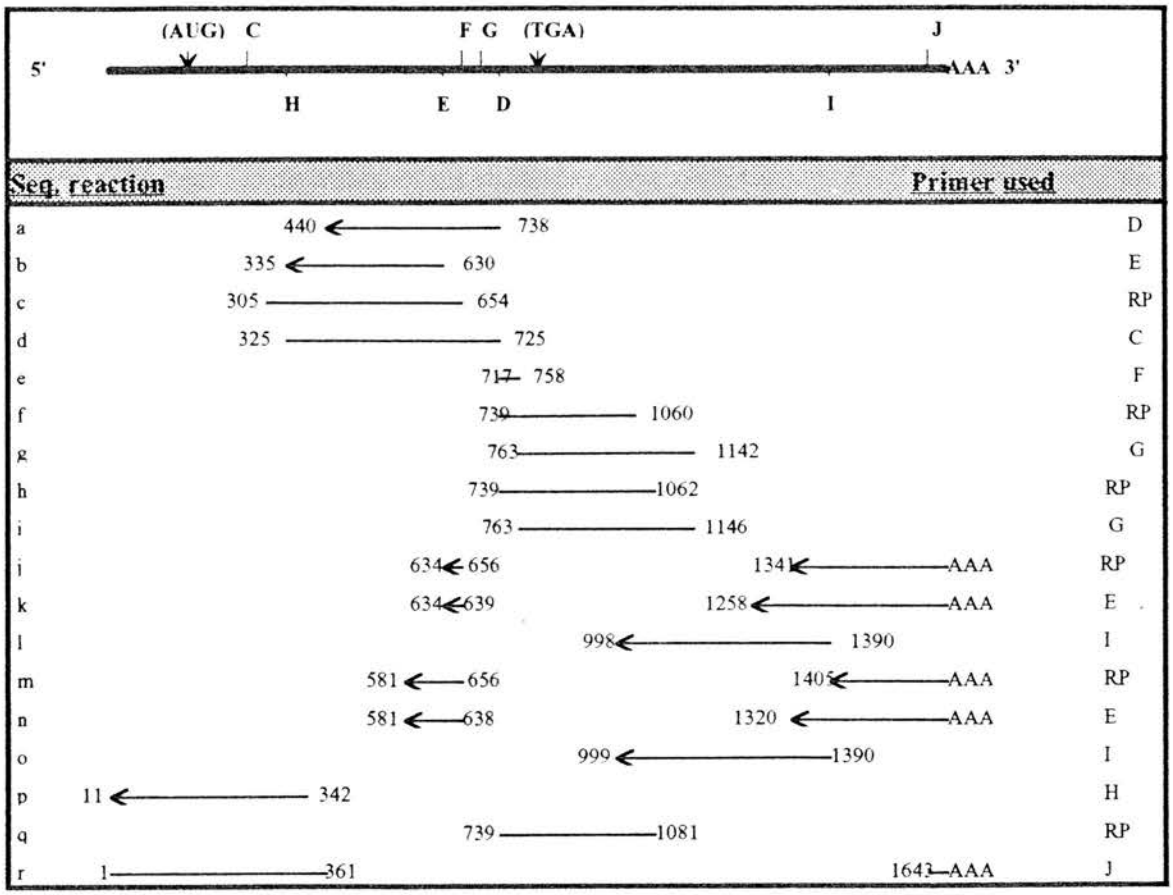


Figure 2.7:- shown at the top of the figure is a linear representation of the complete ovine TNFα cDNA molecule, indicating:- the approximate, annealing positions of sense- & antisense- primers (labelled as in fig.2.2), above & below the line, respectively; the translation initiation (AUG) & termination (TGA) codons; & the polyadenylated tail (AAA). Below this are indicated the relative positions of sequences derived from individual sequencing reactions (with sequence no.s referring to those of fig.2.8; sequences of PCR primers were excluded from results), using the primers indicated (RP= reverse sequencing primer). Templates used in individual reactions were:- c.500bp product of direct PCR (a,b); ss.DNA from povTNF1 (c,d,e); ss.DNA from povTNF2 (f,g); ss.DNA from povTNF3 (h,i); ss.DNA from povTNF4 & 5 (j,k,l; identical results were obtained from each plasmid); ss.DNA from povTNF6 & 7 (m,n,o; identical results were obtained from each plasmid); c.1300bp product of fully-nested inverse PCR (p); & ss.DNA from povTNF8 (q,r). Leftward facing arrows indicate that sequences, as read, were converted to opposite strand form.

Figure 2.8:-

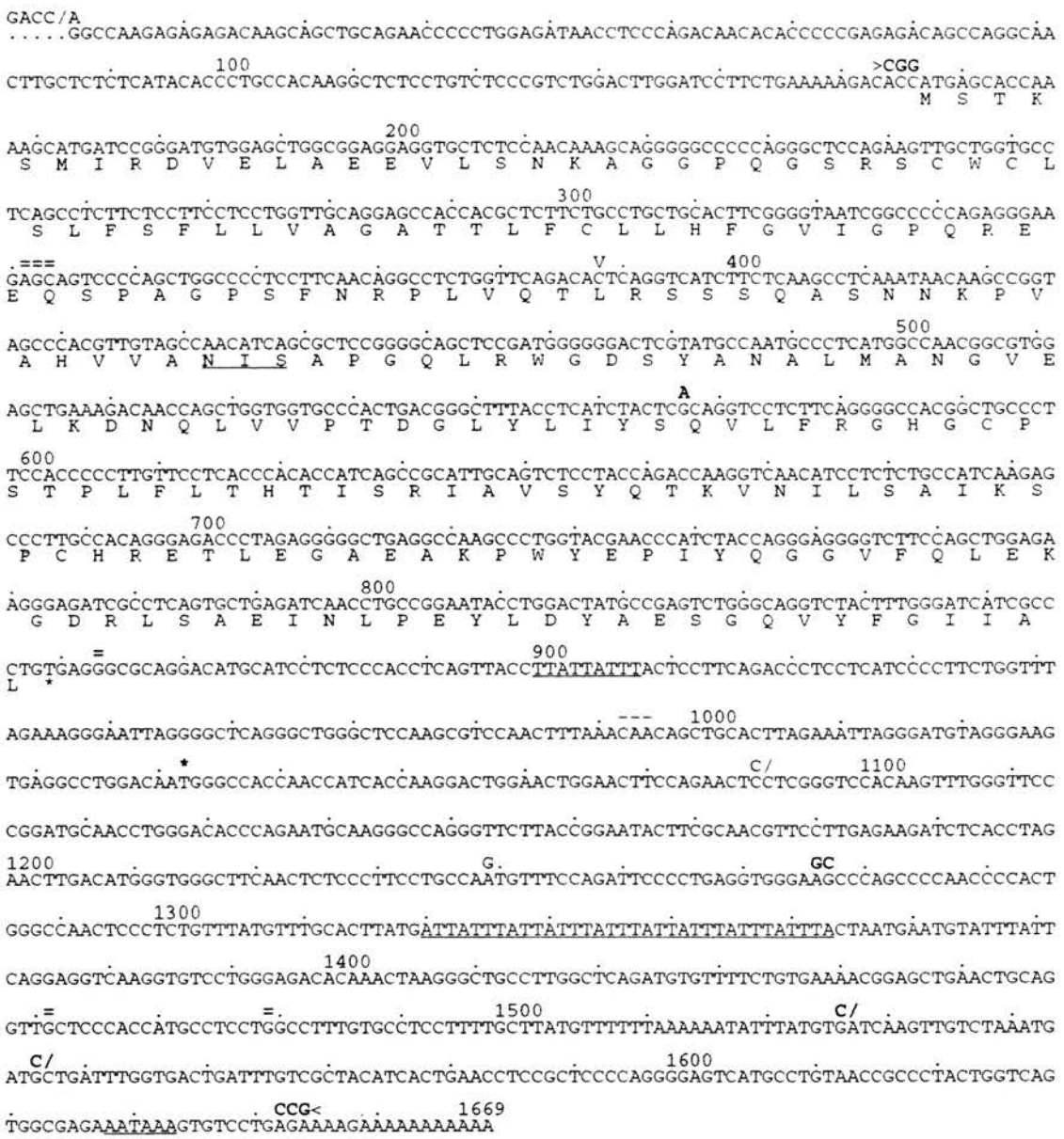


Figure 2.8:- the complete sequence of ovine TNFα cDNA & (below the start of each codon) the deduced amino acid sequence of the corresponding protein. A potential glycosylation site (NIS) & the polyadenylation signal (AATAAA) are underlined, as are two AT-exclusive regions in the 3'UTR. An asterisk underlies the translation termination codon. The presumed start of the mature protein (by analogy with the cleavage site in the human sequence) is marked by an overhead V. Nucleotide no.s are shown as superscripts as are differences from other published ovine sequences. Substitutions and insertions in the sequences of Young et al., (1990) and Nash et al., (1991) are shown in bold and normal types, respectively. Insertions are indicated by an oblique line at the appropriate site, preceded by the additional nucleotide(s). The bold asterisk overlying position no.1042 represents a C residue in each of these sequences. Arrowheads bracket the start & finish of the former sequence and deletions in the former and latter sequences are indicated by double and single overlines, respectively.

2.3.Discussion:-

Several points worthy of note arose during the course of this work. A first concerns the initial failure of direct PCR to produce a discrete product. Indeed, although the annealing temperatures (T_m) of primers A and B were estimated to be 53 and 59-60°C respectively (using the formula $T_m = 3 \times [C + G] + 2 \times [A + T + I]$°C [Bolton & McCarthy, 1962], where C,G,A,T and I represent the number of corresponding bases contained in the primer), no product was formed at all unless the annealing temperature of the PCR was <45°C. Once the final sequence of the ovine cDNA was established, it could be seen that it contained the exact sequence of one of the mixes comprising primer B and that primer A differed from its complementary region (with the exception of its inositol base) by just one base. This base, however, was at the extreme 3' end of the primer, a crucial position for commencing polymerisation, and is likely to account for the difficulties experienced, although the paucity of TNFα encoding molecules suggested by Northern blotting anlysis (see below) may have been a contributory factor.

'Nested' PCR, a procedure adopted by Ohara et al. (1989) to amplify equivalent genes from species of wide evolutionary divergence, was clearly successful in predominantly amplifying the desired fragment. Whilst primer C shared 100% identity with its complementary region in the ovine sequence, primer D contained 2 mismatches. However, these were both contained within the 5' half of the primer.

Several problems also arose during the use of inverse PCR. The non-specific amplification of other molecules by primer F alone may be related to its content of C/G residues, which, at 57%, is slightly higher than the 50% considered ideal and may render this a generally 'sticky' primer. Nevertheless, it could be considered unfortunate that the main product of amplification by this primer alone was of similar size to the main product of primers E and F working together, necessitating further work before the TNFα-specific product could be analysed. The ability to generate significant product using a single primer also serves to illustrate the importance of ensuring sufficient dilution of cDNA during circularisation reactions. Since the PCR is such a powerful amplification device, the joining of only a handful of molecules in head-to-head, or tail-to-tail fashion, with or without

other, intervening molecules, could result in significant contamination of inverse PCR products by undesired products. This scenario clearly becomes more probable when attempting to amplify abundant molecules where such ligations are more likely to occur.

Further problems in the use of inverse PCR were related to the heterogeneous nature of the cDNA produced. In the original development of inverse PCR, Ochman et al. (1988) and Triglia et al. (1988) had worked with homogeneous fragments of genomic DNA. During the course of this work, Huang et al. (1990) subsequently described the successful use of inverse PCR on cDNA but made no mention of encountering any problems.

The smaller-than-anticipated, major, TNFα-specific product of inverse PCR was clearly due to the amplification of incomplete templates. Such templates are more likely to be present when cDNA is synthesised from RNA which has been extracted from a source rich in RNAses (e.g. macrophages) or from lengthy RNA molecules, particularly when they contain regions of strong secondary structure, resulting in polymerase 'pause' sites, and the incomplete extension of first strand cDNA to the 5' terminus. Within a mixed population, shorter templates, with fewer 'pause' sites, are then likely to be preferentially amplified during PCR. On this note, using the methods of Freier et al. (1986) and the sequence determined above, one can demonstrate the potential for complex secondary structure in ovine TNFα mRNA molecules just 5' to the region where primer E would anneal (fig.2.9). Whilst the physiological significance of such a structure is unknown, its presence would account for the fact that the major product of inverse PCR derived from amplification of molecules whose sequence (prior to circularisation) terminated before this region, as demonstrated by the sequencing of povTNF4,5 and povTNF6,7. Although not analysed, it would also seem likely that the c.1000bp major product of fully-nested inverse PCR, using primers G and H, derived from amplification of templates whose sequence terminated (again prior to circularisation) just 5' to the complementary region for primer H. It may have been fortunate that in this instance, having 'by-passed' a few hundred bp of sequence and one or more pause sites, fuller length templates were also substantially amplified.

Figure 2.9:-

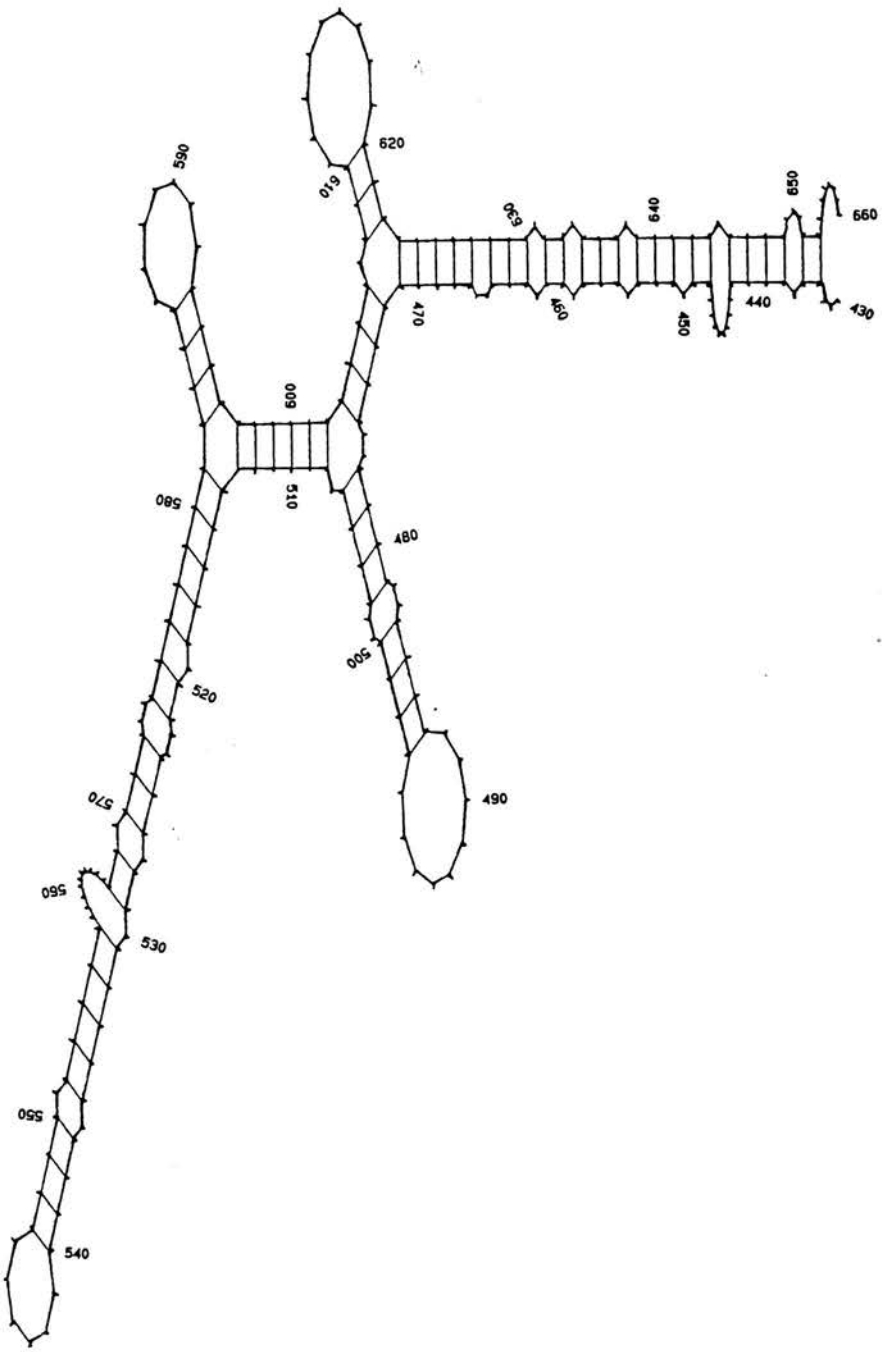


Figure 2.9:- potential secondary structure within the ovine TNFα transcript, as predicted using the method of Freier et al. (1986). Numbering of nucleotides is as in fig.2.8.

An initial response to the problem of trying to amplify fuller length molecules, by carefully separating and further amplifying the lesser amounts of larger molecules produced by inverse PCR, was clearly unsuccessful (fig. 2.6c). Here, a c.1000bp major product again resulted when a c.2000bp band was used as the template. Whilst this could have resulted from preferential amplification of a small number of contaminating c.1000bp molecules, great care was taken to minimise such contamination, by using a small amount of PCR product in slow, preparative electrophoresis, extended to allow wide band separation. The likely implication of this finding is that the c.2000bp product is a concatemer of the c.1000bp product, produced as a result of Taq polymerase working in a 'rolling-circle' fashion (Gilbert & Dressler, 1968) on the original circular template. Whilst there is a shortage of reports concerning the ability of Taq polymerase to cause the strand displacement necessary for rolling-circle replication per se , such displacement would have been favoured by the high temperature of polymerisation. The presence of other positively-hybridising molecules far larger than the maximum anticipated size of an inverse PCR product, including a faint concentration of c.3000bp molecules (as revealed by careful scrutiny of fig.2.5b), lend support to the notion that rolling-circle replication had occurred, though its definitive demonstration clearly requires sequencing of one of these larger products.

The above findings suggest that there are limitations to the usefullness of the inverse PCR in cloning cDNA molecules when a proportion of these templates are likely to be incomplete. Separation of fuller length products on the basis of their size alone may not always be possible, in contrast to the situation one might expect when using anchored PCR. Clearly, the use of a primer complementary to a region as near the 5' extremity of known sequence as possible is to be highly recommended.

Difficulties were also experienced when attempting to clone the three PCR products selected for cloning. In each case only one or two desired clones were obtained from several ligations and transformations. Controls suggested that transformation efficiencies were adequate, the ligase was functional and there was nothing present in the PCR product inhibiting transformation. Furthermore, use of the

same reagents in 'cohesive-ended' ligations when transferring the inserts of povTNF2 and povTNF3 to pTZ18R produced a more than satisfactory result. Others have also experienced poor results following attempts to blunt-end ligate PCR product into cloning vectors (Shuldiner et al., 1990; C.Fiskerstrand, C.Cousens & I.Bennett - personal communications). Approaches to overcome this problem have been described. These include the incorporation of restriction endonuclease sites at the 5' ends of PCR primers, with PCR products being submitted to digestion and 'sticky-ended' ligation, or the incorporation into primers of several bases of sequence complementary to vector sequence, allowing subsequent 'ligase-free' cloning (Shuldiner et al., 1990). Neither is perfectly satisfactory, since the first requires that no similar endonuclease sites exist within the unknown sequence (in order to clone it in entirety in one procedure) and cleavage at the termini of PCR product may not be very efficient (Kaufman & Evans, 1990) whilst the second requires the use of imperfect primers whose bulky nature makes them expensive and less than ideal for use in PCR. There is clearly a place for a more satisfactory alternative. The large number of non-hybridising recombinants present after two cloning attempts was likely to be due to inadvertant contamination of communal reagents.

Given the poor success rate in cloning PCR product it was somewhat fortuitous that much of this was able to be sequenced directly, since double-stranded sequencing may not always give consistently clear results. The protocol adopted, however, was similar to that described as optimal for the sequencing of double-stranded products by Casanova et al. (1990). Failure to obtain readable sequence by direct sequencing of the c.1000bp product of semi-nested PCR using primer E was likely to be due to the heterogeneous nature of this product in respect of the 5' extent of its TNFα sequence and length of poly-A tract, as demonstrated by the sequencing of povTNF4-7. Other failures could have been due to poor specificity of primer annealing, or incomplete extension of complementary strands, leading to the presence of only part of the annealing site and thus poor annealing efficiency.

The final nucleotide sequence presented in fig.2.8 is a composite of direct sequencing of PCR product and the consensus sequence of a handful of clones. In the light of the difficulties encountered in

amplifying the 5' terminus and obtaining sufficient clones, two immediate questions to consider about this sequence are:- 1) is it complete? and 2) is it accurate?

1658 bases of ovine TNFα cDNA sequence were ascertained (excluding the poly-A tail). This compares favourably with the overall lengths of TNFα cDNA sequences from other species (1643b in humans, Pennica et al., 1984; 1644b in mice, Fransen et al., 1985; and 1671b in rabbits, Ito et al., 1986). Furthermore, this ovine sequence can be broken down into an open reading frame, from the first coded methionine residue, of 234 codons, preceded by a 5' untranslated region (5'UTR) of 156 bases. Such a breakdown is similar to that obtained in other species (233 codons, 152b of 5' UTR in humans; 235 codons, 156b of 5' UTR in mice; and 235 codons, 173b of 5' UTR in rabbits). Such homology clearly suggests that most, if not all, of the ovine sequence had been obtained. Northern blotting analysis, however, does cast a little doubt on this conclusion. Several studies in humans and mice have shown that their TNFα transcripts have a similar, or slightly greater, mobility through agarose gels than their respective 18S RNA's (e.g. Pennica et al., 1985; Beutler et al., 1986a; Remick et al., 1989; Sugama et al., 1990). Careful measurement of the gel and blot shown in fig.2.4 clearly demonstrated that ovine TNFα transcripts show slower mobility than ovine 18S RNA. This result was confirmed by a second Northern blotting experiment (see chapter 4, fig.4.2). Whilst this discrepancy could be due to species' differences in the sizes of 18S RNA, the lengths of polyadenosine tailing, secondary RNA structure surviving formamide/formaldehyde denaturation, or any combination of these, a further contributing possibility could clearly be a larger ovine TNFα transcript. A further interesting observation is that the 5' 'UTR' of this ovine sequence is actually an open reading frame, in frame with the 234 codons described above, although it contains no triplets encoding a methionine residue. Questions regarding the possible existence and sequence of more ovine 5' UTR are likely to be answered only by genomic cloning with definitive identification of the transcription initiation site by S1 mapping. The answers would clearly help to ascertain whether the above 'open reading frame' observation

is merely a chance one, or whether an extremely unusual situation exists in the sheep, whereby it could produce a much larger TNFα preprotein than in other species.

Other observations worthy of note were also made from Northern blotting experiments. As in other species, TNFα transcripts in a sheep proved to be rapidly and greatly inducible from a cell population rich in macrophages, reaching a maximum concentration between 90 and 180 minutes with a subsequent rapid decline, when cells were stimulated immediately after preparation. However, similar induction was noted in the absence of added LPS, hence no conclusion can be drawn regarding the induction of ovine TNFα mRNA by LPS (see also fig.4.2). Nor can any firm conclusions be drawn regarding the earlier failure to detect ovine TNFα transcripts when cells were stimulated 24 hours after plating out, since the intended positive control, which had also been prepared following stimulation 24 hours after plating out, did not show detectable hybridisation. However, it can be said that similar amounts of RNA were electrophoresed (as judged by OD measurements prior to electrophoresis and the intensities of fluorescence following electrophoresis and transillumination) and the same reagents (including probe) and techniques were subsequently used as in the successful Northern blotting experiment. In the absence of any inadvertant error(s) in protocol, this would suggest that these samples contained levels of TNFα transcripts below the detection limit of Northern blotting. In the light of the above observation that ovine TNFα transcripts can be greatly induced during cell preparation and the demonstration that macrophages can become tolerant to LPS, with respect to TNFα production, by pre-exposure to only very low levels of LPS (Takasuka et al., 1991), it is possible that the ovine cells were refractory to LPS stimulation 24 hours after plating out. If this interpretation is correct, it could be considered fortunate that PCR proved to be sufficiently powerful at amplifying a small number of 'constitutive' or 'surviving' transcripts for the direct PCR to succeed. Other possible alternative explanations for the original Northern blotting failure would clearly include:- death of cells within 24 hours; insensitivity of ovine cells to the dose or type of LPS used; and/or the original non-adherent fraction of the lung cell population were the source of TNFα transcripts. Each of these

possibilities would appear to be remote. Premature cell death seems unlikely as intact RNA was extracted and several subsequent experiments (see chapter 5) demonstrate the survival of similarly-prepared lung cells for several weeks, whilst the same dose and type of LPS was successfully used in the induction of ovine IL-1ß mRNA from the adherent fraction of a lung-cell preparation (Fiskerstrand et al., 1992). (The above findings will be discussed further in chapter 4.)

On the question of sequence accuracy, it can be seen from fig.2.7 that the sequence from base no.11 to base no.738, as presented in fig.2.8, was derived by directly sequencing PCR products, with further confirmation by the single-stranded sequencing (in the opposite direction) of two overlapping clones. As such there can be few doubts regarding the accuracy of this sequence. Bases 1 to 10 of the presented sequence were arrived at by the sequencing of a single clone and as such could clearly contain a PCR-derived error. Counting povTNF2,4 & 5 and povTNF3,6 & 7 as single clones, sequences from bases 739 to 758, 759 to 1081, 1082 to 1642 and 1643 to 1658 were derived as consensus sequences from only 4,3,2 and 3 clones respectively. No differences were seen between the sequences of any clones from base 739 to 861 (the end of the coding region). Furthermore, this sequence was confirmed by the consensus sequence of five clones obtained for expression purposes and sequenced in both directions (see chapter 3). There can therefore be few doubts regarding the accuracy of this section.

Differences were noticed between the sequences of the three clones in the section from b.862 to b.1081 at four positions. These comprised a T instead of a G residue at position 958 in povTNF2 and, in povTNF3, a C instead of a T residue at positions 910 and 1042, and a 3 base deletion at positions 992-994. The change at position 1042 was confirmed in the sequences of povTNF6 and 7. PovTNF8 contained the consensus sequence. No differences between clone sequences were noted in the remaining sections. Under these circumstances, whilst the majority of the 3' UTR sequence presented is likely to be correct, there exists the possibility that a few of the bases shown may be PCR-derived artefacts. In one survey, Saiki et al. (1988) estimated an error rate of 0.25% for any given base following 30 cycles of amplification of a particular fragment. Whilst error rates may be

dependent on the sequence of DNA amplified, the possibility of sequences here containing errors would have been increased by the fact that as many as 70 rounds of amplification were needed before cloning. Clearly, the differences that were noted between clone sequences could have been due to such PCR errors or, alternatively, allelic polymorphism in an heterozygous individual (see below).

During the course of this work two other groups cloned and sequenced ovine TNFα cDNA and published their data, which was derived following the identification of a single (Young et al., 1990) or two, identical (Nash et al., 1991) cDNA clones. A few differences exist between the three sequences (see fig.2.8). The sequence of Young et al. appears to be incomplete in that it contains only 3 bases of 5' UTR. These 3 bases differ totally to those in the corresponding positions of the other two sequences and, in contrast to these, show no homology to the equivalent positions in the human sequence. Since these bases are at the extreme end of a cloned insert, they may represent a cloning artefact.

With the exception of position 1, the entire 5' UTR sequence presented here is in total agreement with that in the sequence of Nash et al.(which does, however, contain four additional bases at the 5' terminus), as is the sequence of the coding region. 2 differences from the sequence of Young et al. were noted in this region though. These included a conservative 3rd base substitution and a 3 base deletion in the latter sequence, leading to one fewer amino acid in its predicted preprotein structure. As well as being present in the sequence of Nash et al., this additional triplet was present in cDNA amplified by both direct and indirect PCR, and hence in cDNA derived from both of the two sheep used in this work. Furthermore, whilst this extra amino acid has no counterpart in the human sequence, the same residue is present in the same position in a bovine TNFα amino acid sequence (Goeddel et al., 1986; see chapter 4, fig.4.11).

A handful of differences between the 3 sequences are present in the 3' UTR, where most doubt exists concerning my own sequence. However, the other two sequences were in agreement against my sequence in only one of these positions (a C for T substitution at b.1042), so that, with this exception, my sequence, from base 2 onwards, was the same as the consensus of all 3 sequences. It is notable, however, that one of

my three clones covering b.1042 contained the same residue at this position as the two alternative sequences. Also of note was the fact that the sequence of Nash et al. lacked the same 3 bases as povTNF3. Since their sequence derived from cDNA clones, and a survey of multiple PCR-derived errors identified only single base substitions (Saiki et al., 1988), it seems likely that this short deletion represents an allelic polymorphism. Other sequence differences could clearly also be due to other polymorphisms or, alternatively, errors, be they human or polymerase-induced. Whilst further studies would clearly be required to resolve this issue, polymorphism in TNFα genes is not without precedent. Jacob and McDevitt (1988) noted an RFLP in the TNFα genes of different strains of mice (see 1.8.6) which could be linked to differential abilities to produce TNFα, whilst Jongeneel et al. (1990) identified at least eight different alleles, involving a polymorphic microsatellite, in the murine TNFα gene promoter. More recently, Freund et al. (1992) discovered several differences between the TNFα genes of toxoplasmic encephalitis -resistant and -susceptible mice, which could be localised to the promoter, first intron and 3' end of the TNFα gene. Furthermore, polymorphism could perhaps be expected in an immune system gene in a species such as the sheep, which has been line-bred for several centuries, often on the basis of disease resistance. In this regard it can be noted that at least two widely-differing breeds of sheep (Scottish Blackface and Merino, [the breed used by Young et al. was not stated]) from three different continents were used in these studies. Differences also exist in the published sequences of cDNA's encoding other ovine cytokines, such as IL-1β (Fiskerstrand & Sargan, 1990; Andrews et al., 1991) and IFN gamma (McInnes et al., 1990; Radford et al., 1991).

An overall alignment of the ovine and human nucleotide sequences can be made which suggests that the two share 78% homology overall (a similar divergence to that found for human and rabbit or murine sequences), though certain regions show a much higher degree of homology. These include the coding regions, which share 84% homology at the nucleotide level (a discussion on the predicted amino acid sequence of ovine TNFα and its comparison with those of other species will be presented in chapter 4). Another well-conserved region is that immediately preceding the coding region. Here, 11/13 bases share

identity between the two sequences, with the final 6 bases (GACACC) being the same in both cases. These six bases show homology with the sequence identified by Kozak (1987) as a consensus sequence for translation initiation ($GCC^{A}/_{G}CC$), and the conservation of sequence in this region would argue against the possible existence of a larger ovine TNFα preprotein discussed earlier. 2 regions of high homology in the 3' UTR are also worthy of note. These include a typical polyadenylation signal sequence (Proudfoot & Brownlee, 1976) close to the 3' terminus and a 34 base AT-exclusive region, which is perfectly conserved between the two, and most of which is also conserved with the rabbit and mouse sequences. Such AT-exclusive regions have been identified as conferring the properties of instability (Shaw & Kamen, 1986), as well as translational inducibility by LPS (provided the sequence is in appropriate context and in the correct cell type [Han et al.,1990]), on transcripts bearing them (see 1.3.3). With regard to the context of this sequence, it is also apparent that 90% of 50 nucleotides flanking this region share identity between the sequences. The ovine sequence also contains an additional, earlier 10 base AT-exclusive region. One might therefore predict that ovine TNFα transcripts would also display the properties of instability and translational inducibility. The instability of ovine TNFα transcripts is suggested by their rate of disappearance following induction (see fig.2.4). Further studies by Nash et al. (1991), using deletion constructs for protein expression in mammalian cells, suggested that the presence of this region contributed to decreased yields, with transcript instability being one potential explanation. The translational inducibility of ovine TNFα transcripts by LPS will be discussed in chapter 4.

CHAPTER 3:- EXPRESSION AND CHARACTERISATION OF RECOMBINANT OVINE TNFα.

3.1. Introduction:-

A further objective in the study of ovine TNFα was to express recombinant ovine TNFα (rovTNFα), firstly, to be able to answer questions regarding the biological properties of a protein with the sequence of ovine TNFα and secondly, to provide an immunogen for raising antibodies which might be capable of recognising native ovine TNFα. Several factors were considered in the choice of a suitable expression system for this work, including precedents in the production of recombinant TNF'sα, the possible importance of a correct glycosylation pattern, ease of purification and anticipated yields of recombinant protein, and finally, the cost and availability of reagents and equipment.

Recombinant TNF'sα from other species have previously been recovered from a variety of bacterial, yeast and mammalian expression systems and have proven to be highly active when derived from any of these sources (e.g. Pennica et al., 1984; Sreekrishna et al., 1989; Marmenout et al., 1985). Thus a wide choice of potential systems could have been considered. Whilst the function of glycosylation in TNF'sα is unknown, it does not appear to be important for activity, certainly in the murine system, since human TNFα, which is not glycosylated, and unglycosylated recombinant murine TNFα are both highly active on murine cells (Pennica et al., 1984, 1985), even though native murine TNFα is glycosylated (Green et al., 1976). Hence, although the sequence of ovine TNFα predicts one potential Asn-linked glycosylation site (see fig.2.8), the production of a recombinant protein with an authentic glycosylation pattern, as might be achieved using a mammalian expression system with attendant problems of low yields, contamination with other mammalian proteins and possible cytotoxicity of the product, took lower priority than selection of a system for high yield and ease of purification.

The expression system chosen was the yeast Ty-'virus-like particle' (VLP) system originally described by Mellor et al. (1985). An attractive feature of this system is the ease of purification of an expressed protein. Here, proteins are expressed as fusion products with, and downstream from, the Ty element protein P1 (Dobson et al.,

1984), whose ability to self-assemble into VLP's (Adams et al., 1987b) is generally retained in the presence of a fusion partner (Adams et al., 1987a). The particulate nature of the fusion protein can then be exploited in a simple purification process involving centrifugation and/or size-exclusion procedures. By including a specific proteolytic cleavage site between the fusion partners, the protein of interest can subsequently be freed from P1. Reagents for this system were available within the department and it had already proved capable of producing adequate yields and purity of recombinant protein with the equipment available (Reyburn et al., 1992). This system has previously been used to express another recombinant cytokine (human IFNα2), which was found to have biological activity (Mellor et al., 1985) and furthermore, the production of a recombinant TNFα as the carboxy-terminal fragment of a larger precursor, and its subsequent release by proteolytic cleavage, could be considered to be similar to the production and release of mature, native TNFα (Pennica et al., 1984; Scuderi, 1989). Another possible beneficial feature of this system, which has previously been proposed, is that the immunogenicity of the recombinant protein, when inoculated as a fusion protein in uncleaved form, may actually be enhanced by its particulate nature (Adams et al., 1987a).

Several vectors have been constructed for use in producing proteins via the Ty-VLP, including those where expression of the fusion protein is placed under the control of either a constitutive (Adams et al., 1987a) or an inducible promoter (Kingsman et al., 1990). Given the possibility that the presence of a large amount of inappropriate, intracellular TNFα might be detrimental to the host cell (Smith M. et al., 1990), the vector chosen for this work was pOGS40. In this vector, expression of the fusion protein is under the control of a strong galactose-inducible, hybrid promoter (PGK-GAL, Kingsman et al., 1990). Other features of pOGS40 include:- the ability to replicate in both bacteria and yeast; an ampicillin resistance gene allowing selection of bacterial transformants in ampicillin-containing media; a leu2 gene (see below); and an engineered yeast Ty A gene, which encodes P1 and ends in a BamHI restriction endonuclease site (an unique site in this plasmid) followed by termination codons in all three reading frames.

The inducibility of galactose-regulated genes can be limited by the level of GAL4 protein within a cell (Johnston & Hopper, 1982). Co-transformation of yeast with another plasmid, pUG41S, which contains the structural gene for GAL4 (also under the control of a galactose-inducible promotor), can, therefore, be used to increase the yield of fusion protein obtained when using a pOGS40-derived plasmid (Kingsman et al., 1990). pUG41S also contains a ura3 gene. This gene, and the leu2 gene of pOGS40, confer uracil- and leucine- independance respectively, on a uracil- and leucine- dependent host strain of yeast, allowing the selection of double transformants by growth in minimal media. The host strain of yeast used in this work, Saccharomyces cerevisiae, strain BJ 2168, as well as containing incapacitating mutations in its ura3 and leu2 genes, is also protease-deficient, reducing the risk of undesired digestion of the recombinant product (Jones, 1991).

Bovine factor Xa, a protease which specifically cleaves a polypeptide immediately after its recognition sequence (Ile-Glu-Gly-Arg; Magnusson et al., 1975) has previously been used to cleave fusion partners in order to leave a recombinant protein with an authentic amino terminus (Nagai & Thorgersen, 1984). Fortunately, there are no recognition sites for this enzyme, or for the restriction endonuclease BamHI (GGATCC), in the predicted amino acid and nucleotide sequences respectively of mature ovine TNFα (see fig.2.8). The strategy adopted in order to construct an ovine TNFα expression cassette, therefore, was to use 'tailored' primers in a PCR on ovine cDNA. These inward-facing primers were designed to be capable of annealing to the first and last few bases of the coding sequence for mature ovine TNFα but also contained additional, flanking nucleotides, so that in an amplified fragment of DNA the ovine TNFα sequence would be immediately preceded by sequence encoding a factor Xa cleavage site and the whole of this would be flanked at both ends by BamHI sites. (Without knowledge of the actual first amino acid of mature ovine TNFα, this was assumed, by analogy with human TNFα, to be the 78th aa of the preprotein [see figs.2.8, 4.11 and discussion, 3.3] giving the mature protein [without glycosylation] a predicted molecular weight of 17kDa.)

Fragments of DNA thus amplified, then digested with BamHI, would be inserted into the BamHI site of pOGS40 (after first cloning and sequencing via the 'phagemid pTZ18R to eliminate potential errors in sequence introduced by PCR [see chapter 2]). Following co-transformation (with pUG41S) and culture of yeast, then purification of VLP's, rovTNFα would be freed from P1 by digestion with factor Xa and characterised, in order to ascertain whether it shows properties similar to TNF'sα from other species.

3.2.Results:-

3.2.1.Production of a 'rovTNFα cDNA expression cassette':-

The following primers (with positions of complementary sequences and recognition sites indicated overhead) were selected for use in the production of an ovine TNFα expression cassette:-

Sense primer-

	***	BamHI	FactorXa	ovine TNFα*	
5'	AAG CTT GGA TCC ATA GAA GGT AGA CTC AGG TCA TCT TCT CAA GCC				3'

Anti-sense primer-

	***	BamHI	(Stop)	ovine TNFα**	
5'	AAG CTT GGA TCC TCA CAG GGC GAT GAT CCC AAA GTA GAC C				3'.

(* positions 388-408, ** positions 861-834 [no.s according to fig.2.8]; *** additional nucleotides were included 5' to the BamHI sites to help extension of the opposite strand through these regions during PCR, hence ensuring the synthesis of complete endonuclease recognition sites.)

When PCR's were performed using these primers on templates of 10ng of first strand or ds cDNA (see 2.2.1 and 2.2.6), at an annealing temperature of 48°C, a product of anticipated size (c.510bp) was produced in either case (fig.3.1a).

Figure 3.1:-

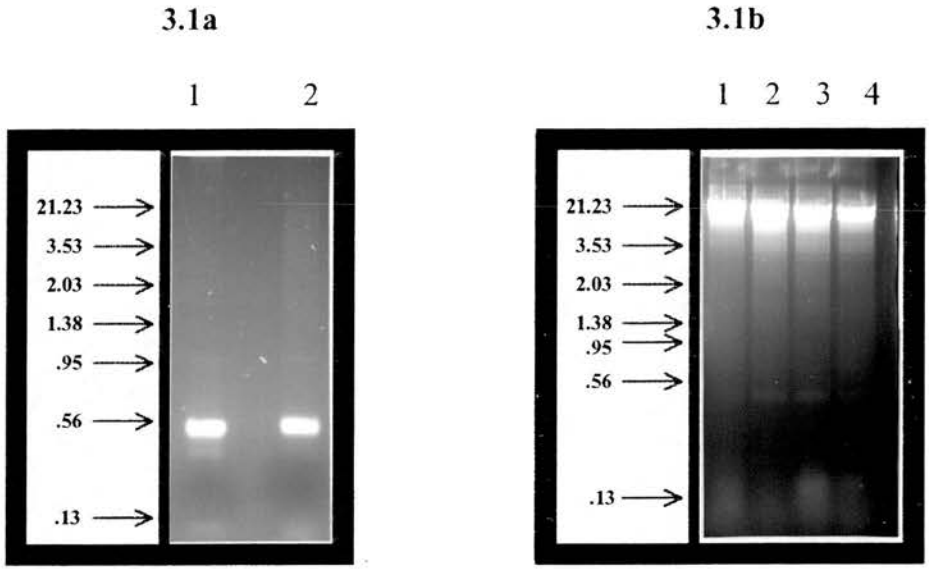


Figure 3.1:- photographs of 1.2% agarose gels following electrophoresis & transillumination, with the positions of molecular size markers (in kb) indicated. Samples submitted to electrophoresis comprised 10% of the product of PCR's, involving the tailored primers described in the text, on templates of double stranded- or first strand- cDNA (3.1a, lanes 1 & 2, respectively) & plasmid preparations, after digestion with BamHI, of four selected colonies which had been transformed with pOGS40-derived plasmids (3.1b, lanes 1-4). Colonies whose extracts are shown in fig.3.1b, lanes 2 & 3 were subsequently designated pOGSTNF1 & 2, respectively.

Table 3.1:-

COLONY	1	2
ScTNF12	0.95	2
ScTNF13	0.65	1.55
ScTNF14	1	1.8
ScTNF15	1.1	2.2

Table 3.1:- the OD's$_{600}$ of four selected yeast colonies after 24 h growth in glucose-containing medium (Sc-glc) (column 1) & 24 h after transfer to galactose-containing medium (Sc-glc-gal)(column 2).

After purifying the PCR product of one of these reactions (first strand template), digesting it with BamHI, repurifying and submitting it to preparative electrophoresis through LGT agarose, an estimated 1µg of c.510bp product was available for 'cohesive-ended' ligation into the BamHI site of pTZ18R. When 25 or 50ng of this were used in ligations with 50ng of linearised 'phagemid and the products used to transform E.coli, >100 recombinant colonies resulted from either ligation. >90% of these hybridised to an ovine TNFα cDNA probe (c.500bp product of direct PCR, 2.2.3). It was also noted that most positively-hybridising colonies were pale blue rather than white in colour.

21 positive colonies were randomly selected for further analysis involving agarose gel electrophoresis of miniplasmid preparations after their digestion by BamHI. Suitably-sized inserts were excised from 15 of these preparations. Undigested miniplasmid preparations were also analysed to help assess overall plasmid size, thereby hopefully avoiding clones with multiple inserts. 5 clones (designated povTNF 9-13), with similarly-sized plasmids from which a c.510bp insert could be excised, were chosen for sequencing after preparation of ssDNA.

Initial sequencing reactions using reverse sequencing primer, and their analyses, showed that povTNF9 & 11 contained a similar insert in one orientation, extending from the sense primer (above) through sequence of ovine TNFα cDNA 3' to the complementary region for this primer, and that povTNF10,12 & 13 contained insert in the other orientation, starting with the sequence of the antisense primer. Subsequent sequencing reactions using primers E or F (fig.2.2) on preparations from povTNF10,12 & 13 or povTNF9 & 11, respectively, confirmed these sequences in the regions of overlap and extended the known sequences of these inserts through their full extent. In each case plasmids contained only a single insertion of the PCR product and the consensus sequence of all 5 clones (having converted the sequences of povTNF10,12 & 13 to sense form) was in agreement with the appropriate region of sequence presented in fig.2.8. However, povTNF9 contained a C instead of a T residue at position 560 and povTNF11, a C instead of a G residue at position 424, whilst povTNF12 contained approximately 30 bases of DNA of unknown origin inserted between the

sense primer and the multiple cloning site of pTZ. Since either of these base alterations would have resulted in a different amino acid from the consensus sequence, and the presence of an extra fragment of DNA might have interfered with 'clean' insertion of DNA, only two clones were considered suitable for providing an insert for ligation into pOGS40.

An estimated 1µg of insert, ready for use as the rovTNFα cDNA expression cassette, was obtained from a large scale plasmid preparation of povTNF13, after BamHI-digestion and purification by agarose gel electrophoresis and electroelution.

3.2.2. Preparation of a rovTNFα expression vector:-

When 50, 100 or 250 ng of the above rovTNFα cDNA expression cassette were incubated with 200ng of BamHI-cut, CIP-treated pOGS40 in cohesive-ended ligation reactions, and the purified ligation reactions used to transform E.coli, strain JM83, >100 colonies resulted from each ligation. However, a similar number resulted from a control ligation reaction involving vector with no DNA for insertion. (Strain JM83 [Messing, 1979] was used here because of its greater susceptibility to transformation, likely to be important when using a relatively large plasmid such as pOGS40.)

After 200 randomly-selected colonies were screened by colony hybridisation to an ovine TNFα cDNA probe (c.500bp product of direct PCR, 2.2.3), 14 colonies gave a positive signal, with four giving a much stronger signal than the rest. When plasmids from these colonies were analysed on agarose gels alongside a preparation of uncut pOGS40, the four strong-signal colony plasmids showed significantly faster mobilities than pOGS40, whilst those derived from the 10 weaker signal colonies showed slightly slower mobilities than pOGS40.

When 4 of these 10 latter preparations were randomly selected and further analysed following digestion with BamHI, it was found that a c.510bp insert could be excised from at least 3 of the 4 (fig.3.1b). An estimated 150µg of plasmid DNA were then obtained from a large scale preparation of each of these 3 plasmids. Following submission of these preparations to double-stranded sequencing reactions, using a pOGS40-specific primer (chosen for its complementarity to a site 50-30 bases upstream from the BamHI site) the sequences obtained comprised

some 20 bases of the TyA gene through to the BamHI site, followed by c.100 bases of readable sequence derived from the ovine TNFα expression cassette. It was found that 2 plasmids (pOGSTNF1 & 2) contained this expression cassette inserted in the correct orientation and in the same reading frame as the TyA gene, whilst in the third, it was in reverse orientation.

3.2.3.Transformation of yeast:-

When 5 or 10µg each of pOGSTNF1 and pUG41S, with or without 20µg of salmon sperm carrier DNA, were used to co-transform aliquots of yeast spheroplasts, a total of 75 colonies were counted on leucine- and uracil- deficient plates 6 days later. By contrast no colonies were seen on similar negative control plates (spheroplasts with 10µg pUG41S only) whilst hundreds of colonies were seen on a plate which included leucine and uracil as a positive control for regeneration. Notably more colonies derived when salmon sperm DNA had been used as a carrier (50 cf 25), though similar numbers derived whether 5 or 10µg of plasmid had been used (43 cf 32).

3.2.4.Verification of the presence of P1-fusion protein in yeast transformants:-

4 of the largest colonies (designated ScTNF12-15 and derived from transformations involving 5 or 10µg of plasmid without carrier [ScTNF12,13], or 5 or 10µg of plasmid with carrier [ScTNF14,15, respectively]) were selected for further work. After a single fast-growing sub-clone derived from each was used to initiate a small-scale broth culture, yeast cells continued to grow and divide, even after transfer to galactose-containing medium, as assessed by optical density measurements and phase-contrast microscopy (table 3.1, fig.3.2).

Crude extracts of each of the four cultures were then analysed by SDS PAGE with visualisation of proteins by Coomassie blue staining and by Western blotting using an anti-P1 polyclonal antiserum as the primary antibody (fig.3.3). On Coomassie-stained gels, a dense band, which could not be seen in an extract of untransformed yeast, was seen at the anticipated position of a P1-TNF fusion protein (c.67kDa) in 3 of the 4 extracts, whilst a dense band seen at the expected position

Figure 3.2:-

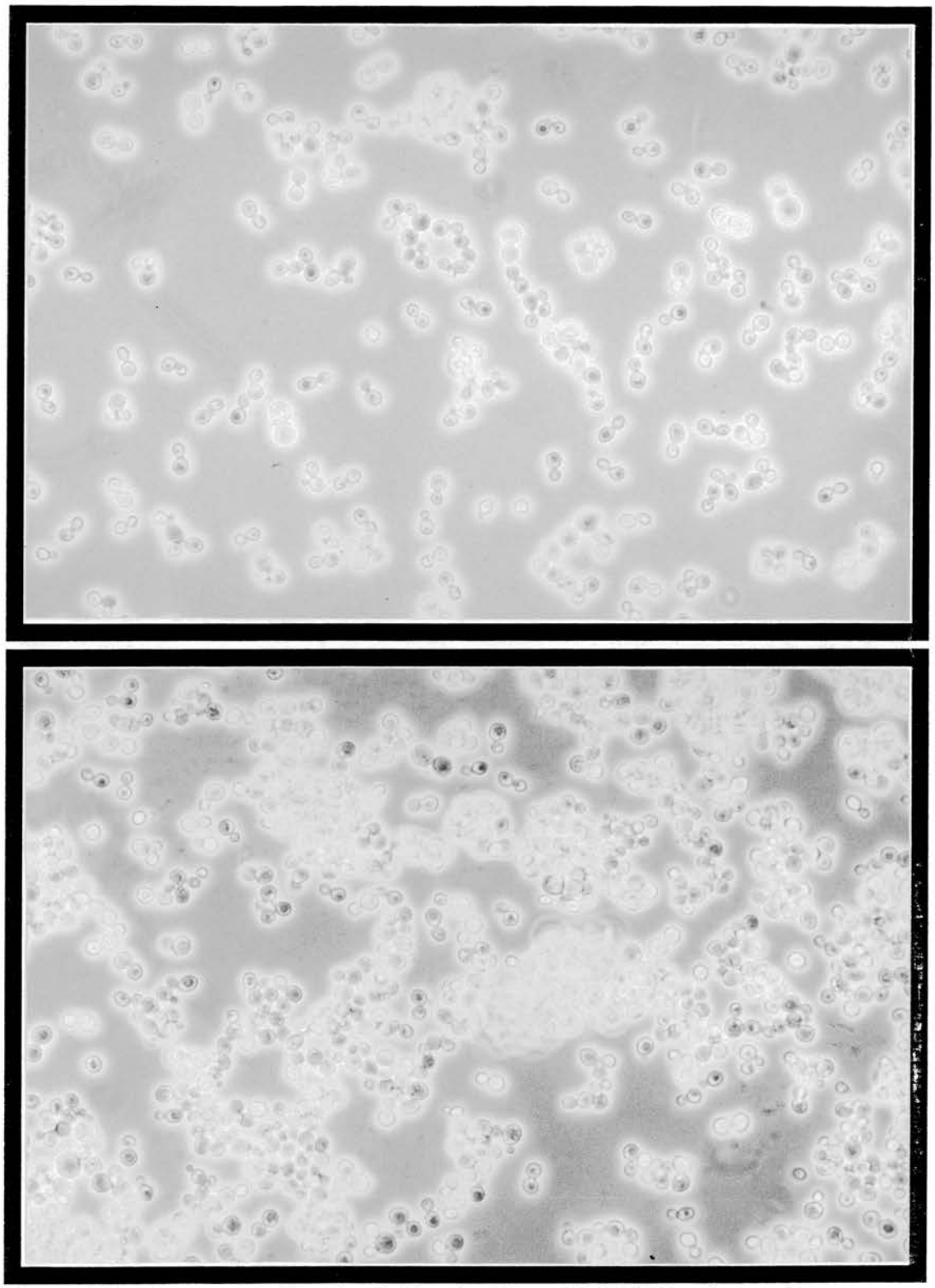
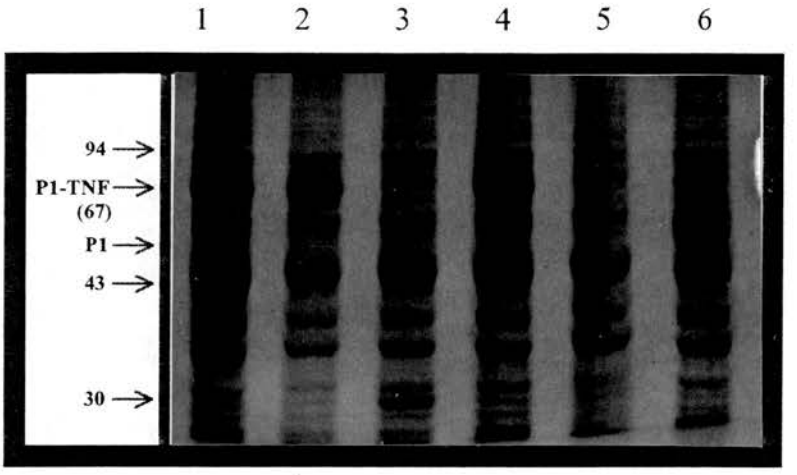


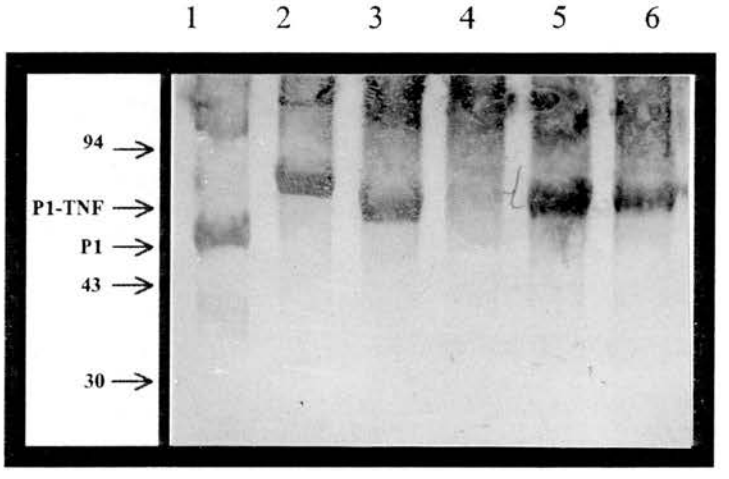
Figure 3.2:- shows representative fields of a drop of ScTNF12 culture, when examined by phase contrast microscopy, 24 h after growth in glucose-containing medium (above) and 24 h after transfer to galactose-containing medium (below). Note the generally increased cell density in the latter & the continued presence of budding, phase-bright cells.

Figure 3.3:-

3.3a



3.3b



Figures 3.3a & b:- photographs of a Coomassie blue-stained gel & Western blot, respectively, following electrophoresis, under reducing conditions, of extracts of assorted yeast cultures through SDS- 10% polyacrylamide gels. The primary detection reagent used for the Western blot was an anti-P1 polyclonal rabbit antiserum, used at a recommended dilution of 1:1000. The positions of molecular weight markers are indicated (in kDa), as are the anticipated positions of P1 & a P1-TNF fusion protein. Samples submitted to electrophoresis comprised:- 7µls of extracts from ScTNF12,13,14 & 15, untransformed yeast & yeast transformed with pMA5260 (fig.3.3a, lanes 1-6, respectively) & 2µls of extracts from the latter colony, yeast expressing a c.75kD fusion protein (P1-P25, Reyburn et al., 1992) & ScTNF 15, 14, 13 & !2 (fig.3.3b, lanes 1-6, respectively).

of P1 (c.50kDa) in an extract of yeast transformed with pMA5620 (which constitutively expresses P1 [Adams et al., 1987a]), could not be seen in extracts of ScTNF12-15 (fig.3.3a). Western blotting confirmed that in the three extracts with an extra, dense band there was an apparent decrease in electrophoretic mobility of P1 consistent with the formation of a P1-TNF fusion protein, whilst the fourth (ScTNF14) was not apparently expressing P1 at all (fig.3.3b).

3.2.5.Large-scale preparation of hybrid VLP's:-

48 hours after a glycerol stock of ScTNF12 was used for its initiation, a larger scale yeast culture had reached an OD_{600} of 1.4 and, following a 5-fold dilution, it had regained an OD_{600} of 1.2, 24 hours later. Having ascertained that a shift into galactose-containing media did not appear to prevent yeast growth (see above), further culture then involved simple dilution into this medium. 24 hours after such a 9-fold dilution, the culture had regained an OD_{600} of 1.0. Electron microscopy demonstrated the presence of large numbers of VLP's within these cells at this stage (fig.3.4).

4 rounds of vortexing with glass beads were required to achieve >85% cell destruction. After low speed spins to remove gross debris from the crude extracts of these cells, a large amount of material was collected on and within a 60% sucrose cushion following a subsequent higher speed spin. When a dialysate of this material was separated by centrifugation through a sucrose density gradient, large amounts of protein(s) of appropriate size for a P1-TNF fusion protein collected in the bottom half of this gradient, whilst some other yeast proteins remained in the top half (fig.3.5). This pattern was consistently seen on each gradient. When fractions 9-18 (fig.3.5) were pooled and dialysed into a recommended buffer for factor Xa, a flocculent precipitate was formed. Analysis of dialysate and supernatant suggested that further purification could be achieved by precipitate removal (fig.3.6a) and, having done so, the putative fusion protein was left at an estimated purity of c.80%, in a preparation containing 700µg protein/ml. Electron microscopy of this final preparation revealed the presence of large numbers of VLP's with the typical morphology described by Adams et al. (1987a,b) (fig.3.6b,c).

Figure 3.4:-

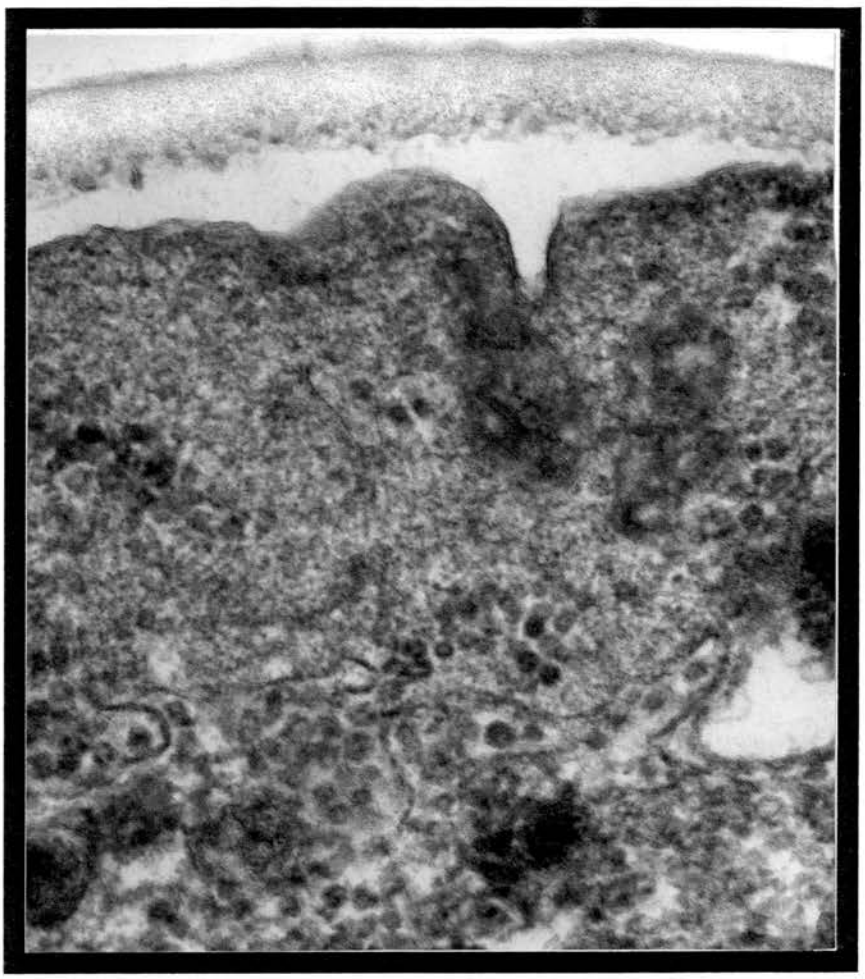


Figure 3.4:- an electron micrograph of a yeast cell taken from a culture of ScTNF12, after growth in galactose-containing medium (magnification x 60,000). Note the presence of large numbers of virus-like particles within the cytoplasm.

Figure 3.5:-

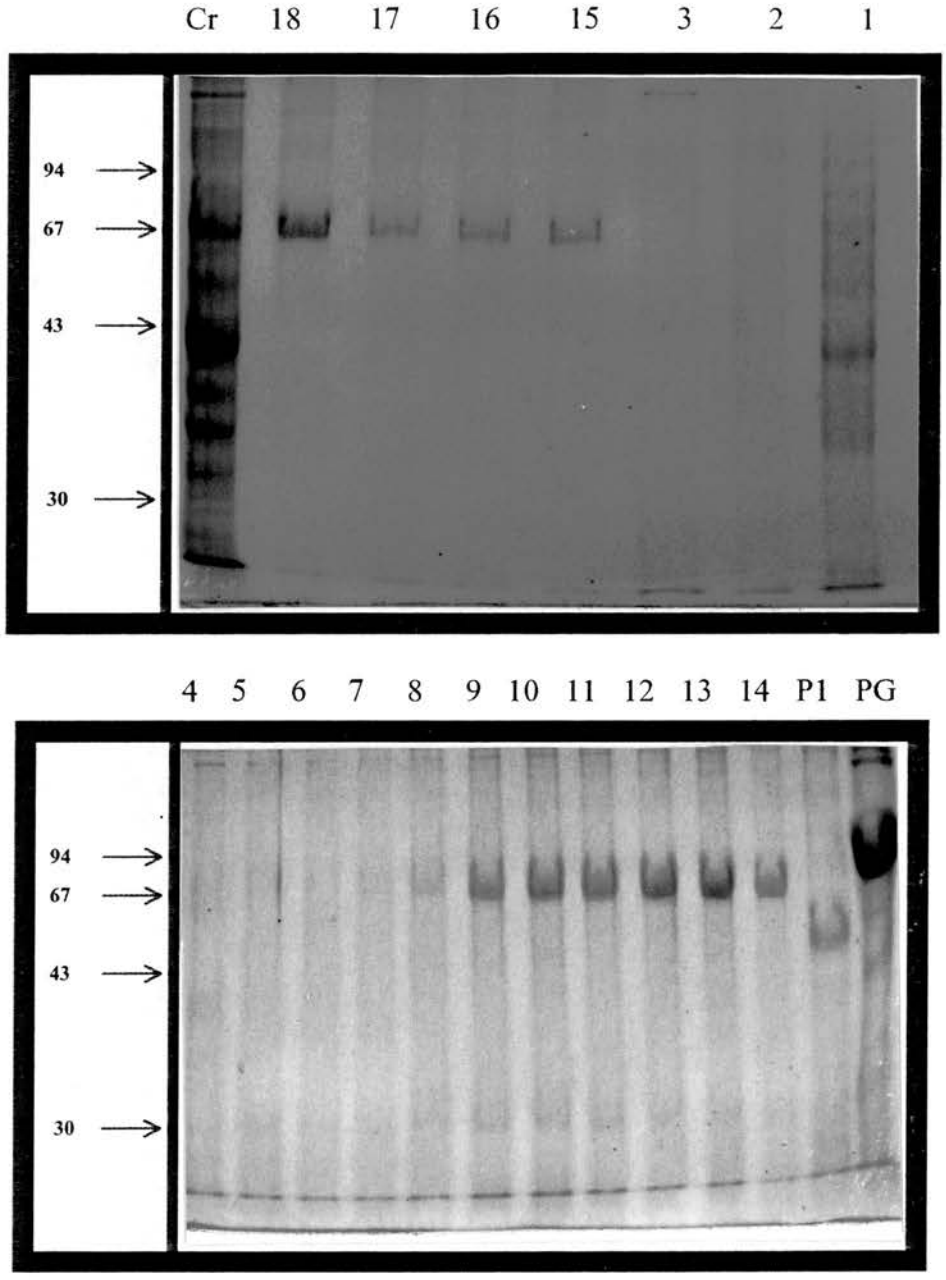


Figure 3.5:- photographs of 2 Coomassie blue-stained, SDS-10% polyacrylamide gels, following electrophoresis under reducing conditions. The positions of molecular size markers are indicated (in kDa). Samples subjected to analysis comprised:- 7µls of crude extract of ScTNF12 at the end of culture (lane Cr);5µl aliquots of 2ml fractions (numbered from top to bottom) removed from a sucrose density gradient following centrifugation (see text)(lanes 1 -18; lane no.s correspond to fraction no.s); an estimated 3µg of a preparation of P1 (lane P1); & an estimated 10µg of a preparation of a c.75kD fusion protein (P1-P25, Reyburn et al., 1992)(lane PG).

Figure 3.6:-

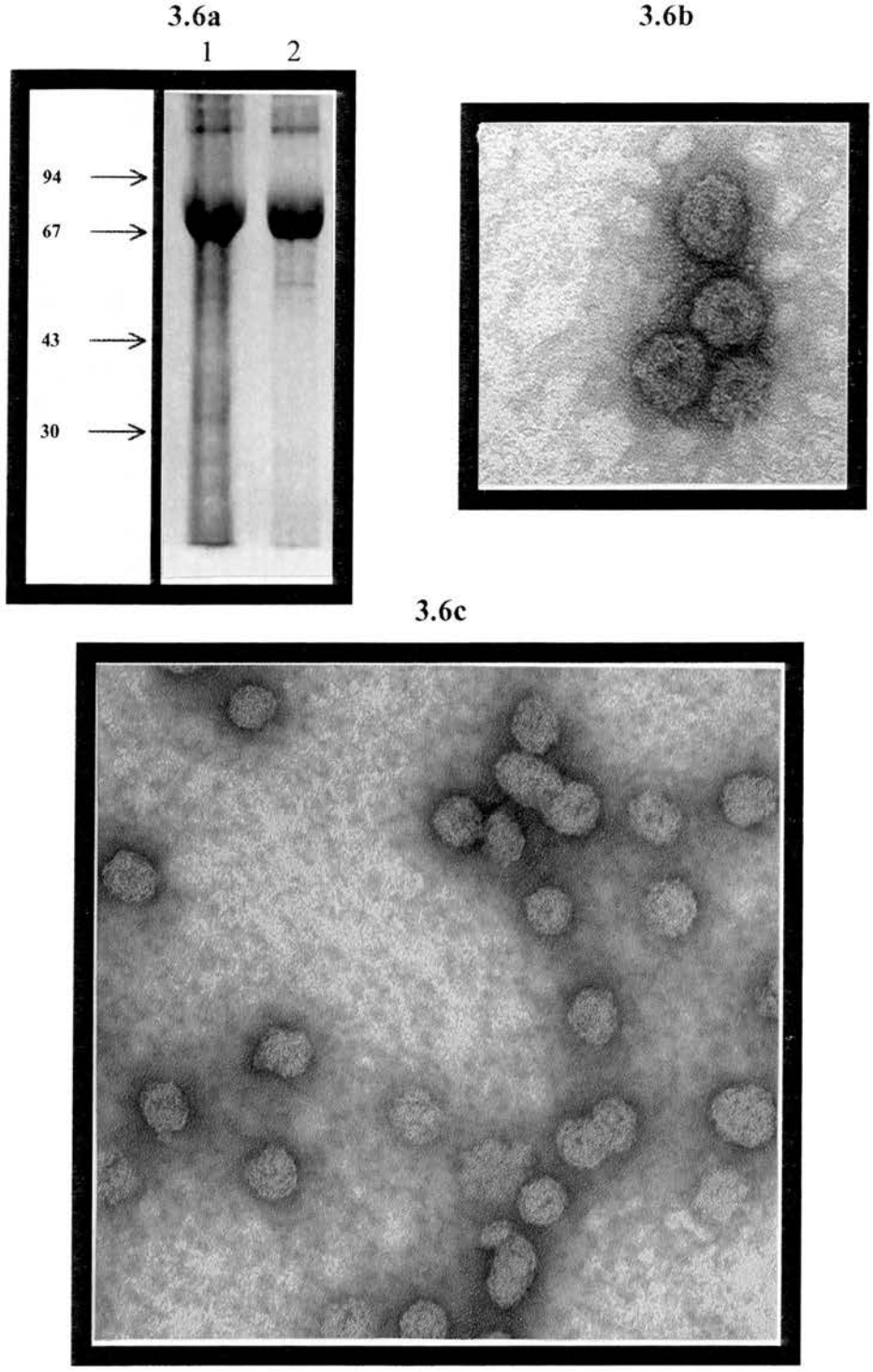


Figure 3.6a:- an SDS-10% polyacrylamide gel following electrophoresis, under reducing conditions, of 20µl samples removed from pooled & dialysed sucrose density gradient fractions (9-18, fig.3.5), before (lane 1) & after (lane 2) removal of precipitates formed during dialysis. The positions of molecular size markers are indicated (in kDa). Figures 3.6b & c:- electron micrographs (magnifications x165,000 & x100,000, respectively) of the final P1-TNF hybrid VLP preparation.

3.2.6.Cleavage of fusion protein:-

It has previously been found necessary to add chaotropic agents to VLP suspensions to enable the cleavage of some P1-fusion proteins by factor Xa (Braddock et al., 1989; Gilmour et al., 1989) and indeed no cleavage of fusion protein in aliquots of the above P1-TNF suspension occurred when no other additions were made, even using a high concentration of factor Xa and an extended incubation time. Nor was any cleavage achieved when urea or the detergents CHAPS or deoxycholate (DOC) were added on their own (to 1M, 0.05% or 0.05% respectively) (fig.3.7a). Partial cleavage was achieved using 0.5% Triton-X-100. However, full cleavage could be obtained when 0.05% DOC was added in addition to Triton-X-100 or CHAPS (fig.3.7b). Since they can both be removed by dialysis, the 0.05% CHAPS/ 0.05% DOC combination was then used for subsequent cleavages. A further experiment was conducted to ascertain the minimum concentration of factor Xa that would effect c.100% cleavage in an overnight incubation. The results (fig.3.8a) showed that the appearance of c.50kDa (P1) and c.17kDa (rovTNFα) proteins required the presence of both the P1-TNF preparation and factor Xa, and suggested that 1 part Xa to 50 parts fusion protein would be the most cost-effective concentration for cleavage. This concentration was therefore subsequently used to cleave the rest of the VLP suspension.

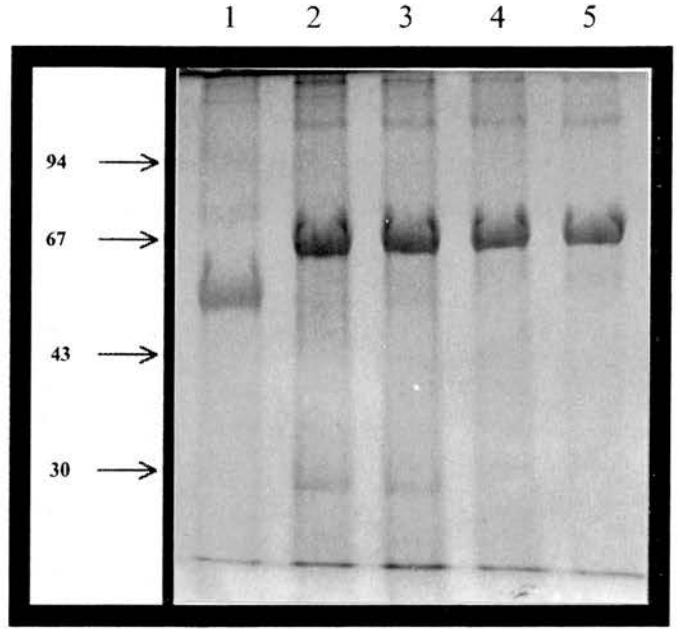
c.27kDa and lesser amounts of c.24kDa proteins were also visible in some of these experiments [see fig.3.7a and especially b] but only where high concentrations of factor Xa had been added (see discussion, 3.3).

3.2.7.Final purification of the recombinant protein:-

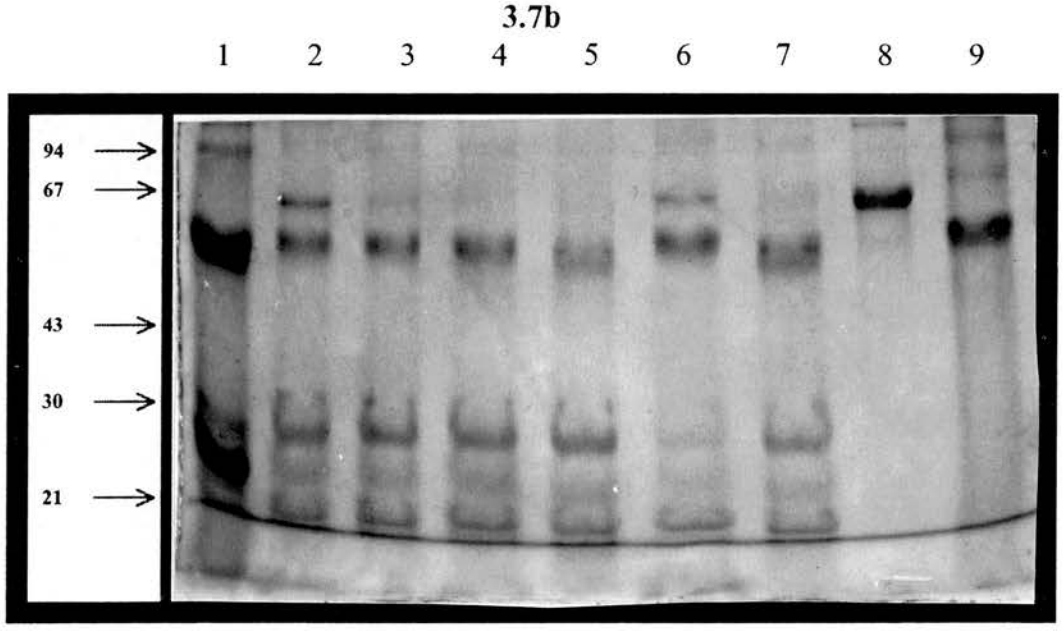
After cleaved suspensions were submitted to high speed centrifugation and the supernatants dialysed into PBS, the c.17kDa putative rovTNFα protein was the only protein clearly visible when 10µl of supernatant were analysed by SDS PAGE and Coomassie blue staining (fig.3.8b, lane 2). Most of the P1 protein appeared to be contained in the pellet formed after high speed spinning (fig.3.8b, lane 3).

Figure 3.7:-

3.7a



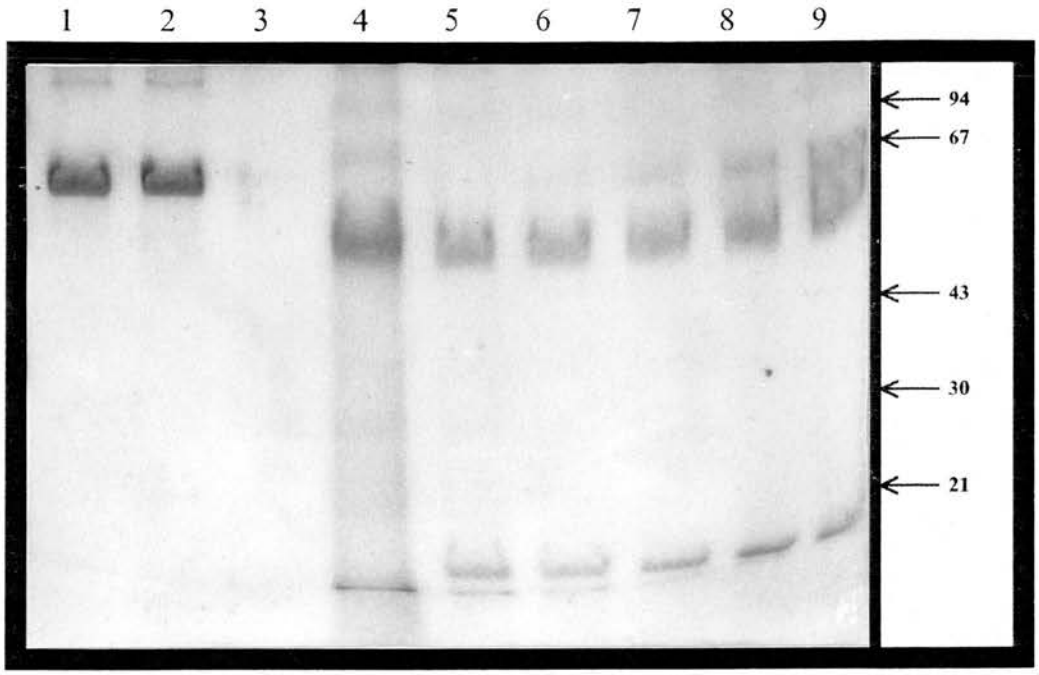
3.7b



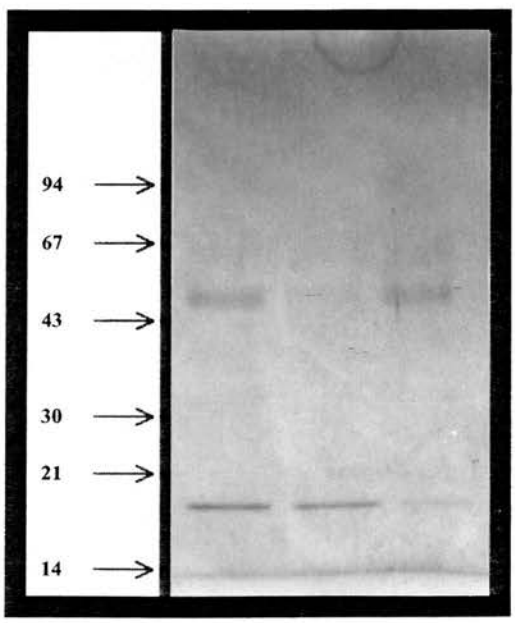
Figures 3.7a & b:- Coomassie blue-stained, SDS-12% polyacrylamide gels run under reducing conditions. Positions of molecular weight markers (in kDa) are indicated for each. Samples analysed comprised:- a P1 preparation (3.7a, lane 1; 3.7b, lane 9); 10µl of P1-TNF preparation with no additions or incubation (3.7b, lane 8) or following incubation for 18 h with 1 part factor Xa to 10 parts P1-TNF, w/w, (3.7a, lanes 2-5; 3.7b, lanes 2-7); & a P1-P25 preparation (Reyburn et al., 1992) following similar incubation to serve as a positive control (3.7b, lane 1). Other additions, prior to incubation,were as follows:- urea to 1M (3.7a, lane 3); CHAPS, to 0.05% (3.7a, lane 4; 3.7b, lanes 1 & 5), 0.5% (3.7b, lane 4) or 0.01% (3.7b, lane 6); DOC, to 0.05% (3.7a, lane 5; 3.7b, lanes 3,5 & 7). 0.5% (3.7b, lane 4) or 0.01% (3.7b, lane 6); & Triton-X-100 to 0.5% (3.7b, lanes 2 & 7) or 0.05% (3.7b, lane 3).

Figure 3.8:-

3.8a



3.8b



Figures 3.8a & b:-Coomassie blue-stained, SDS- 12%- & 5-20% gradient- poly-acrylamide gels, run under reducing- & non-reducing- conditions, respectively. Positions of size markers are shown (in kDa) for each. Samples analysed in 3.8a comprised:- 10µl of P1-TNF preparation, without (lane 1), or following (lanes 2 & 5-9), 18 h incubation & a similarly-incubated P1 preparation (lane 4) or buffer (lane 3). Prior to incubation, all samples had CHAPS & DOC added (each to 0.05%) and factor Xa was added to the samples analysed in lanes 5-9 (to 1 part per 12.5, 25, 50, 100 & 200 parts [w/w] of P1-TNF, respectively) as well as to the samples of lanes 3 & 4 (to the same final concentration [w/v] as the sample in lane 5). Samples analysed in 3.8b comprised:- 10µl of cleaved P1-TNF preparation (lane 1) & the precipitate (lane 3) & dialysed supernatant (lane 2) formed by centrifugation of the same.

3.2.8.A second, large-scale preparation of rovTNFα:-

Because of the necessarily protracted nature of its preparation, this initial batch of rovTNFα was only used in immunisations. In order to study the biological activity of rovTNFα, a second batch was prepared following the same methods, but with no delays in its preparation, whilst fewer aliquots were removed for analysis.

The rates of growth of this second large-scale culture were virtually identical to the first and similar levels of fusion protein were present in the same fractions of sucrose density gradients following centrifugation of the yeast extracts. Once again a 17kDa protein was the only protein clearly visible following separation of the final preparation by SDS PAGE and staining by Coomassie blue (fig.3.16a, lane 1), though faint concentrations of higher molecular weight proteins could also be seen.

The final protein concentration in this second preparation was 125μg/ml in a total of 80mls of preparation. Because of the presence of other faint concentrations of protein, the 17kDa protein was estimated to comprise 80% of the total content, giving a final yield of 2mg rovTNFα/l of yeast culture. All subsequently quoted rovTNFα concentrations are therefore based on this estimate. Multiple aliquots of the second preparation were stored at -70°C, immediately after the final preparative step, either with the addition of 0.1% BSA as a stabilising agent for subsequent use in bioassays, or without for use in analysis and immunisations.

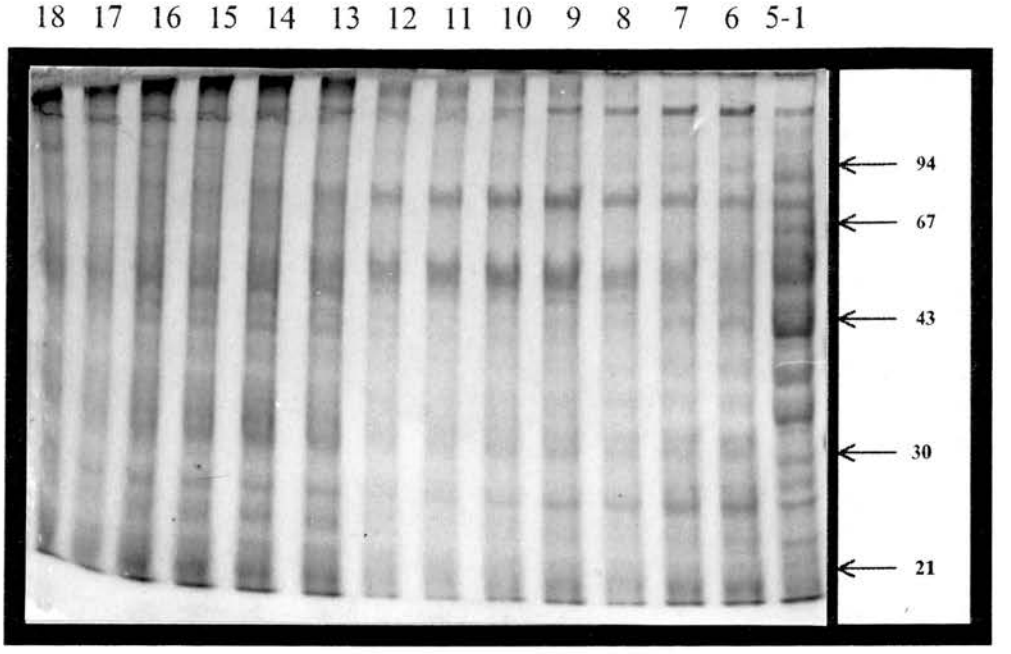
3.2.9.A preparation of yeast extract for use as a negative control:-

Advantage was taken of departmental stocks of yeast transformed with plasmid pMA5620, which constitutively produce P1 (Adams et al., 1987a), to make a negative control preparation of yeast extract with factor Xa. Somewhat fortuitously, these yeast grew at exactly the same rate as ScTNF12 in a parallel incubation with the second large-scale culture.

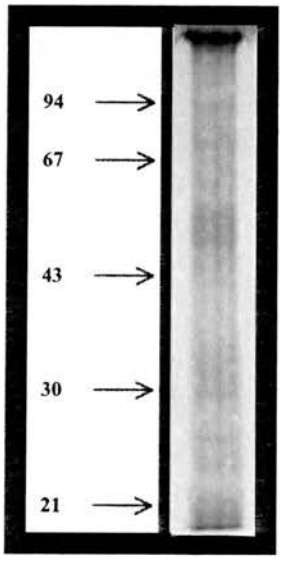
When extracts of these yeast were submitted to identical purification procedures as used in rovTNFα preparation, it was found that most of the P1 VLP's collected in fractions 9-12 of the sucrose density gradients and were therefore included when fractions 9-18 were

Figure 3.9:-

3.9a



3.9b



3.9c

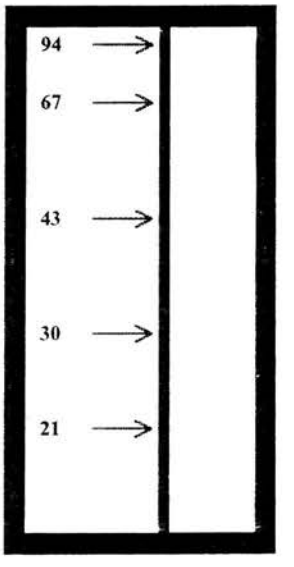


Figure 3.9:- Coomassie blue-stained, SDS-10%- (3.9a,b) & 15%- (3.9c) poly-acrylamide gels, run under reducing- & non-reducing- conditions, respectively, showing stages in the preparation of a yeast extract/factor Xa negative control preparation. The positions of size markers are indicated (in kDa) for each gel. Samples submitted to electrophoresis comprised:- 5µl aliquots of 2ml fractions removed from a sucrose density gradient (3.9a)(fractions [& corresponding lanes] were numbered from top to bottom of the gradient; 5-1 represents a pooling of the top 5 fractions); 10µl of fractions 9-18 after they were pooled, dialysed & centrifuged (3.9b); & 10µl of the final negative control preparation after the addition of factor Xa, mock cleavage, centrifugation & dialysis of the sample shown in 3.9b (3.9c).

pooled (fig.3.9a). Once these pooled fractions had been dialysed and microfuged, however, the final concentration and purity of P1 was poor in comparison to that achieved for P1-TNF (fig.3.9b).

After mock cleavage (having added the same concentration [w/v] of factor Xa as used for P1-TNF cleavage), centrifugation and dialysis of this preparation into PBS, faint concentrations of proteins were visible when 10µl were separated by SDS PAGE and stained by Coomassie blue (fig.3.9c). The total protein content of this preparation, which was immediately stored in identical ways to the rovTNFα preparation, was 25µg/ml, a similar concentration to that estimated for contaminants in the rovTNFα preparation, and it was subsequently used in assorted procedures at equivalent dilutions as the rovTNFα preparation.

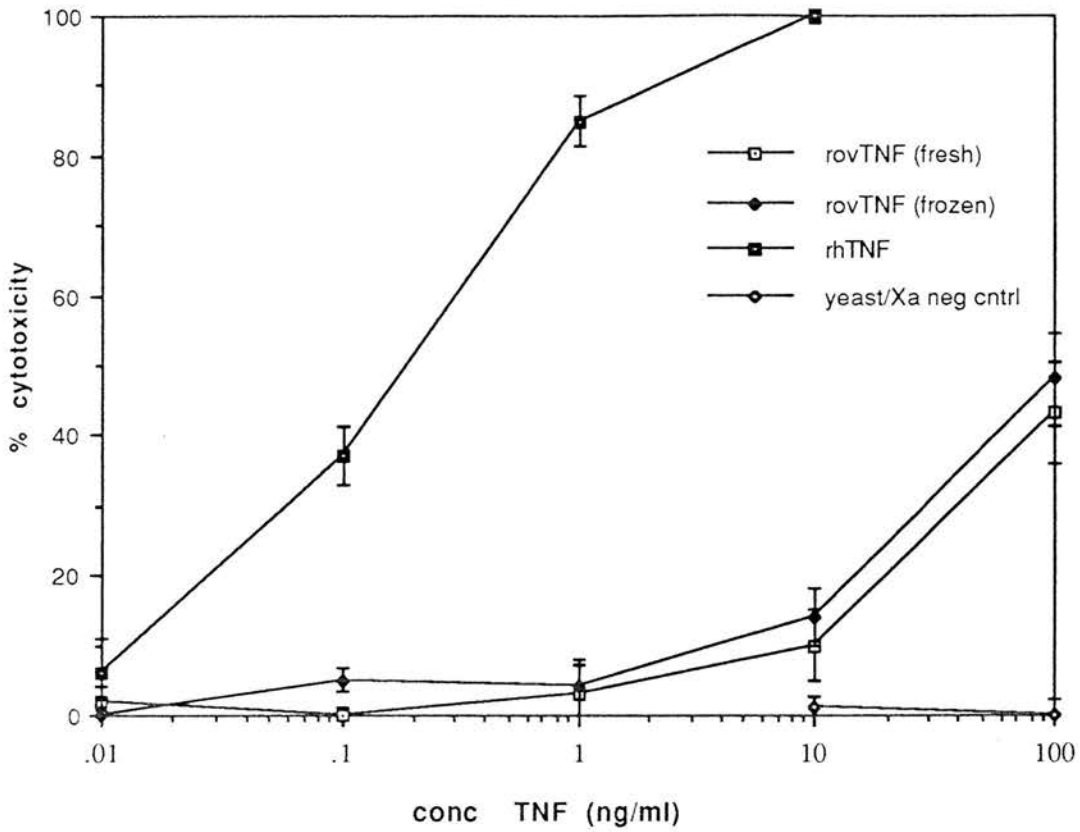
3.2.10.The cytotoxic activity of rovTNFα:-

Initial assessments of the activity of the rovTNFα preparation were made using 'classical' cytotoxicity assays on TNF-sensitive murine cells (Meager et al., 1989). In a preliminary experiment, the activity of one aliquot of rovTNFα, which had not been frozen but simply stored at 4°C (for 3 days after preparation prior to assay), was found to be similar to that of an aliquot which had been stored frozen (fig.3.10a). Since this implied that one cycle of freeze/thawing had not been particularly detrimental to activity, only aliquots which had been stored frozen, and thawed once, were used in all subsequent bioassays involving rovTNFα.

However, whilst it did induce cell death when used at high concentrations, in each of 4 such experiments the rovTNFα preparation consistently showed much less of this activity on a weight-for-weight basis than a preparation of rhTNFα (fig.3.10a-d). Typically, approximately 1000-fold more rovTNFα was required to achieve the same effect as rhTNFα, either in 18 hour incubations with L929 cells in the presence of actinomycin D, or in a 3 day incubation with WEHI 164:clone 13 cells without actinomycin D. For example, in one experiment (see fig.3.10c) the levels of rovTNFα and rhTNFα required to kill 50% of L929 cells were 200 and 0.2 ng/ml respectively.

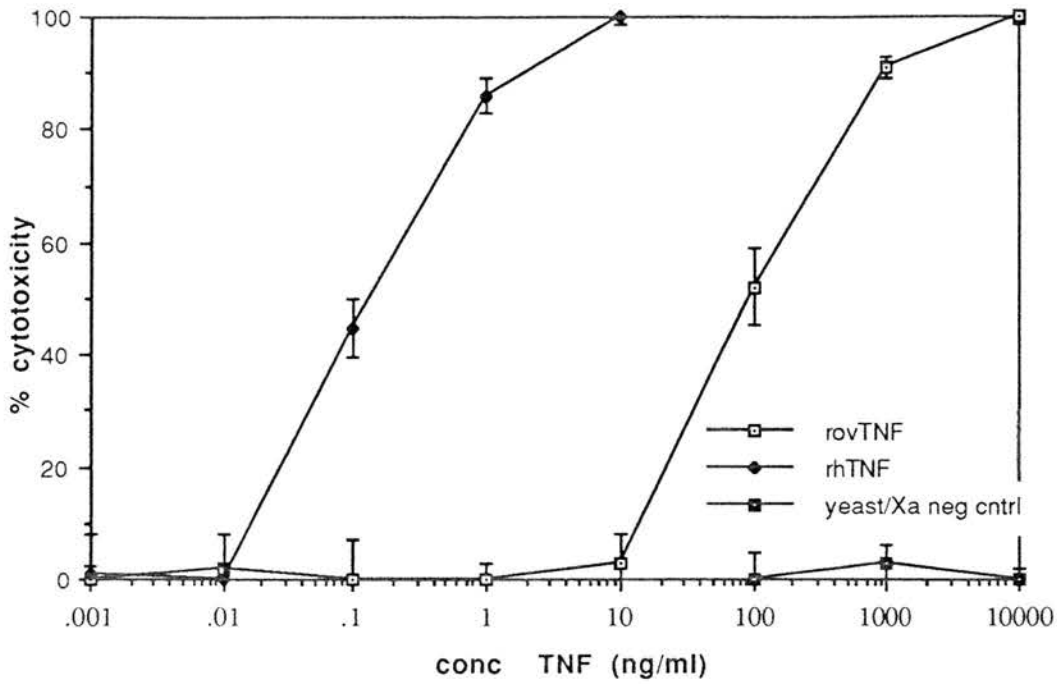
Figure 3.10:-

3.10a

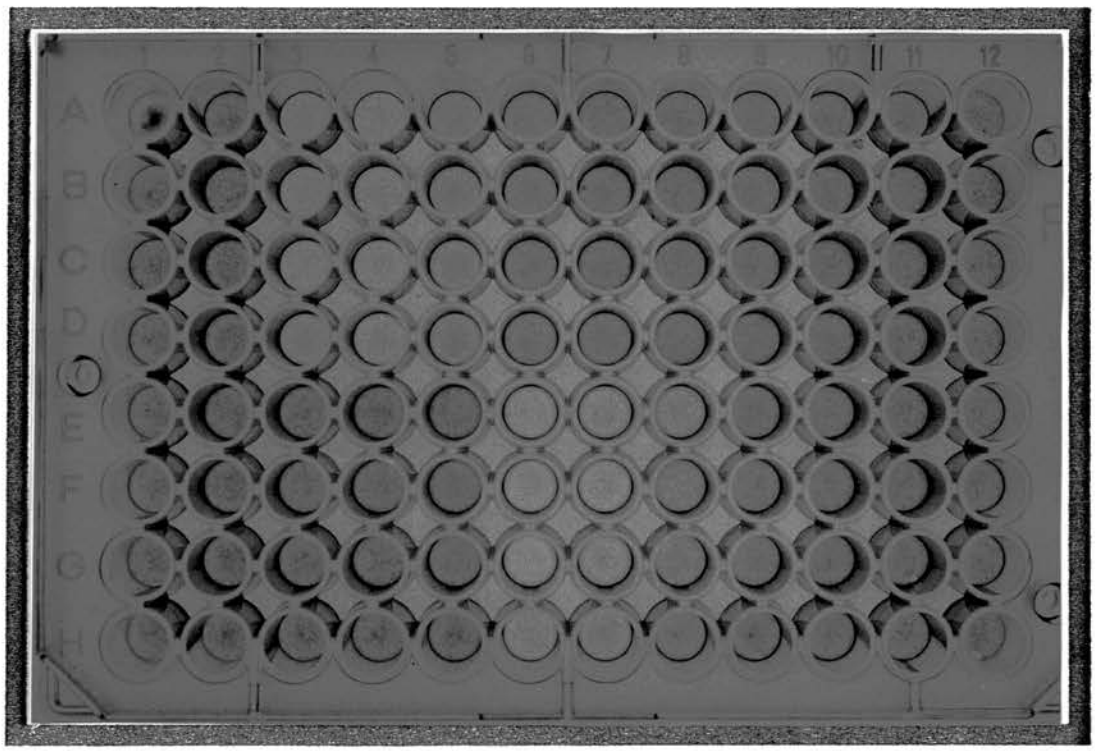


Figures 3.10 a & (see over) b(i), c &d:- the results of cytotoxicity assays (expressed as mean percentage cell death, +/- standard deviations, of quadruplicate wells) performed on L929- (3.10a-c) & WEHI 164:clone 13- (3.10d) cells. Concentrations of TNFα refer to those in added samples (see symbol legends for individual figures), which comprised 25 %(3.10a-c) or 50% (3.10d) of the final volume. Results for a yeast extract/factor Xa negative control preparation are plotted at equivalent dilutions to rovTNFα. Figure 3.10b(ii) (see over):- a photograph of the tissue culture plate from which the results shown in 3.10b(i) were derived. Here, added samples contained PBS + 0.1%BSA only (columns 1, 2, 11 & 12) or rovTNFα (rows A-D in columns 3-10), the negative control preparation (rows E-H in columns 3-5) & rhTNFα (rows E-H in columns 6-10). Recombinant proteins were diluted in PBS + 0.1% BSA to 10^4, 10^3, 10^2, 10, 1, 10^{-1}, 10^{-2} or 10^{-3} ng TNFα/ml (or equivalent dilutions for the negative control) to samples added in columns 3-10 respectively.

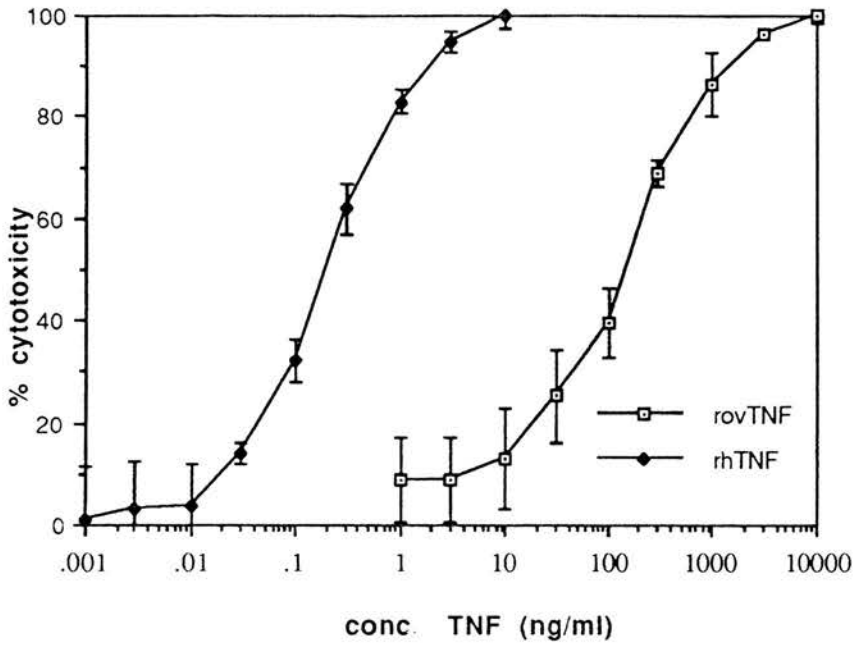
3.10b(i)



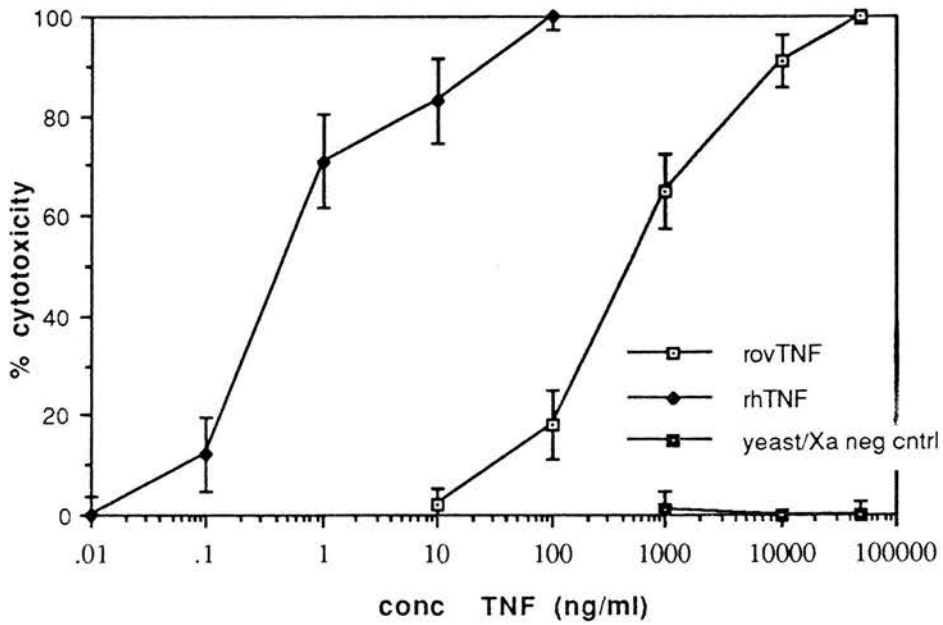
3.10b(ii)



3.10c


100
80
60
40
20
0
% cytotoxicity
.001
.01
.1
1
10
100
1000
10000
conc TNF (ng/ml)
rovTNF
rhTNF

3.10d


100
80
60
40
20
0
% cytotoxicity
.01
.1
1
10
100
1000
10000
100000
conc TNF (ng/ml)
rovTNF
rhTNF
yeast/Xa neg cntrl

No cytotoxicity was seen when either recombinant protein was used on the TNF-resistant cell line L929L/R (Matthews & Watkins, 1978) at concentrations sufficient to induce 100% cytotoxicity on L929 cells (table 3.2), nor was any cytotoxicity seen when the negative control preparation was used on any of the above cell lines at any concentration tested (fig.3.10a,b,d).

In attempts to ascertain whether the preparation might show significantly increased activity, relative to the human preparation, when used on ovine cells, the cytotoxic actions of rovTNFα on transformed ovine cells were also assessed. 2 such cell lines were available for this study.

No detectable cytotoxicity was caused by either recombinant TNFα at any concentration when used on ST6 cells, an adherent cell line derived from a sheep adenocarcinoma, in a 3 day incubation without actinomycin D (table 3.3). (Low concentrations of actinomycin D can kill these cells per se, Dr.G.Entrican, personal communication.)

Since the other cell line tested, line 5, was a non-adherent line of ovine peripheral blood mononuclear cells transformed by sporozoites of Theileria annulata (Entrican et al, 1991), a ^{3}H-thymidine incorporation assay was conducted to determine whether TNF might induce either cytotoxicity/cytostasis or cell proliferation. No significant effect was seen using any of the recombinant proteins at any concentration, under the conditions employed (fig.3.11).

3.2.11.Induction of ovine thymocyte proliferation by rovTNFα acting as a comitogen:-

Since the above results implied that the two ovine cell lines tested were insensitive to the cytotoxic actions of TNFα (see discussion, 3.3) the ability of the rovTNFα preparation to act in other ways on ovine cells was also tested.

TNFα can act as a comitogen for thymocytes in the presence of phytohaemagglutinin (Ranges et al, 1988). When tested in such assays on material derived from 3 different lambs, rovTNFα consistently caused more enhancement of ovine thymocyte proliferation (as assessed by ^{3}H-thymidine uptake during the final 18 hours of a 3 day

Table 3.2:-

Added sample	Mean OD_{540}	Standard deviation
PBS + 0.1% BSA	_0.206	_0.024
rovTNFα, 10μg/ml	_0.201	_0.011
rhTNFα, 10ng/ml	_0.209	_0.014

Table 3.2:- the results of cytotoxicity assays performed on L929L/R cells, expressed as the mean OD's$_{540}$ (& standard deviations of mean OD's) of quadruplicate wells, after incubation with the quoted samples (which comprised 25% of the final volume), washing & staining with crystal violet. Monolayers of intact cells were visible in all wells when examined by light microscopy at the end of the experiment.

Table 3.3:-

Added sample	Mean OD_{540}	Standard deviation
PBS + 0.1% BSA	_0.415	_0.032
rovTNFα, 50μg/ml	_0.393	_0.036
" , 10μg/ml	_0.438	_0.048
" , 1μg/ml	_0.409	_0.027
" , 100ng/ml	_0.362	_0.055
" , 10ng/ml	_0.390	_0.041
rhTNFα, 1μg/ml	_0.417	_0.052
" , 100ng/ml	_0.433	_0.039
" , 10ng/ml	_0.398	_0.026
neg. cntrl, 50μg/ml eq.	_0.381	_0.043
" , 1μg/ml eq.	_0.412	_0.040
" , 10ng/ml eq.	_0.378	_0.049

Table 3.3:- the results of cytotoxicity assays performed on ST6 cells, expressed as the mean OD's$_{540}$ (& standard deviations of mean OD's) of quadruplicate wells, after incubation with the quoted samples (which comprised 50% of the final volume), washing & staining with crystal violet. Results for a yeast extract/factor Xa negative control preparation are quoted at equivalent dilutions to rovTNFα. All wells contained monolayers of intact cells when examined by light microscopy at the end of the experiment.

Figure 3.11:-

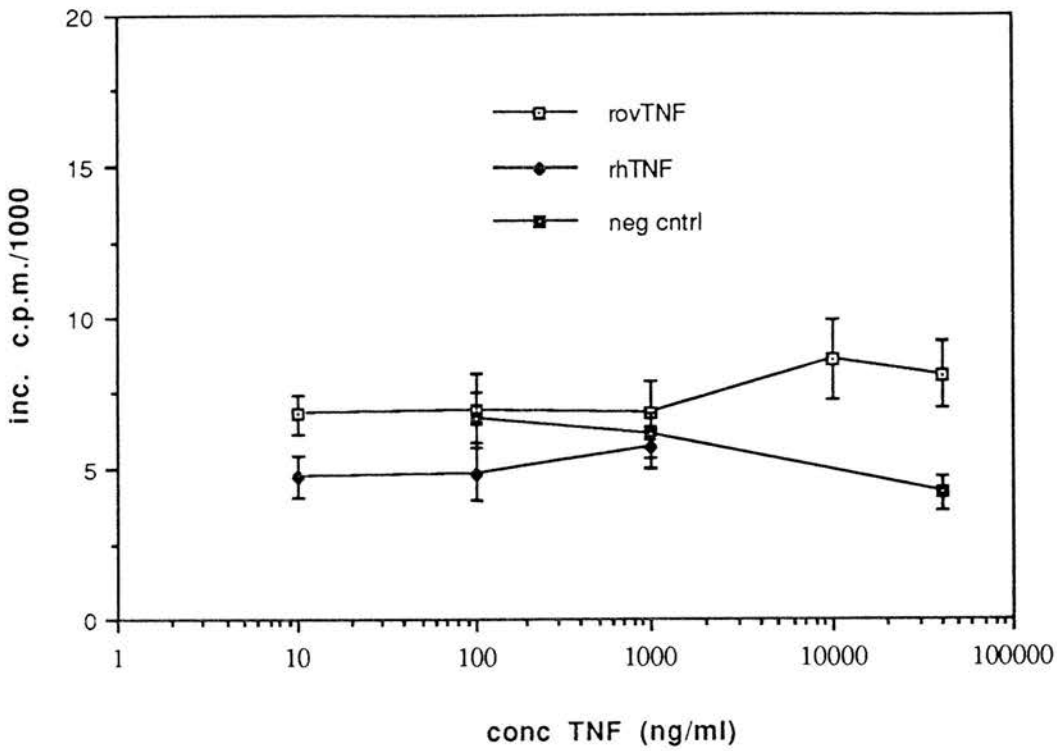


Figure 3.11:- shows the (lack of) activity of recombinant TNF's-α on ovine PBL's, transformed by T.annulata (line 5 cells). Results are expressed as mean incorporated counts per minute per well (+/- standard deviations) of quadruplicate wells, after incubation with samples of recombinant proteins (see symbol legends). The concentrations quoted are those of the added samples, which comprised 50% of the total incubation volume, prior to the addition of ^{3}H-thymidine for the final 18 hours. The results for a yeast extract/factor Xa negative control preparation are plotted at equivalent dilutions of rovTNFα.

incubation), than equivalent concentrations of rhTNFα (fig.3.12). The negative control preparation showed no activity over the use of medium alone at any concentration tested.

3.2.12.Cartilage-degrading activity of rovTNFα:-

To further confirm the activity of the rovTNFα preparation on material of ovine origin, cartilage degradation assays (Saklatvala, 1986) were performed on discs of ovine, xiphoid cartilage. In each of three assays to assess the release of glycosaminoglycans from the cartilage of different individuals the rovTNFα performed very similarly to rhTNFα (fig.3.13). Again the negative control preparation showed no activity at any concentration tested.

3.2.13.Influence of contaminants in the rovTNFα preparation:-

To test the possibility that contaminants likely to be present in the rovTNFα preparation (such as factor Xa) might be affecting its activity, the negative control preparation was mixed with rhTNFα (25µg yeast/Xa proteins / 100µg rhTNFα, a similar level to that estimated to contaminate rovTNFα) and the adulterated rhTNFα preparation was then compared to pure rhTNFα. Contamination of rhTNFα did not significantly affect its activity in any of the above assays (fig.3.14).

3.2.14.Induction of fibroblast proliferation by rovTNFα:-

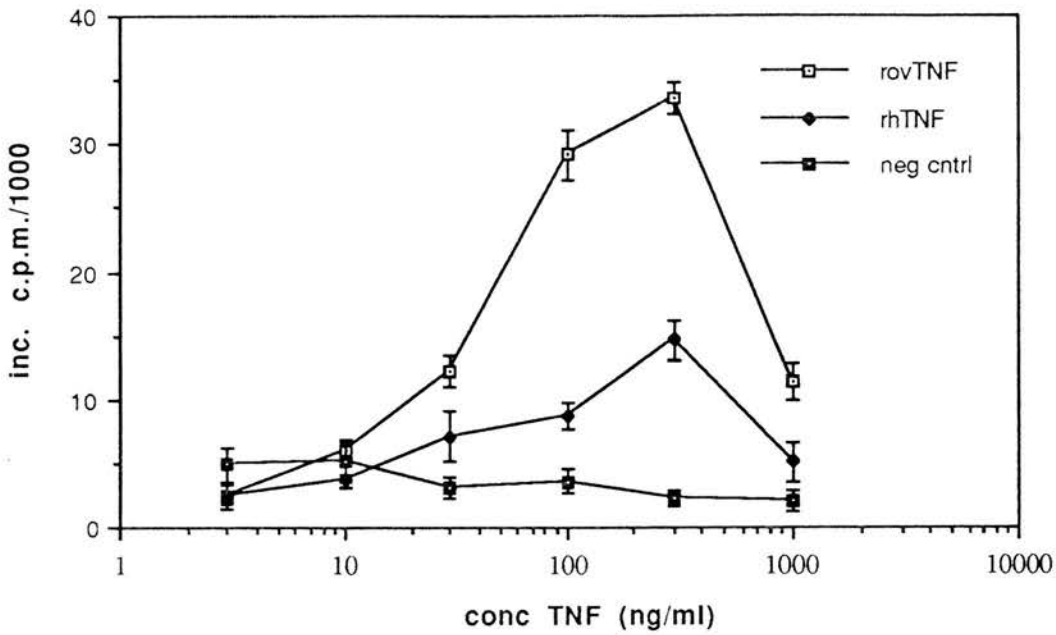
The ability of rovTNFα to enhance the proliferation of fibroblasts (Vilcek et al., 1986) of ovine origin was also assessed. RovTNFα was indeed able to increase the uptake of ^{3}H-thymidine by Weybridge Sheep Choroid Plexus cells (WSCP's) (a fibroblast-like cell type [Harter & Choppin, 1967]) over control levels (fig.3.15). No such activity was seen from the negative control preparation at any concentration tested. (The ability of rovTNFα to enhance WSCP cell density was later confirmed in a subsequent experiment [see chapter 5].) RovTNFα was not compared with rhTNFα in this assay owing to a shortage of the latter.

3.2.15.Is rovTNFα present in multimeric form?:-

Since the active form of TNFα is believed to be a non-covalently bound, self-associating homotrimer (Smith & Baglioni, 1987), it was of interest to see whether the rovTNFα produced here was present as a

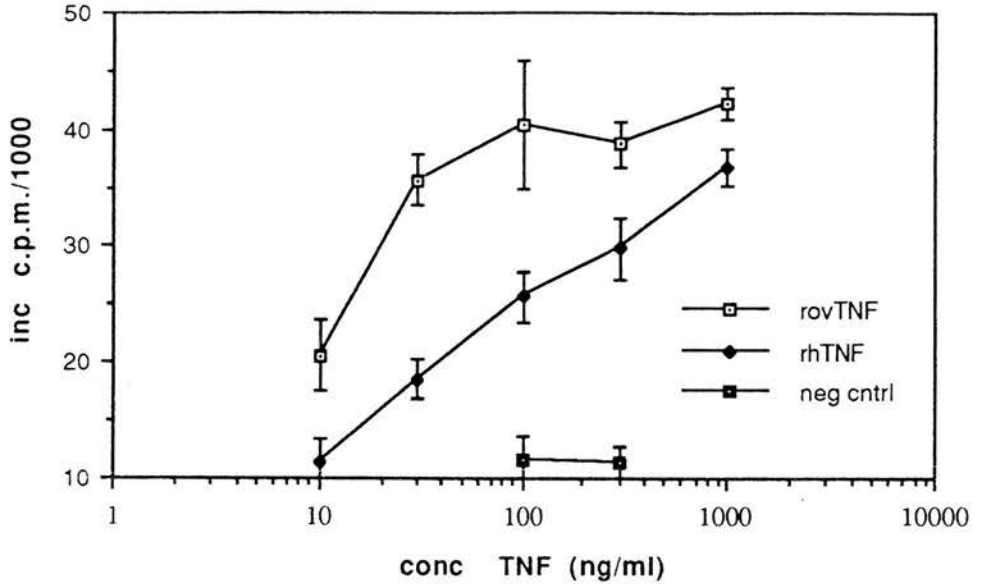
Figure 3.12:-

3.12a



Figures 3.12 a & (see over) b & c:- the results of 3 separate co-mitogen proliferation assays, performed on ovine thymocytes, expressed as the mean incorporated counts per minute per well (+/- standard deviations) of quintuplicate wells, after incubation with samples of recombinant proteins (see symbol legends). The concentrations quoted are those in added samples, which comprised 50% of the incubation volume prior to the inclusion of ^{3}H-thymidine for the final 18 hours of incubation. Results for a yeast extract/factor Xa negative control preparation are plotted at equivalent dilutions to rovTNFα. Mean incorporated c.p.m. x 10^{-3}, associated with the addition of samples comprising PBS + 0.1% BSA only, were:- 2.5 (SD 0.5), 11.0 (SD 1.8) & 25.6 (SD2.7) in the experiments shown in fig.s 3.12 a,b & c, respectively.

3.12b


50
40
30
20
10
inc c.p.m./1000
1
10
100
1000
10000
conc TNF (ng/ml)
rovTNF
rhTNF
neg cntrl

3.12c

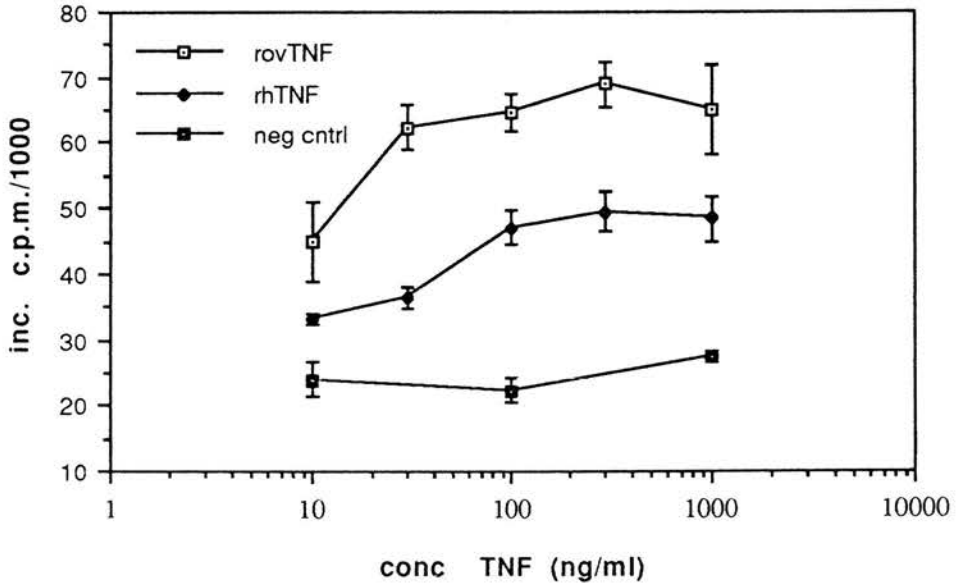
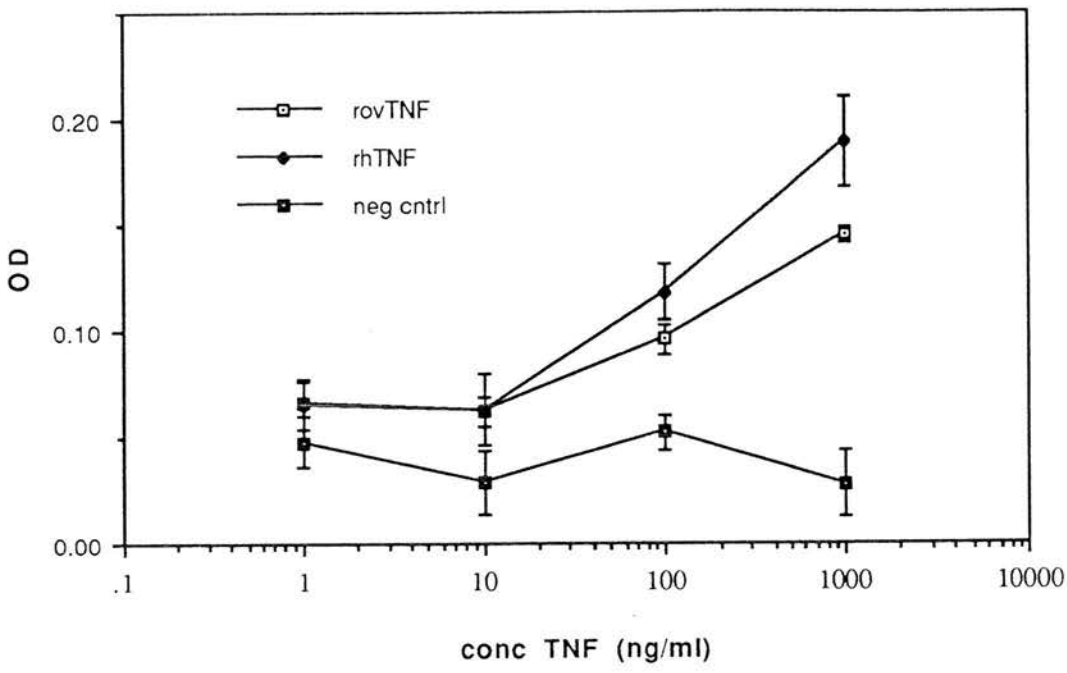

80
70
60
50
40
30
20
10
inc. c.p.m./1000
1
10
100
1000
10000
conc TNF (ng/ml)
rovTNF
rhTNF
neg cntrl

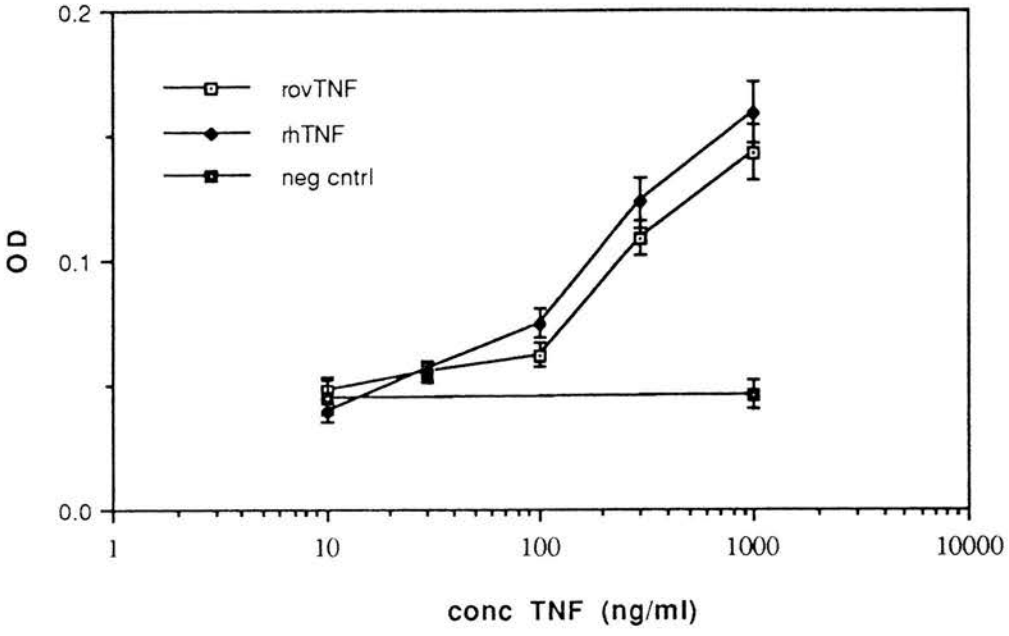
Figure 3.13:-

3.13a



Figures 3.13 a & (see over) b & c:- the results of 3 separate degradation assays, performed on discs of ovine xiphoid cartilage. Results are expressed as the mean OD's$_{540}$ (+/- SD's) when the supernatants of discs which had been incubated, in quintuplicate, with samples of recombinant proteins (see symbol legends) were analysed following the addition of dimethyl-methylene blue. Results for a yeast extract/factor Xa negative control preparation are plotted at equivalent dilutions to rovTNFα. The concentrations quoted are those in the added samples which comprised 25% of the incubation volume. The OD$_{540}$ associated with similar analysis of a mix of 75% medium and 25% PBS/0.1%BSA has been subtracted from each mean figure. No difference in this `baseline' OD was caused by the addition of 1μg/ml of rovTNFα, or rhTNFα, to the PBS/BSA, nor was any difference noted in the final OD$_{540}$ of a supernatant from a disc incubated with medium alone, whether a sample added to it prior to similar analysis (to 25% of final volume) contained 0 or 1μg/ml of these proteins. The increases in OD$_{540}$ over baseline associated with the incubation of discs with samples containing PBS/BSA alone were 0.057 (SD 0.007), 0.035 (SD 0.007) & 0.017 (SD 0.003) in the experiments shown in figures 3.13 a, b & c, respectively.

3.13b


0.2
0.1
0.0
OD
rovTNF
rhTNF
neg cntrl
1
10
100
1000
10000
conc TNF (ng/ml)

3.13c

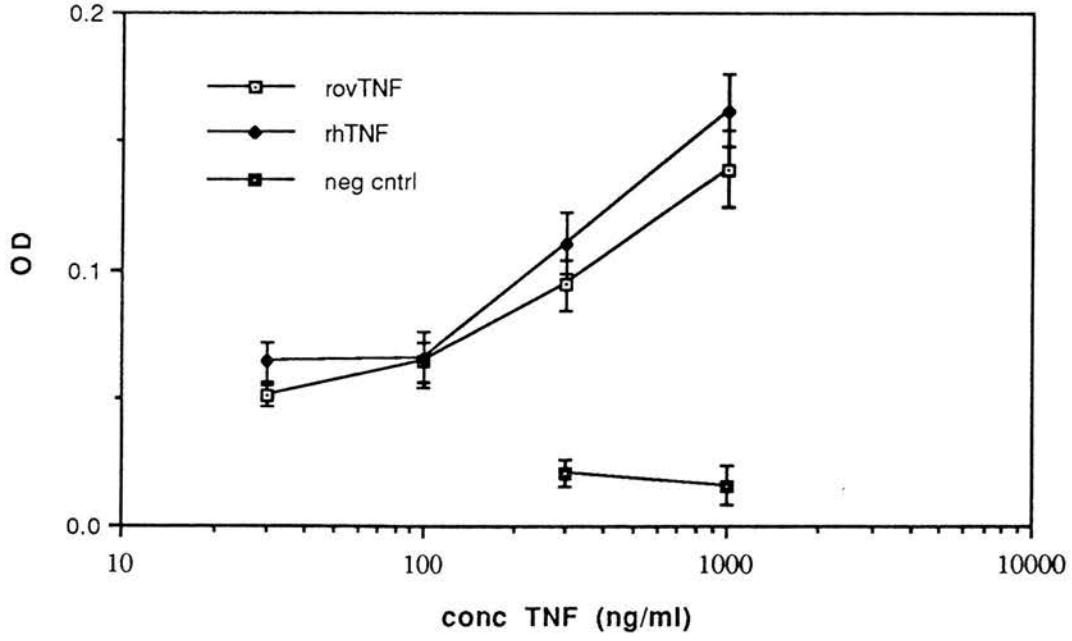
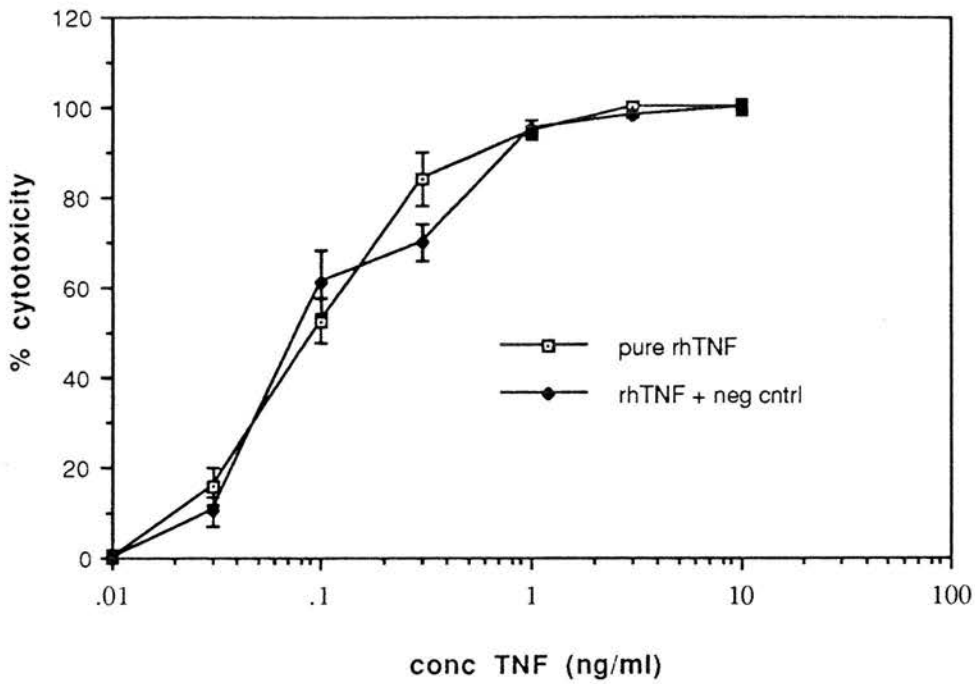

0.2
0.1
0.0
OD
rovTNF
rhTNF
neg cntrl
10
100
1000
10000
conc TNF (ng/ml)

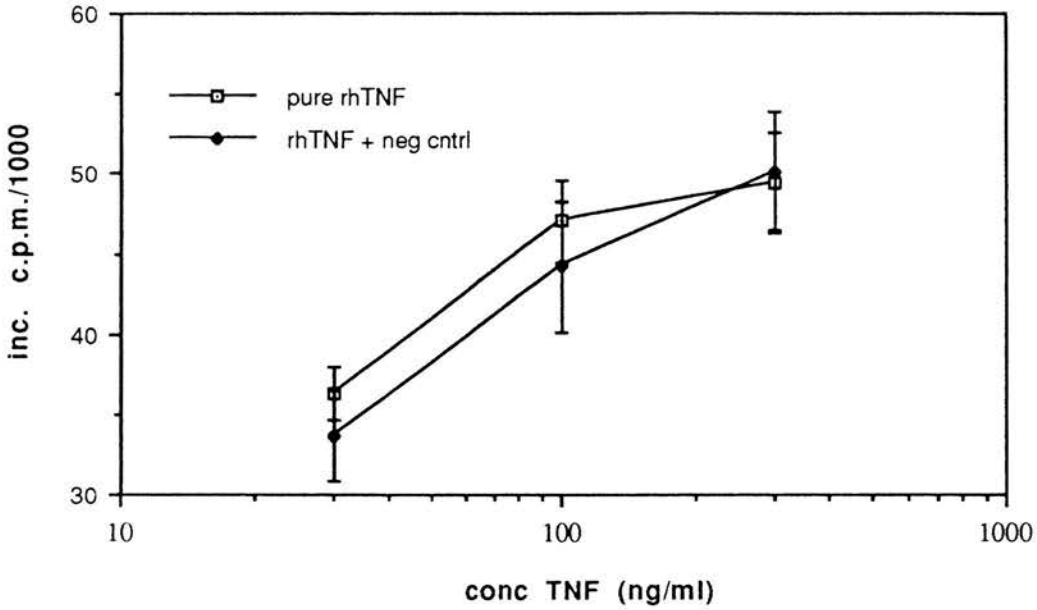
Figure 3.14:-

3.14a



Figures 3.14 a & (see over) b & c:- the results of a cytotoxicity assay (on L929 cells), a co-mitogen proliferation assay (on ovine thymocytes) & a cartilage degradation assay (on ovine cartilage), respectively, demonstrating the failure of adulteration of rhTNFα with the yeast extract/factor Xa negative control preparation to influence the activity of the former (see symbol legends). Assays were conducted, & results are expressed, as in previous assays (see figures 3.10, 12 & 13).

3.14b


60
50
40
30
inc. c.p.m./1000
pure rhTNF
rhTNF + neg cntrl
10
100
1000
conc TNF (ng/ml)

3.14c

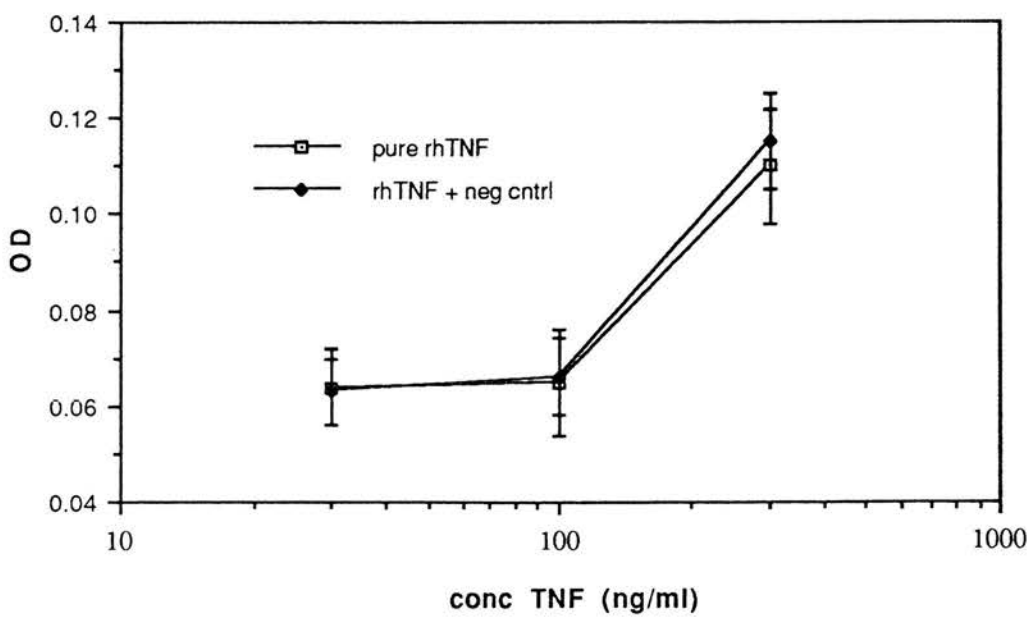

0.14
0.12
0.10
0.08
0.06
0.04
OD
pure rhTNF
rhTNF + neg cntrl
10
100
1000
conc TNF (ng/ml)

Figure 3.15:-

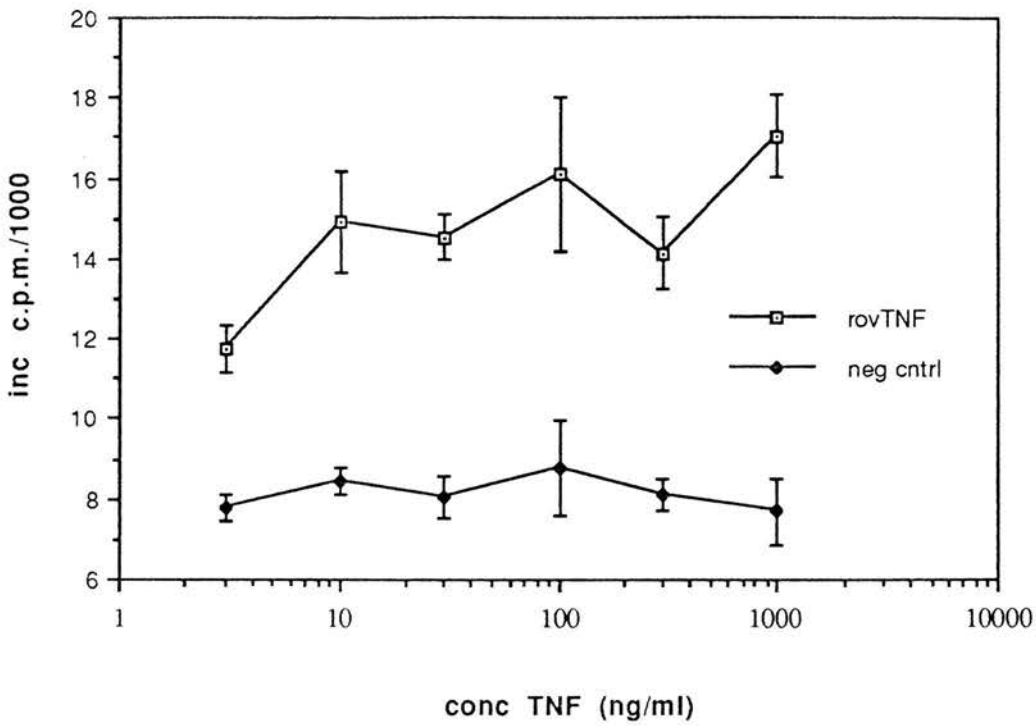


Figure 3.15:- the results of a fibroblast proliferation assay, performed on WSCP cells, expressed as the mean incorporated counts per minute (+/- standard deviations) of quintuplicate wells. The concentrations of rovTNFα quoted are those in the added samples, which comprised 33.3% of the incubation volume, prior to the inclusion of ^{3}H-thymidine for the final 18 hours of incubation. Results for a yeast extract/factor Xa negative control preparation are plotted at equivalent dilutions of rovTNFα. The mean incorporated c.p.m. x 10^{-3} associated with a sample containing PBS + 0.1% BSA only was 7.79 (SD 0.32).

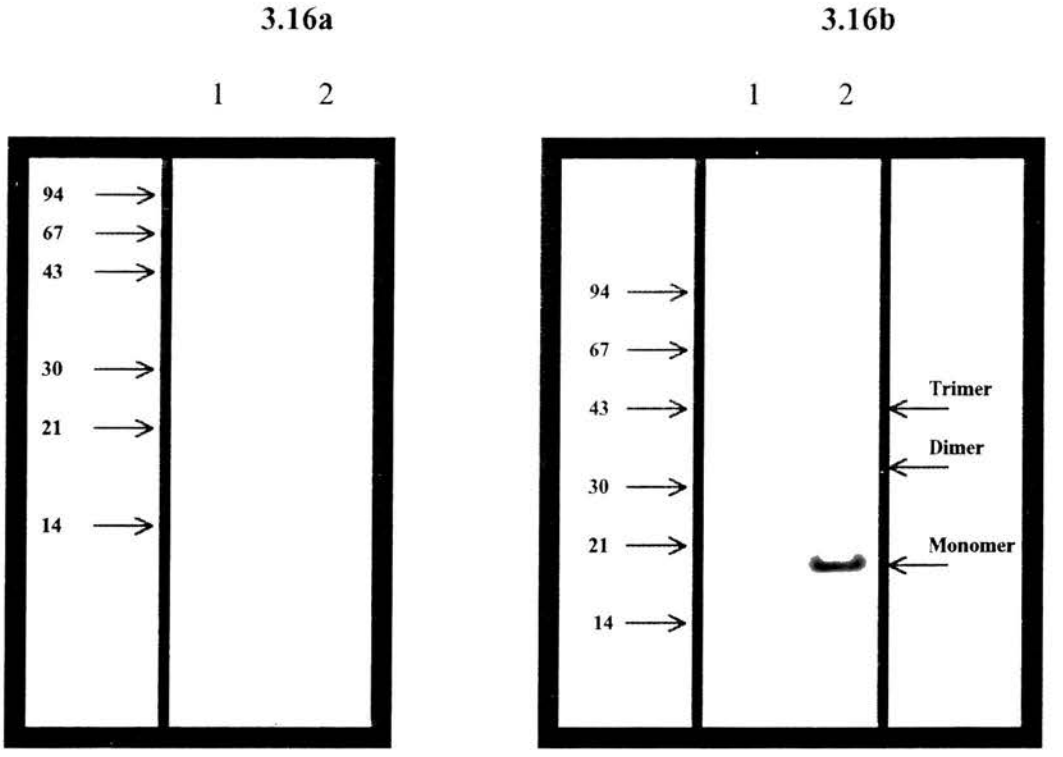
multimer. The homobifunctional cross-linking agent BSOCOES has previously been used to cross-link rhTNFα in order to identify trimers, which then typically show eqivalent mobility to a 42kDa protein during SDS PAGE (Van Ostade et al., 1991). When rovTNFα was cross-linked by this reagent and similarly analysed, the 17kDa protein virtually disappeared and an increased density of 30-50kDa proteins was observed, although distinct bands could not be clearly seen (fig.3.16a). (No such increased density was noted when the negative control sample was submitted to cross-linking.) However, when a similar gel was analysed by Western blotting, using a rovTNFα-specific monoclonal antibody, **cyt1** (see chapter 4), as the primary detection agent, distinct bands with the anticipated mobilities of dimers and trimers could be observed in cross-linked samples (fig.3.16b). As well as the distinct monomeric band, faint bands with the anticipated mobilities of dimers and residual, uncleaved, P1-TNF fusion protein were also present in non cross-linked samples. A small amount of very high molecular weight material, which may correspond to cross-linked residual fusion protein, or aggregates of rovTNFα (see discussion, 3.3), was also detected in the cross-linked sample. No bands were seen either when negative control samples (whether cross-linked or not) were submitted to the same procedure or when a duplicate blot was incubated with an irrelevant monoclonal antibody preparation (**VPM53**, see chapter 4) as the primary antibody, when used at the same dilution as the **cyt1** preparation.

3.3.Discussion:-

Having obtained a final preparation with appropriate activity (cytotoxicity to TNF-sensitive but not TNF-resistant cells) and a major component of appropriate size (17kDa - suggesting that no glycosylation had occurred, see chapter 4), one can conclude that a recombinant TNF with some biological activity was successfully produced. Knowledge of the sequence of cDNA employed defines this rTNF as rovTNFα. However, some observations made during its production are worthy of discussion.

The sequencing of several clones of the initial PCR product confirmed its slightly heterogeneous nature and underlined the necessity of careful selection from such a product in order to express

Figure 3.16:-



Figures 3.16 a & b:- a Coomassie blue-stained, SDS-15% polyacrylamide gel (3.16a) & a Western blot (using a 1:30 dilution of ***cyt1*** *saturated supernatant [see chapter 4] as the primary, ovTNFα-detecting- antibody) of an SDS- 5-20% gradient polyacrylamide gel (3.16b) showing the effect of cross-linking rovTNFα. Both gels were run under non-reducing conditions & the positions of molecular size markers are indicated (in kDa) for each, as are the positions of putative mono-, di- and tri-mers in 3.16b. Samples submitted to electrophoresis comprised 10μl aliquots of rovTNFα, incubated without (3.16a, lane 1; 3.16b, lane 2), or with (3.16a, lane 2; 3.16b, lane 1) the cross-linking reagent BSOCOES.*

a protein with 100% authentic primary structure. Whilst an error rate of 2 residues in some 2,130 bases of cloned material, following 35 cycles of amplification, compares favourably with the rate of 0.25% quoted by Saiki et al. (1988) following 30 cycles, inadvertant selection of 2 out of 5 clones could have had a significant effect on the sequence of the recombinant protein.

The pale blue appearance of these clones, in the presence of X-Gal and IPTG, also demonstrated that the blue/white colour selection method, which is frequently used to identify recombinant plasmids, may not always be a clear-cut process. If the inserted DNA contains a perfect number of codons, with no termination codons in the lac Z reading frame, then it can still be possible for a partially functional LAC Z protein to result.

In spite of careful preparation of the rovTNFα cDNA expression cassette for ligation into pOGS40, some smaller plasmids (probably povTNF13 'carry-over' from a consideration of their size and positive hybridisation) appeared to contaminate the ligation reactions. Given the likely higher transforming efficiency of these plasmids in comparison to the larger pOGS40, the effects of such contamination can be out of proportion to its degree and could have posed problems in the selection of desired colonies. Others have also encountered similar 'carry-over' problems (Dr.N.Carey, C.Cousens, C.Fiskerstrand & Dr.H.Reyburn, personal communications). Fortunately, their stronger hybridisation signal (possibly a reflection of plasmid copy number) and obviously smaller plasmid size did help to distinguish colonies transformed by these contaminants. The fact that similar numbers of colonies were seen on plates derived from control ligations involving plasmid without added DNA may reflect the presence of other contaminants or simply poor efficiency of CIP treatment.

Although a carrier DNA was employed to help in case of difficulty, yeast transformation was not found to be a problem, with several transformants becoming available for characterisation and control plates giving predicted results. It was surprising, therefore, to find that one of four transformants selected for analysis did not appear to be expressing P1 at all. Whilst this could have been a wild-type contaminant or leu2 revertant, the facts that no colonies were seen on a control plate derived from transformations without pOGSTNF1 and that

BJ2168 contains a double mutation in its leu2 gene (Jones, 1991) reduce the likelihoods of these possibilities. Examination of a crude extract of a culture of this yeast did reveal that it contained a c.28kDa protein, which was neither expressed by the others (fig.3.3a) nor recognised by a polyclonal anti-P1 antiserum (fig.3.3b). A third possibility, therefore, is that this was a transformant containing a frame-shifting mutation early in the TyA gene, which led to the production of a truncated, irrelevant protein.

The prominence of a putative P1-TNF fusion protein in crude extracts of the other three chosen transformants suggested a potentially high yield of recombinant product. Indeed the estimated final yield of rovTNFα (2mg/l) compares favourably with those of other proteins expressed via this system under similar conditions (e.g. 0.1mg/l for rovIL-1ß, <0.05mg/l for Maedi-Visna virus rev and gp41 proteins [C.Fiskerstrand, M.Fotheringham, C.Cousens, personal communications] and 15mg/l for Maedi-Visna virus core protein P25 [Reyburn et al, 1992]). Given that much of the success of this system lies in the ability of a fusion protein to self-assemble into VLP's (usually mediated solely by the P1 component), one can speculate that the known structural homology of TNFα to a viral coat protein (Eck & Sprang, 1989) and its ability, as well as that of Maedi-Visna virus P25, to aggregate into multimers may actually help to stabilise the structure of the VLP and contribute to the relatively high yields of these proteins in this system.

Several possibilities existed for the purification of fusion protein and final product, with the initial choice being a simple method previously employed to produce a satisfactory result (Reyburn et al., 1992). A considerable degree of purification was clearly achieved since only putative P1 and rovTNFα proteins were distinct on analysis of final cleavage suspensions. Although c.27kDa and c.24kDa proteins were visible following a few experimental cleavages, these were only seen when high concentrations of factor Xa were added. Given that factor Xa comprises covalently-bound 27kDa and 17kDa subunits (Fujikawa et al., 1972) it seems likely that the c.27kDa protein seen in these gels was the larger component of Xa, with the c.24kDa protein being either a breakdown product or contaminant of the Xa preparation. Although no putative Xa proteins were seen following the analysis of

other preparations which utilised lesser amounts of this protein, all subsequent gels were run under non-reducing conditions to avoid possible confusion of rovTNFα with the smaller subunit of Xa.

Even though the final preparation contained a 17kDa entity as the only band clearly visible in Coomassie-stained gels, the purification methods used could undoubtedly have been improved upon. The wide spread of fusion protein throughout the sucrose density gradient meant that it became more dilute and less pure than might have been achieved with a tighter banding pattern and, with the further addition of factor Xa (and no specific method for removing it), it is probable that there was still significant contamination of the final rovTNFα preparation (for further consideration of the purity of this preparation see below and chapter 4). Nevertheless, whilst greater purification might have been achieved by adjustments to protocol and/ or additional chromatographic or affinity procedures, the level of purity attained was considered adequate for the initial characterisation of rovTNFα (and proved sufficient for the raising of antibodies [see chapter 4]). Since the activity of TNFα declines with time, particularly when stored at temperatures above freezing (Aderka et al., 1992) the main aim during the production of a second batch of rovTNFα, therefore, was to reach the same level of purity as quickly as possible, in order to test the activity of the preparation.

Several possibilities were considered when initial experiments demonstrated that the rovTNFα preparation showed poor cytotoxic activity, when compared with rhTNFα, towards TNF-sensitive murine cells. These included:- 1) there was a potential contribution from innaccuracy in the estimation of rovTNFα concentration; 2) the choice of starting amino acid for mature rovTNFα might have been inappropriate; 3) inhibitors of ovine, but not human TNFα might be present in the media used; 4) contaminants in the rovTNFα preparation might be conferring a protective effect on the cells; 5) the methods employed had created a preparation with generally poor activity and 6) a species 'barrier' might exist, reducing the activity of ovine TNFα on murine cells.

1) Purity:- Since the estimated purity and hence concentration of rovTNFα was based on the visual assessment of a gel, these could clearly have been subject to errors of perhaps +/- 25%. However, a 25% overestimate of rovTNFα concentration could make only a very limited contribution towards the 1000-fold discrepancy in activity between the two rTNF'sα noted. (Improvement to the estimate of purity by, for example, densitometric scanning, was complicated by the continued presence of dimers as well as monomers on gels [fig.3.16b] a phenomenon seen previously in other TNFα analyses [e.g. Arbustini et al., 1991].)

2) Starting amino acid:- Some thought had been given to the authentic start site of mature TNFα and the amino acid selected was chosen because of its homologous position to the start site of mature human TNFα. Although all factors involved in the cleavage of human TNFα preprotein to release the mature form are not understood, Scuderi (1989) demonstrated that one or more serine proteases, which cleave the second bond prior to an arginine-serine link, are involved. The ovine sequence also contains such a pairing in the same position as the human sequence (see chapter 4, fig.4.11). Furthermore, 3 dimensional analyses of human TNFα have revealed that all but the first 4-5 amino acids of the mature protein are folded and held in a compact structure (Jones et al., 1989, Eck & Sprang, 1989). Given the homology between the ovine and human sequences (again see fig.4.11), and the demonstration by cross-linking analysis that rovTNFα can, like human TNFα, also self-associate into trimers, it seems likely that ovine TNFα has a similar secondary structure. Whilst the presence of 10 extra amino acids at this terminus can significantly reduce the activity of the molecule (Cseh & Beutler, 1989), those present in the small 'loose end' referred to above can be removed without inactivation (Creasey et al., 1987) and there may, therefore, be a little leeway either side of the true starting position for the production of a molecule with genuine activity.

3) Inhibitors:- The third possibility was considered feasible since soluble TNF receptors can bind to TNFα and inhibit its actions (Engelmann et al., 1990b), are present at significant levels in serum

(Aderka et al., 1991) and any present in the bovine serum used in these experiments would presumably have greater affinity for TNF'sα from more closely-related species. However, the fact that dose-response curves, in the presence of a fixed concentration of FCS, were parallel on logarithmic not linear scales (fig.3.10) tended to argue against significant effects caused by such a specific inhibitor.

4) Contaminants:- Although failure to kill TNF-resistant cells confirmed that the rovTNFα preparation's cytotoxic activity was due to a TNF, the possible influence of contaminants could not initially be ruled out. For example, though much of the factor Xa activity was likely to have been exhausted by an overnight incubation, it was possible that any residual proteolytic activity could be damaging TNF-R's, hence conferring a protective effect on the cells. Whilst several different methods of producing a negative control preparation were considered, it is unlikely that any method could produce a perfect combination of the contaminants present. Likely contaminants included factor Xa, residual P1 not removed by centrifugation and other, unknown, yeast constituents. Although use of a pOGS40/pUG41S double-transformant would have been preferred, expediency dictated that the negative control preparation was prepared from a pMA5620-transformed yeast, a constitutive producer of P1 (Adams et al., 1987a). Nevertheless, the final negative control preparation was likely to contain similar levels of Xa, as well as some P1 protein and many other yeast components as found in the rovTNFα preparation. (This was subsequently confirmed, to an extent, by immunoblotting, see chapter 4.) Since this preparation neither showed activity of its own at any concentration in each of the assays described here, nor affected the activity of rhTNFα when mixed with it, it seems unlikely that contaminants could have contributed greatly to the effects described in this chapter.

5) General activity:- Because the activity of TNFα does decline with time, particularly at higher temperatures (Aderka et al., 1992), it is possible that a more active (though more costly and contaminated) preparation could have been produced by using higher concentrations of factor Xa and shorter incubation times for cleavage. However, such

decline in activity is generally associated with the breakdown of trimers into monomers and the subsequent formation of high molecular weight aggregates (Petersen et al., 1989; Aderka et al., 1992). The relative densities of the bands seen in the cross-linking studies described here, and the purity of the rovTNFα preparation, tend to rule out an association of TNF monomers with contaminants and therefore suggest that rovTNFα, in common with TNF'sα from other species, is capable of self-association into multimeric forms. Furthermore, although a proportion of rovTNFα may have been present as monomers and high molecular weight aggregates, more than half was present in trimeric or possibly dimeric forms (assuming an equal affinity of **cyt1** for each). (The extents to which dimeric forms represent incompletely cross-linked trimers, or trimeric forms possibly represent 'over' cross-linked dimers are unclear [Petersen et al, 1989].) Such results, as well as those of experiments performed on ovine cells (see below), tend to rule out suspicions that a highly degraded and inactive form of rTNFα had been produced (see below).

6) Species barrier:- Although murine cells have been used to detect and assay TNF's from a wide range of species (see chapter 4), with only minor degrees of species specificity noted for the induction of cytotoxicity and interaction with the murine TNF-RI (Smith et al., 1986, Lewis et al., 1991), the possible contribution of a species barrier was further examined by looking at the activity of rovTNFα on ovine cells.

Little significance was attached to the failure to detect any cytotoxicity towards the two transformed, ovine cell lines, since, in one survey, Sugarman et al. (1985) found that only 30% of transformed, human cell lines were susceptible to the cytostatic/toxic effect of TNFα. De Martini & Baldwin (1991) have since demonstrated that rhTNFα also shows no toxicity towards Theileria-infected bovine cells.

RovTNFα clearly showed a much increased activity relative to rhTNFα in two other types of assay on ovine tissues, however, including consistently superior activity in thymocyte proliferation assays. Although rhTNFα has previously been shown to have very significant effects on ovine tissue at doses as low as 10μg/kg in vivo (Johnson et al., 1989; Redl et al., 1990) a possibly reduced affinity of ovine

cells for human TNFα could clearly contribute to the improvement in relative activity seen on these cells, particularly in an assay such as the thymocyte proliferation assay, known to involve a high degree of species specificity in murine systems (Ranges et al., 1988; Ehrke et al., 1988). However, the concentrations of rovTNFα displaying activity in these assays, as well as in the fibroblast proliferation assay, are similar to those of other TNF'sα described as displaying activity in similar systems using similar methods. For example, in thymocyte co-mitogen proliferation assays performed on a murine thymocyte cell line, Ranges et al. (1988) and Ehrke et al. (1988) each found that recombinant murine TNFα began to have an effect at approximately 3ng/ml and the latter workers also found that activity peaked at a concentration between 30 and 300 ng/ml: converting the concentrations shown in fig.3.12 to final concentrations reveals that, in these assays, rovTNFα began to have an effect at <5ng/ml and showed peak activity in 2 out of 3 experiments between 50 and 500 ng/ml. Furthermore, Vilcek et al. (1986) found that rhTNFα's proliferating activity towards human fibroblasts reached a plateau between 1 and 10ng/ml, which also compares favourably with the final concentration of c.3.33ng/ml at which the fibroblast proliferating activity of rovTNFα appears to plateau (fig.3.15). These results suggest that an appropriately-active rTNFα preparation had indeed been produced (unless ovine cells are both exquisitely sensitive to rovTNFα in comparison to other syngeneic systems and greatly insensitive to rhTNFα). Consequently, these findings help to strengthen the argument that murine cells may be relatively insensitive to the cytotoxic actions of ovine TNFα.

One other group have expressed rovTNFα, using a mammalian expression system (Nash et al., 1991), and, although they did not examine their preparation for activity on ovine cells, they too found that rovTNFα showed very poor cytotoxic activity on TNF-sensitive, murine cells.

Some other observations were also made from these experiments. Whereas murine thymocyte co-mitogen proliferation assays demonstrate complete insensitivity to human TNFα (Ranges et al., 1988; Ehrke et al., 1988), caused by total failure of human TNFα to interact with the murine TNF-RII which is responsible for mediating this action (Lewis

et al., 1991; Tartaglia et al., 1991), ovine thymocytes clearly show some response to rhTNFα. Furthermore, whilst the dose-response curves and sensitivities of these assays were all slightly different (possibly reflecting individual variation of genetically-unrelated sheep and/or differences in pre-existing in vivo stimulation of these cells), the peak level of enhanced proliferation caused by rhTNFα never reached that caused by rovTNFα and tended to occur at similar concentrations of the two (see fig.s 3.12a,c). Assuming that ovine thymocyte proliferation is also mediated via type II receptors, this would suggest that the species specificity demonstrated here is not simply a reflection of a reduced affinity of ovine type II TNF receptors for rhTNFα, which one might expect to be overcome by increasing the rhTNFα concentration, but may involve a more complex ligand-receptor interaction.

RovTNFα and rhTNFα clearly showed virtually identical activities in degradation assays on ovine cartilage. In the light of the above observation of some ovine TNF-RII species specificity, one would expect that this activity is likely to be predominantly mediated via the generally less species-specific type I receptor (see 1.6). This fits with the demonstration by Keffer et al. (1991) that in transgenic mice, uncontrolled in vivo production of human TNFα (able to act only via type I receptors) leads to multifocal cartilage destruction and polyarthritis (though here possible effects of induced murine TNFα, contributing via type II receptors, cannot be ruled out). Such activity, as well as activity in the induction of fibroblast proliferation, which is known to be mediated via type I receptors (Engelmann et al., 1990a), also argue against another considered possibility:- that the yeast system had folded rovTNFα in such a way as to produce a protein able to interact normally with type II receptors but only poorly with type I receptors.

One last possibility considered was that the emphasis of TNFα function in the sheep might be different, so that it is generally less cytotoxic. Slightly different activities of a cytokine in different species would not be without precedent. For example, murine, but not human, IL-3 has the ability to stimulate mast cells (Arai et al., 1990). However, when assayed for cytotoxic effect on PK15-1512 cells

(cloned from a TNF-sensitive, porcine cell line) rovTNFα showed cytotoxic activity which was more comparable to that of rhTNFα (Dr.E. Peterhans, personal communication).

Since, in sensitive murine cells at least, TNF-R'sI only need to be cross-linked for the induction of cell-death (Engelmann et al., 1990a, see 1.6) and rovTNFα, with its multimeric make-up, can apparently react 'normally' with ovine type 1 receptors, the conclusion drawn from these results is that the murine TNF-RI seems likely to have a generally poor affinity for ovine TNFα. Although binding studies were not performed to support this conclusion, further evidence of the poor cytotoxic activity of ovine TNFα on TNF-sensitive murine cells, and the possible reasons for this, will be presented and discussed in the following chapter.

CHAPTER 4:- THE DETECTION AND PRELIMINARY CHARACTERISATION OF OVINE TNFα.

4.1.Introduction:-

Whilst useful information can be gleaned by the use of a probe to detect TNFα transcripts, their presence does not necessarily imply their translation (Beutler et al., 1986a), hence an ability to also detect ovine TNFα protein was clearly essential to studies of its induction. TNF'sα from other species have previously been detected by both biological and immunological assays (Meager et al., 1989). However, the large number of different, currently-employed assays in itself suggests that no method is likely to be ideal (see below).

Two of the most commonly-used forms of biological assay for TNFα derive from early in vitro studies, which demonstrated its ability to kill susceptible transformed cells (Carswell et al., 1975) or to suppress LPL activity in adipocytes (Kawakami et al., 1982). Significant improvements in the sensitivity and duration of cytotoxicity assays were soon made by including metabolic inhibitors such as actinomycin D (Ostrove & Gifford, 1979) and protocols have since been improved in order to provide optimum assay conditions (e.g. Flick & Gifford, 1984). These highly-sensitive assays, employing widely-available cells, such as murine L929 cells, have thus become the most popular form of bio-assay (Meager et al., 1989).

An additional feature which has contributed to their widespread use is that such assays have displayed only a limited degree of species specificity (Smith et al., 1986). Thus murine cells have proven capable of detecting TNF-like activity not just from the mouse itself (Carswell et al., 1975) and other rodents or lagomorphs such as the guinea pig (Zuckerman & Bendele, 1989) and rabbit (Matthews & Watkins, 1978), but also from primates, such as the human (Matthews, 1981a) and, more recently, from ungulates such as the ox (Adams & Czuprynski, 1990), horse (MacKay et al., 1991a) and pig (Baarsch et al., 1991). Other murine transformed cell lines, such as WEHI 164: clone 13 have also been selected for use in cytotoxicity assays with the aim of further increases in sensitivity, reportedly achieved even in the absence of metabolic inhibitors (Espevik & Nissen-Meyer, 1986).

There are problems associated with such assays, however. These include concerns about the specificity and relevance of results. Apart from TNF's, other factors, such as high concentrations of IL-1 (Onozaki et al., 1985) and ligand(s) for Fas antigen (Yonehara et al., 1989), are also known to kill several types of transformed cell, as may some yet-to-be-discovered factors. An assessment of the specificity of action can be made using TNF-resistant sub-clones of these lines, such as L929L/R cells (Matthews & Watkins, 1978), though failure to kill these cells cannot establish whether cytotoxicity to the parent cell line is due to TNF-α or β. Blocking studies, using specific antibodies, have been used to attribute such cytotoxicity to either TNF (e.g. Nedwin et al., 1985b). However, quantitatively this method too is not without problems. Synergy with other cytokines is a common feature of TNF activity (see 1.7) and has been demonstrated with IFN gamma in cytotoxicity assays without actinomycin D (Sugarman et al., 1985), possibly working via an induced upregulation of TNF-Rs (Aggarwal et al., 1985a). Clearly, any such synergistic action could mean that the concentration of TNFα in a given sample is significantly less than comparisons with TNFα standards and the degree of protection afforded by anti-TNFα antibodies in blocking studies might initially suggest.

Furthermore, given that soluble TNF-R's may be present in variable concentrations in body fluids (Aderka et al., 1991), can be released from certain cells by some pathophysiological stimuli (Porteu & Nathan, 1990) and can inhibit TNF activity (Engelmann et al., 1990b) or in some prolonged assays actually stabilise it (Aderka et al., 1992), it is also apparent that these assays provide only a crude assessment of the overall, accumulated, TNF-like activity of a biological fluid, which depends on the net balance of several factors. Thus, the results of such assays may bear little relevance to the potential activity of TNFα at particular times during its production and in certain micro-environments, especially when one considers the added complication that biologically-active TNFα may be present in membrane-bound (Kriegler et al., 1988) as well as soluble forms.

A variety of immunologically-based methods have also been applied to the detection of TNFα, including RIA, sandwich or other forms of ELISA (Meager et al., 1989) and Western blotting (e.g. Beutler et al.,

1986a). Fortunately, in spite of the fact that TNF's-α and ß share approximately 30% amino acid homology (Pennica et al., 1984), cross reactivity with the latter rarely seems to have been a problem. However, these assays too are not without their drawbacks, since they may detect biologically-inactive and therefore, possibly 'irrelevant' forms of TNF. Furthermore, some of these assays, such as RIA or ELISA, which detect TNFα in its native form may also be influenced by the presence of other proteins. Several TNF-binding proteins have now been described, including the soluble forms of the two receptors (Engelmann et al., 1990b), α2-macroglobulin (Wollenberg et al., 1991), heparin (Lantz et al., 1991), uromodulin (Hession et al., 1987) and auto-antibodies to TNFα (Fomsgaard et al., 1989). Clearly, unless the anti-TNFα antibodies employed only recognise epitopes which are not potentially masked by these proteins, such assays could seriously, and variably, underestimate sample concentrations of TNFα. By comparison, the use of a technique requiring denaturation of a protein prior to its detection, Western blotting, for example, though less sensitive and more difficult to quantify, circumvents the problem of interference by other proteins and can also define the molecular size(s) of TNFα detected.

In spite of their associated problems, however, bio- and immuno-assays for TNFα have undoubtedly yielded a great deal of invaluable information about its production and, in the absence of superior alternatives, can still provide meaningful results, provided they are interpreted carefully and preferably in conjunction with one another.

Although the induction of TNFα has now been documented in many cell types using a variety of stimuli (see 1.2), it is generally agreed that the LPS-stimulated macrophage remains one of the most potent known producers of TNFα (Jäättelä, 1991, Vassalli, 1992). Therefore, early attempts to detect ovine TNF-like activity concentrated on examining supernatants (SN's), collected after adding LPS to cultures rich in ovine alveolar macrophages, for cytotoxic activity towards L929 or WEHI 164: clone 13 cells. Later, advantage would be taken of the availability of rovTNFα (see chapter 3) to raise both poly- and mono- clonal antibodies for use in the immunological detection of ovine TNFα, primarily by Western blotting.

4.2.Results:-

4.2.1.Preliminary cytotoxicity assays:-

An estimated 3 x 10^8 cells were obtained by lavage from the lungs of a freshly-slaughtered Scottish Blackface ewe. Approximately 70% of cells used remained adherent to the bottom of 24-well, tissue culture plates 4 days after plating out at 3 x 10^5 cells/well, when the medium in each well was completely replaced with 1ml of fresh medium containing 0, 0.1 or 1µg LPS/ml. Separate SN's were collected from three wells of each LPS concentration 0,2,4,8,16 and 24 hours later and analysed for their cytotoxic activity on L929 cells. No detectable cytotoxicity was induced by any of these SN's, whatever the time point or concentration of LPS added (table 4.1a). A positive control for the assay comprised rhTNFα at 10ng/ml in the same medium. In contrast to test sample wells, no intact cells were visible by light microscopy in positive control wells at the end of the assay.

This experiment was repeated, using cells from a different sheep, although this time they were stimulated 3 days after plating out. Once again no detectable cytotoxicity was induced by any SN and 100% cell death/detachment was induced by the positive control (table 4.1b).

Since WEHI 164: clone 13 cells are reported to be more sensitive to TNFα than L929 cells (Espevik & Nissen-Meyer, 1986) both assays were repeated using the former cell line. Again no cytotoxicity was noted in any wells where SN's had been added (table 4.1c,d). A few intact cells were visible in some of the positive control wells, suggesting that these cells might in my hands be less TNF-sensitive than the L929 cells. The results of a separate experiment (fig.4.1) using a range of rhTNFα concentrations confirmed that this was the case and, since they also proved to grow faster and were more strongly adherent, only L929 cells were used in subsequent cytotoxicity assays.

4.2.2.Further cytotoxicity assays, in cases of proven TNFα transcript induction:-

Whilst several different factors could have contributed to the failures to detect cytotoxic activity in the above SN's, it was unclear whether any ovine TNFα had even been induced. Adams and Czuprynski (1990) suggested that the ability of bovine monocytes to

Table 4.1:-

Sample	4.1a	4.1b	4.1c	4.1d
UM	_0.247(0.023)	_0.213(0.011)	_0.176(0.021)	_0.184(0.013)
UM+1μg LPS/ml	_0.236(0.029)	_0.221(0.020)	_0.162(0.013)	_0.182(0.019)
10ng rhTNFα/ml	_0.019(0.004)	_0.015(0.003)	_0.032(0.004)	_0.034(0.006)
" + 1μgLPS/ml	_0.023(0.007)	_0.015(0.002)	_0.037(0.006)	_0.033(0.005)
SN, no LPS, 0h	_0.243(0.010)	_0.231(0.014)	_0.177(0.011)	_0.178(0.014)
" " , 2h	_0.239(0.021)	_0.219(0.011)	_0.169(0.015)	_0.180(0.017)
" " , 4h	_0.252(0.024)	_0.226(0.017)	_0.181(0.013)	_0.169(0.010)
" " , 8h	_0.221(0.017)	_0.218(0.012)	_0.179(0.019)	_0.184(0.011)
" " , 16h	_0.246(0.015)	_0.220(0.010)	_0.171(0.010)	_0.173(0.022)
" " , 24h	_0.246(0.020)	_0.215(0.009)	_0.180(0.009)	_0.179(0.013)
", 100ngLPS/ml, 0h	_0.254(0.018)	_0.233(0.016)	_0.176(0.014)	_0.166(0.015)
" " , 2h	_0.263(0.023)	_0.226(0.015)	_0.165(0.012)	_0.168(0.009)
" " , 4h	_0.239(0.011)	_0.229(0.015)	_0.168(0.011)	_0.173(0.016)
" " , 8h	_0.243(0.015)	_0.217(0.021)	_0.170(0.013)	_0.177(0.013)
" " , 16h	_0.254(0.019)	_0.222(0.011)	_0.168(0.013)	_0.184(0.017)
" " ,24h	_0.247(0.012)	_0.232(0.012)	_0.164(0.012)	_0.185(0.016)
", 1μgLPS/ml, 0h	_0.238(0.023)	_0.216(0.018)	_0.173(0.018)	_0.191(0.018)
" " , 2h	_0.241(0.016)	_0.227(0.007)	_0.168(0.011)	_0.190(0.014)
" " , 4h	_0.246(0.014)	_0.225(0.015)	_0.179(0.017)	_0.181(0.012)
" " , 8h	_0.235(0.019)	_0.214(0.022)	_0.185(0.014)	_0.176(0.010)
" " , 16h	_0.252(0.012)	_0.223(0.013)	_0.178(0.010)	_0.174(0.011)
" " , 24h	_0.240(0.017)	_0.217(0.010)	_0.182(0.011)	_0.174(0.008)

Table 4.1a-d:- results of 4 separate assays to determine the cytotoxic potential of ovine lung-cell supernatants (SN's), collected at indicated times after the addition of stated concentrations of LPS to 2 different cell preparations. Samples used in 4.1a & b correspond to those of 4.1c & d respectively. Results are expressed as the mean OD's$_{540}$ (with standard deviations in brackets) of quadruplicate wells, after L929 cells (4.1a,b) or WEHI 164 clone 13 cells (4.1c,d) were incubated with samples, & residual cells then stained with crystal violet. RhTNFα was diluted in unconditioned medium (UM).

Figure 4.1:-

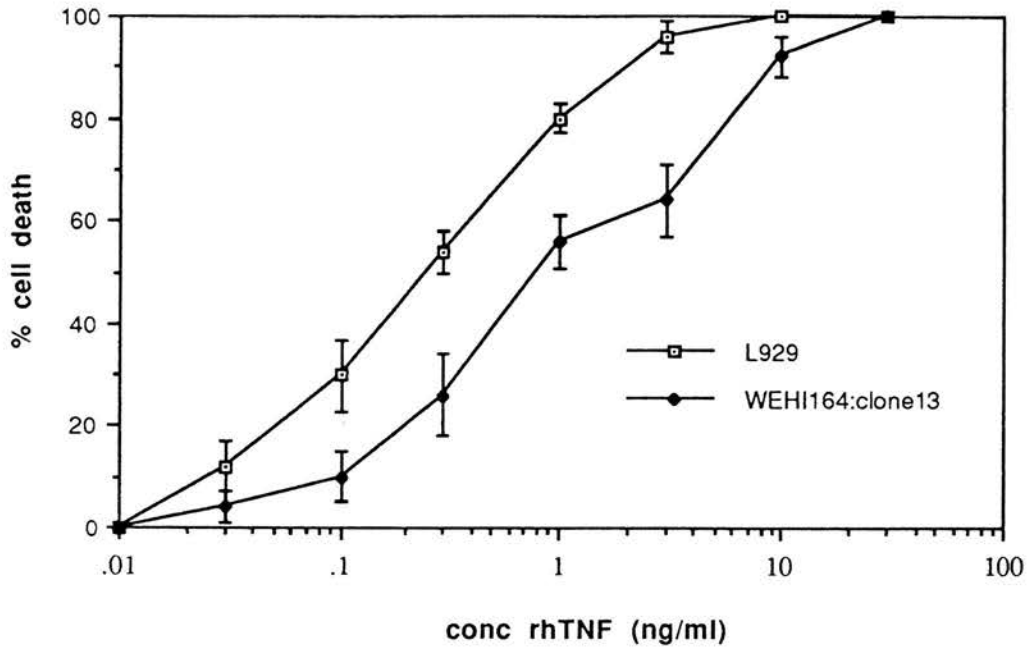


Figure 4.1:- a comparison of the sensitivity of two different cell lines (see symbol legends) to rhTNFα. Results are expressed as the mean percentage cell death (+/- standard deviations) induced in quadruplicate wells by the quoted concentrations of rhTNFα in added samples.

secrete TNFα decreased markedly with time spent in culture. After Northern blotting studies also suggested that ovine lung cells might be refractory to stimulation for a while after plating out (see 2.2.5), subsequent studies concentrated on the use of freshly-isolated cells, where TNFα mRNA at least was known to have been induced.

When all the lung-cell SN's generated during the Northern blotting experiment shown in fig.2.4 were retained and analysed in cytotoxicity assays, once again no detectable cell death was induced. In this experiment, a range of concentrations of rhTNFα were also added and the lower limit of detection was c.30pg rhTNFα/ml (table 4.2).

A further Northern blotting experiment was conducted to confirm some of the results of fig.2.4. Once again the cell source, which yielded 1.6 x 10^8 cells, was a pair of lungs from a Scottish Blackface ewe. These cells were divided between 8 x 75cm^2 flasks filled with 25mls of medium containing 0 or 100ng LPS/ml and RNA was collected from 1 flask of each type 0,1,2 and 3 hours later. 10µg of RNA from each time-point were then submitted to Northern blotting using an ovine TNFα cDNA probe (povTNF1 insert, conditions as before [see 2.2.5]). The result (fig.4.2) verified that the size of an inducible RNA detected by this probe was slightly larger than 18S. Furthermore, it again demonstrated induction of this transcript in the absence or presence of added LPS and here the concentration of ovine TNFα mRNA peaked between 0 and 2 hours.

When the SN's of these cells were tested for the presence of cytotoxicity towards L929 cells, once again none could be found. The lower limit of detection for rhTNFα was similar to the previous assay, c.30pg/ml (table 4.3).

In spite of proven induction of TNFα mRNA, it was still conceivable that these failures of ovine SN's to display detectable cytotoxicity towards L929 cells, was due to an absence of secreted ovine TNFα protein. (For example, any pre-slaughter stress could have subjected these macrophages to the influence of corticosteroids, a situation known to allow the accumulation of TNFα transcripts but prevent TNFα secretion [Beutler et al., 1986a]). With the production of rovTNFα, however, came the opportunity to raise specific antibodies which might be of use in detecting native ovine TNFα.

Table 4.2:-

Sample	Conc^n or time	Mean OD_{540} (SD)	% cytotoxicity(SD)
rhTNFα (diluted in Iscove's medium with 100ng LPS /ml).	10ng/ml	_0.012 (0.002)	_100
	3ng/ml	_0.025 (0.002)	_95 (0.8)
	1ng/ml	_0.061 (0.004)	_81 (1.6)
	300pg/ml	_0.114 (0.015)	_60 (5.8)
	100pg/ml	_0.160 (0.009)	_42 (3.5)
	30pg/ml	_0.232 (0.010)	_14 (3.9)
	10pg/ml	_0.265 (0.017)	_1 (6.6)
	0pg/ml	_0.268 (0.013)	_0
ovine lung-cell supernatants (with 100ng LPS/ml)	0 h	_0.263 (0.021)	_2 (8.2)
	0.75 h	_0.268 (0.012)	_0 (4.7)
	1.5 h	_0.271 (0.014)	_-1 (5.5)
	2.25 h	_0.276 (0.016)	_-3 (6.2)
	3 h	_0.265 (0.009)	_1 (3.5)
	24 h	_0.260 (0.011)	_3 (4.3)
SN (no LPS)	2.25 h	_0.278) (0.018)	_-4 (7.0)

Table 4.3:-

Sample	Conc^n or time	Mean OD_{540} (SD)	%cytotoxicity (SD)
rhTNFα (diluted in Iscove's medium).	10ng/ml	_0.008 (0.001)	_100
	3ng/ml	_0.013 (0.003)	_98 (1.2)
	1ng/ml	_0.069 (0.010)	_74 (3.9)
	300pg/ml	_0.130 (0.021)	_48 (8.2)
	100pg/ml	_0.170 (0.009)	_31 (3.5)
	30pg/ml	_0.219 (0.012)	_10 (4.7)
	10pg/ml	_0.245 (0.018)	_-1 (7.1)
	0pg/ml	_0.243 (0.012)	_0
ovine lung-cell supernatants (without LPS).	0 h	_0.234 (0.007)	_4 (2.7)
	1 h	_0.238 (0.013)	_2 (5.1)
	2 h	_0.250 (0.014)	_-3 (5.5)
	3 h	_0.241 (0.012)	_1 (4.7)
ovine lung-cell supernatants (with 100ng LPS/ml).	0 h	_0.245 (0.020)	_-1 (7.8)
	1 h	_0.231 (0.009)	_5 (3.5)
	2 h	_0.238 (0.016)	_2 (6.3)
	3 h	_0.240 (0.011)	_1 (4.3)

Tables 4.2 & 4.3:- results of assays to analyse the cytotoxic potential to L929 cells of supernatants generated in the Northern blotting experiments depicted in fig.s 2.4 & 4.2, respectively (means [&, in brackets, SD's] are of quadruplicate wells).

Figure 4.2:-

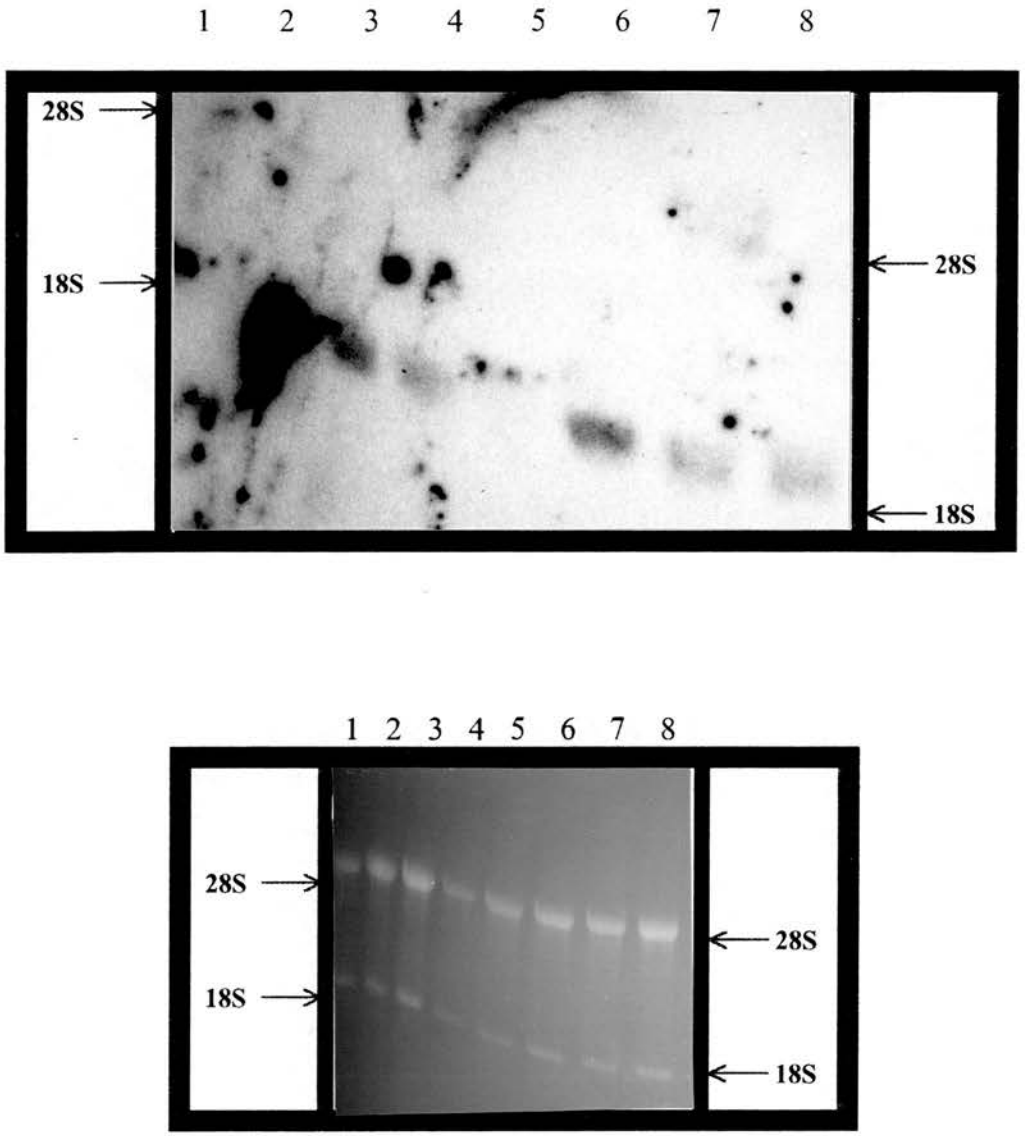


Figure 4.2:- a Northern blot (above) following hybridisation to an ovine TNFα cDNA probe (povTNF1 insert)& the transilluminated gel (below) from which the blot was taken (hence with corresponding lane numbers), after the electrophoresis of an estimated 10μg RNA per lane. The positions of putative 28S & 18S RNA are indicated for each. RNA was extracted from ovine lung-cells which had been cultured in the absence (lanes 1-4) or presence (lanes 5-8) of 100ng LPS/ml for 0 (lanes 1 & 5), 1 (lanes 2 & 6), 2 (lanes 3 & 7) or 3 (lanes 4 & 8) h.

4.2.3.Raising polyclonal antisera to rovTNFα:-

Since the particulate nature of the VLP may enhance the immunogenicity of a recombinant protein (Adams et al., 1987a) an initial attempt to raise a rabbit antiserum to rovTNFα was made by giving a primary inoculation of uncleaved, purified P1-TNF fusion protein in complete Freund's adjuvant (CFA) followed by a second inoculation of purified rovTNFα in incomplete Freund's adjuvant (IFA) three weeks later. However, whilst this rabbit developed a strong response to higher molecular weight contaminants in rovTNFα, serum taken two weeks after the second immunisation failed to react with any proteins of c.17kDa when utilised at a dilution of 1:50 in a Western blot against 100ng of rovTNFα.

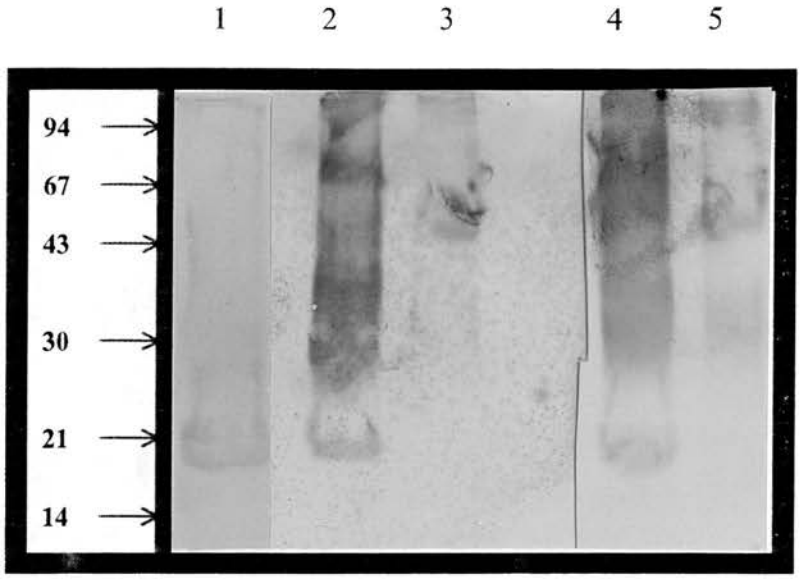
Following this attempt, two other rabbits (R198 and R199) were immunised solely with purified rovTNFα. 2 weeks after second inoculations (given in IFA, 3 weeks after primary inoculations given in CFA), sera from each of these rabbits, when used at a dilution of 1:50 as primary antibodies in Western blots, demonstrated significant affinity for a c.17kDa protein in 100ng of the rovTNFα preparation, but not in an equivalent volume of the yeast extract/ Xa negative control preparation (fig.4.3a). A larger proportion of the antibodies in each serum, however, reacted with the smaller amounts of higher molecular weight contaminants in rovTNFα, some of which shared immunological identity with proteins in the negative control preparation. No activity against any of these proteins was seen when preimmune sera were tested at the same concentration. These rabbits were further 'boosted' by one more inoculation of rovTNFα, before larger amounts of sera were harvested and, following heat inactivation of complement, aliquoted and stored for subsequent characterisation (see below).

4.2.4.Preparing monoclonal antibodies to rovTNFα: i) immune response of mice:-

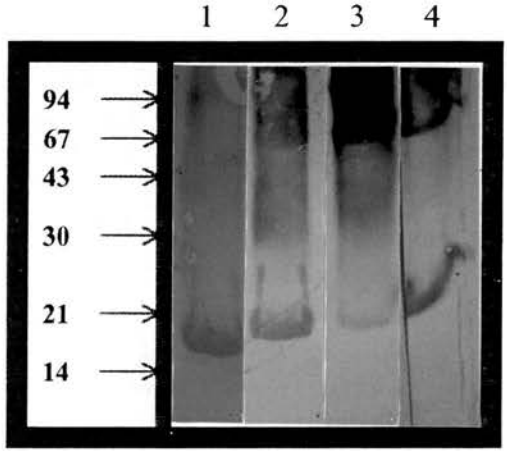
3 mice were also immunised, with the aim of producing monoclonal antibodies (Köhler & Milstein, 1975) specific for rovTNFα. When sera from each was collected after 4 rovTNFα inoculations and tested for immunoreactivity with 100ng rovTNFα by Western blot, all 3 were capable of reacting with the component of interest when used at a

Figure 4.3:-

4.3a



4.3b



Figures 4.3a & b:- blots of SDS- 15% polyacrylamide gels, run under non-reducing conditions, with the positions of molecular size markers indicated (in kDa). Samples submitted to electrophoresis comprised:- 1μg rovTNFα (4.3a, lane 1; 4.3b, lane 1), 100ng rovTNFα (4.3a, lanes 2 & 4; 4.3b, lanes 2,3 & 4) & 1μl of a yeast extract/factor Xa negative control preparation (4.3a, lanes 3 & 5). Strips shown in lane 1 of each figure were stained with amido black. Other strips were submitted to immunodetection using a 1:50 dilution of serum (post 2nd inoculation) from rabbits 198 (4.3a, lanes 2 & 3) or 199 (4.3a, lanes 4 & 5), or a 1:1000 dilution of serum from mice no.s 3, 2 or 1 (4.3b, lanes 2,3 & 4, respectively), as the primary antibodies.

dilution of 1:1000. (This can be considered an acceptable response before attempting a cell fusion [Fuller et al., 1988]). They all, however, also showed significant response to the higher molecular weight contaminants in this preparation (fig.4.3b).

4.2.5.Preparing monoclonal antibodies: ii) an ELISA for screening hybridoma supernatants:-

Given the strong response of these mice to the contaminants in rovTNFα, it seemed probable that anti-rovTNFα antibody-producing colonies would comprise only a small proportion of the hybridomas which might be obtained following fusion of splenocytes with myeloma cells. Therefore a suitable, sensitive screening method, preferably capable of analysing many samples simultaneously, was likely to be required for their preferential selection. An experiment was conducted to determine an appropriate protocol for screening potential hybridoma SN's by ELISA (Engvall & Perlmann, 1972).

Proteins show differing abilities to adhere to plastic under different conditions. When murine anti-rovTNFα antiserum was used at a dilution of 1:1000 as the primary detecting antibody in an ELISA against immobilised rovTNFα, it was found that similar responses were obtained whether rovTNFα was diluted in PBS, pH 7.2, or in carbonate coating-buffer, pH 9.6 prior to coating (table 4.4). By comparison, although significant responses were obtained in ELISA's against the yeast extract/Xa negative control preparation when it had been immobilised following dilution in PBS, the responses seen when the same preparation had been diluted to the same concentration in carbonate coating-buffer were considerably less. Thus, under these conditions a large proportion of the control preparation (and therefore probably rovTNFα contaminants) coated relatively poorly. Negligible responses were noted, whatever the coating conditions, when irrelevent antibodies (normal spleen-cell SN's) were used as the primary detecting agent against rovTNFα.

Consequently, the assay adopted for initial screening of SN's was an ELISA performed against rovTNFα, immobilised following dilution to 300ng/ml in carbonate coating-buffer - conditions which might reduce the number of 'false' positive results, caused by antibodies recognising contaminants, yet retain good sensitivity to antibodies

Table 4.4:-

Conc^{n} of rovTNFα (ng/ml)	1	2	3	4	5	6
3,000	_1.112	_1.188	_0.726	_0.767	_0.056	_0.057
1,000	_1.207	_1.454	_0.607	_0.868	_0.097	_0.065
300	_1.104	_1.280	_0.354	_0.826	_0.078	_0.078
100	_0.871	_0.923	_0.190	_0.498	_0.052	_0.092
30	_0.557	_0.552	_0.160	_0.276	_0.083	_0.087

Table 4.4:- results of an ELISA to determine suitable coating conditions for rovTNFα. Results are expressed as mean OD's$_{492}$ of duplicate wells. Primary detecting antibodies were antiserum from mouse no.3, diluted 1:1000 (columns 1-4), or supernatant from normal murine splenocytes (columns 5,6). Protein coating conditions were:- rovTNFα, diluted to the stated concentration in carbonate coating buffer (columns 1 & 5) or PBS (columns 2 & 6); or a yeast extract/factor Xa negative control preparation (plotted at equivalent dilutions to rovTNFα), diluted in carbonate coating buffer (column 3) or PBS (column 4).

Table 4.5:-

Test batch no. >>	1	2	3	4	5	6
Day post-fusion of test	13	16	22	23	27	29
No. wells tested	22	11	27	10	23	11
Test results (controls)						
+ve control	1.91	1.76	1.6	1.72	1.64	1.8
Medium	0.1	0.09	0.07	0.09	0.07	0.1
Monoclonal no.1	0.13	0.06	0.04	0.08	0.03	0.09
" no.2	0.15	0.08	0.08	0.04	0.04	0.1
" no.3	0.11	0.08	0.08	0.04	0.06	0.09
Splenocyte SN	0.73	0.64	0.37	0.33	0.44	0.64
Test results (specific wells)	**1G10**: 1.03 **6E7**: 0.81	**1E2**: 1.70				
Summary of remainder	20wells > 0.37 < 0.72	10 wells >0.29 <0.62	17 wells >0.23 <0.37 10 wells <0.21	4 wells >0.23 <0.33 6 wells <0.23	6 wells >0.20 <0.44 17 wells <0.20	4 wells >0.30 <0.61 7 wells <0.30

Table 4.5:- a summary of the results of a fusion. Results are expressed as the mean OD's$_{492}$ of duplicate wells following ELISA's testing hybridoma supernatants for immunoreactivity against rovTNFα. The positive control was antiserum from mouse no.3, diluted 1:1000 in medium. `Irrelevent' monoclonals no.s 1 (anti-CD8), 2 (anti-CD4) & 3 (anti-ovine MHC) were of IgM, IgG1 & IgG2a isotypes, respectively.

reacting with rovTNFα. Secondary screening would then include an assay against the negative control preparation, coated following a lesser dilution in PBS.

4.2.6.Preparing monoclonal antibodies: iii) results of a fusion:-

Mouse no.3 was selected for use in a fusion, since this appeared to show the strongest response to rovTNFα (fig.4.3b). It provided a total of 1.8 x 10^8 splenocytes. Following fusion to NSO myeloma cells (Galfre & Milstein, 1981), hybridomas appeared in 120/350 wells. 104 of these continued to grow to the point of confluency, when their SN's were analysed. These tests were performed in 6 batches conducted over the course of 16 days (summarised in table 4.5). Whilst 64 SN's gave results which might be considered positive (> 3x the background reading obtained using medium only), 2 (from wells 1G10 and 6E7) in the first batch tested, and 1 (from well 1E2) in the second, gave strongly-positive results (> the result obtained using the SN of splenocytes only). Cells from these 3 wells were submitted to immediate cloning procedures.

4.2.7.Preparing monoclonal antibodies: iv) cloning of hybridomas:-

Cells from wells 1G10 and 6E7 were initially cloned by limiting dilution whilst cells from well 1E2 were cloned by simple dilution (see 6.4.13). Whilst all tested SN's of cells derived from 6E7 gave positive results in an ELISA against rovTNFα, they also gave positive results in an ELISA against the yeast extract/Xa negative control preparation. In contrast, no SN's of clones derived from wells 1E2 or 1G10 gave positive results when analysed against the negative control, although 10/10 of the former and 3/6 of the latter gave positive results against rovTNFα.

Cells from a well which had demonstrated healthy cell growth and given one of the strongest results for each clone source were then subjected to a further cloning procedure, by simple dilution. All wells with single colonies derived from the '1E2' clone gave positive results in ELISA's against rovTNFα, in contrast to only 5/7 single-colony wells derived from the '1G10' clone.

When cells from a single similarly-selected well from each original source were subjected to a third cloning procedure, by limiting dilution, all wells developing single colonies gave positive results in ELISAs against rovTNFα, though the SN's from all 1E2-derived clones gave consistently greater ELISA results than those from 1G10-derived clones. Large numbers of cells from a clone from each original source were grown and stored in frozen aliquots before some were retrieved for the production of monoclonal antibodies (designated **cyt1** [1E2-derived] and **cyt2** [1G10-derived]) in the form of saturated tissue-culture SN's.

Whilst both of these saturated SN's (SSN's) gave positive results in the ELISA against rovTNFα, a stronger response, greater than that of the positive control (1:1000 dilution of antiserum from mouse no.3), was noted for **cyt1**. (OD's$_{492}$ were 1.86, 1.34, 1.76 and 0.04 for **cyt1**, **cyt2**, positive control and medium only, respectively.)

4.2.8.Characterisation of antibodies raised to rovTNFα: i) isotype of monoclonals:-

Use of a commercial kit determined that the monoclonal antibodies **cyt1** and **cyt2** were of immunoglobulin isotypes G2a and G1 respectively (fig.4.4a).

4.2.9.Characterisation of antibodies: ii) use in Western blotting:-

The polyclonal antisera and monoclonal antibodies **cyt1** and **cyt2** were tested over a range of dilutions for their ability to detect rovTNFα on Western blots (fig.4.4b). Both antisera could react with a c.17kDa protein at dilutions as great as 1:4000.

Both monoclonals also recognised the same size protein. However, whilst **cyt1** retained detectable affinity for rovTNFα at dilutions of SSN as low as 1:100, the ability of **cyt2** to react with rovTNFα on Western blot was lost at a dilution between 1:2 and 1:10. **Cyt1** also reacted with a c.32kDa protein (figs.4.4b and, more noticeably, 4.4c), putatively rovTNFα dimer (see chapter 3).

Because it gave a stronger response and could be used at greater dilutions, **cyt1** was considered the more suitable antibody for use in further Western blotting studies. Whilst, when used at a dilution of 1:30, **cyt1**-SSN proved capable of detecting as little as 1ng of

Figure 4.4:-

4.4a

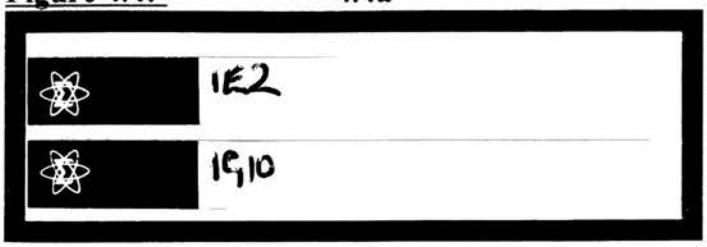
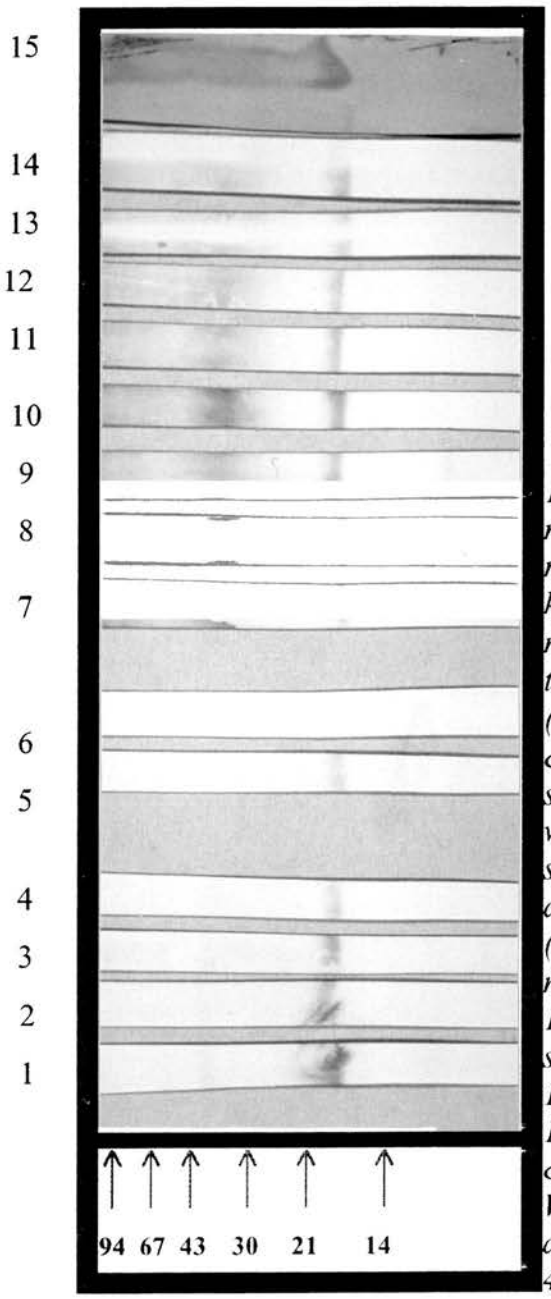
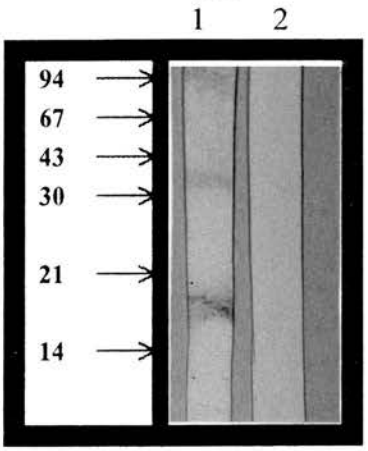


Figure 4.4a:- the result using a commercial kit (Sigma) to determine the isotypes of monoclonal antibodies ***cyt 1*** *(1E2) &* ***cyt 2*** *(1G10).*

4.4b



4.4c



Figures 4.4b & c:- Western blots of SDS-15% polyacrylamide gels, run under non-reducing conditions. The positions of molecular size markers are indicated (in kDa). Each strip contained c.10ng of rovTNFα (assuming 100% transfer from the gel) with the exception of strip 15 (4.4b), which contained 1μg of rovTNFα & was stained with amido black. All other strips were submitted to immuno-detection with primary antibodies as follows:- saturated supernatant (SSN)of ***cyt 1*** *diluted 1:2, 1:10, 1:30, 1:100 & 1:30 (4.4b, strips 1,2,3,4 & 4.4c, strip 1, respectively); SSN of* ***cyt 2*** *diluted 1:2 & 1:10 (4.4b, strips 5 & 6, respectively); serum (post 3rd inoculation) from rabbits 198 & 199, diluted 1:500, 1:1,000, 1:2,000 & 1:4,000 (4.4b, strips 7-10 [198] & 11-14 [199], respectively); & SSN from* ***VPM53*** *(an irrelevent control monoclonal antibody) diluted 1:30 (4.4b, strip 2). In 4.4c, colour development was continued for a prolonged period.*

rovTNFα, it failed to detect any proteins in 100x the equivalent volume of yeast extract/Xa negative control preparation (fig.4.7a, lanes 3 & 4). Furthermore, an 'irrelevant' monoclonal antibody of the same G2a isotype, **VPM53** (whose specific ligand is a component of a Campylobacter-like organism [Dr.S.McOrist, personal communication]), failed to detect any proteins in rovTNFα, when used in SSN form (of proven activity [E.Mills, personal communication]) at the same dilution (fig.4.4c), suggesting that the recognition of these proteins by **cyt1** was a specific phenomenon.

It was also noted that whilst the polyclonal antisera displayed affinity for high molecular weight contaminants in rovTNFα, they too showed a strong response to a c.32kDa protein (fig.4.4b).

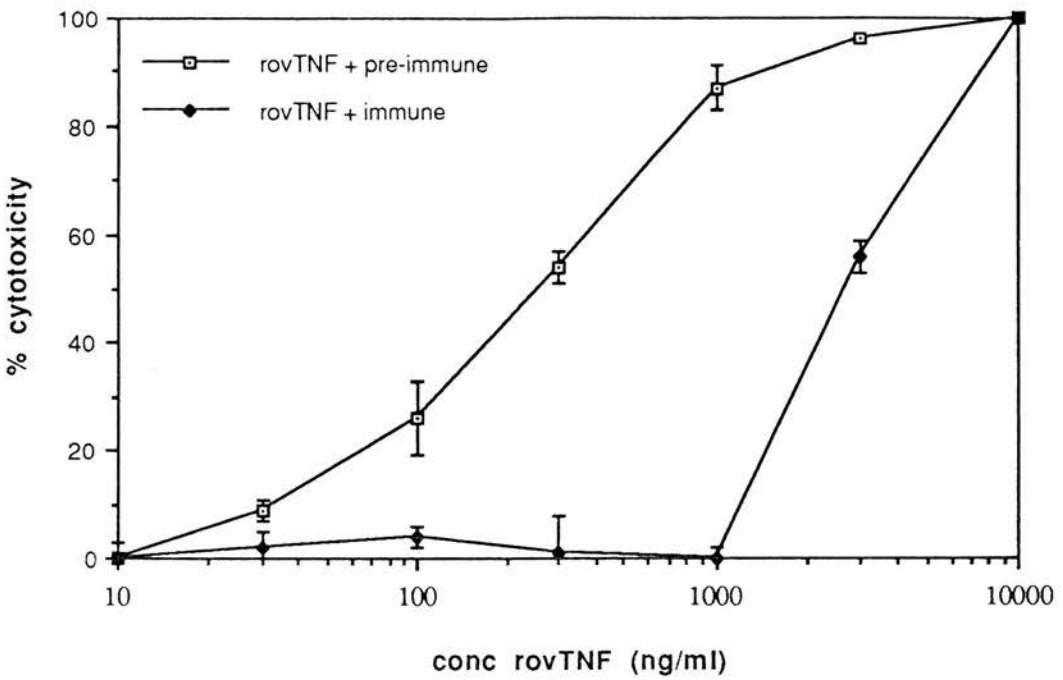
4.2.10.Characterisation of antibodies: iii) neutralising abilities:-

The abilities of both polyclonal antisera and the monoclonal antibodies **cyt1** and **cyt2** to neutralise the activity of rovTNFα were initially assessed in cytotoxicity assays on L929 cells. Whilst a 1:50 dilution of antisera from rabbits 198 and 199 were able to completely neutralise the activity of 1 and 3μg rovTNFα /ml respectively (fig. 4.5a,b) (a protective effect which could be overcome by the addition of surplus rovTNFα), neither of the monoclonal antibodies caused the slightest reduction in activity of any concentration of rovTNFα tested, when used at a 1:2 dilution of SSN (fig.4.5c).

Since it is conceivable that an anti-TNFα antibody might be capable of interfering with TNFα binding to one, but not the other, type of receptor, the abilities of these antibodies to neutralise rovTNFα activity in thymocyte co-mitogen proliferation assays were also assessed. Both rabbit antisera also proved capable of neutralising rovTNFα activity in this assay (fig.4.6a,b). Neither monoclonal antibody displayed any neutralising ability whatsoever (fig.4.6c). It was also surmised that, since there was no significant difference in the levels of proliferation induced by medium alone, whether immune or pre-immune sera were added to the assay, endogenous TNFα seems not to play a role in 'background' proliferation.

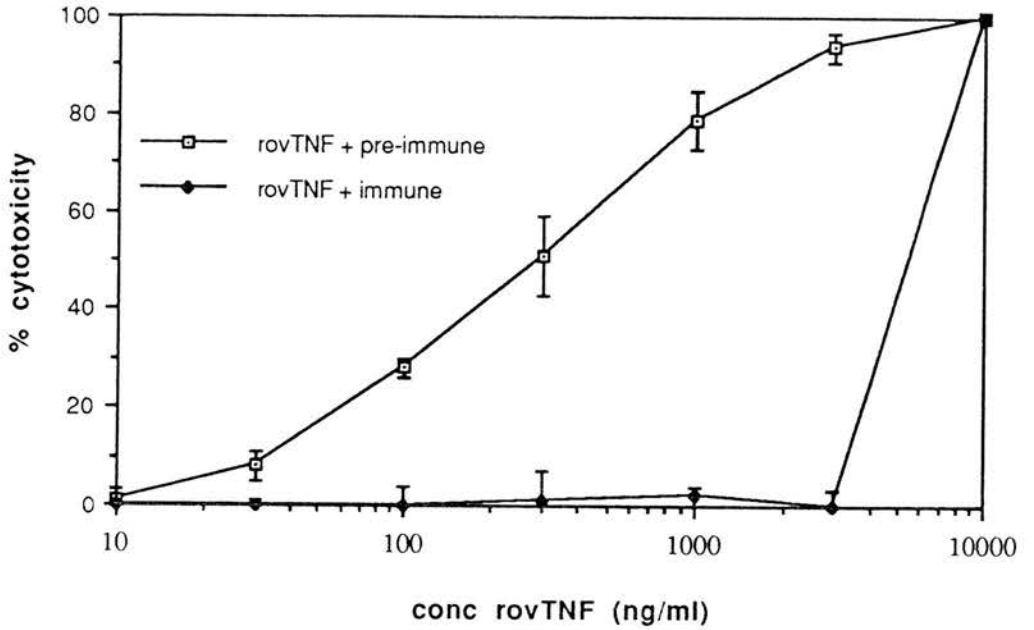
As in the cytotoxicity assay, the serum from rabbit 199 displayed slightly greater neutralising potential than that from rabbit 198. The specificity of this action was confirmed following incubation of pre-

Figure 4.5:- **4.5a**

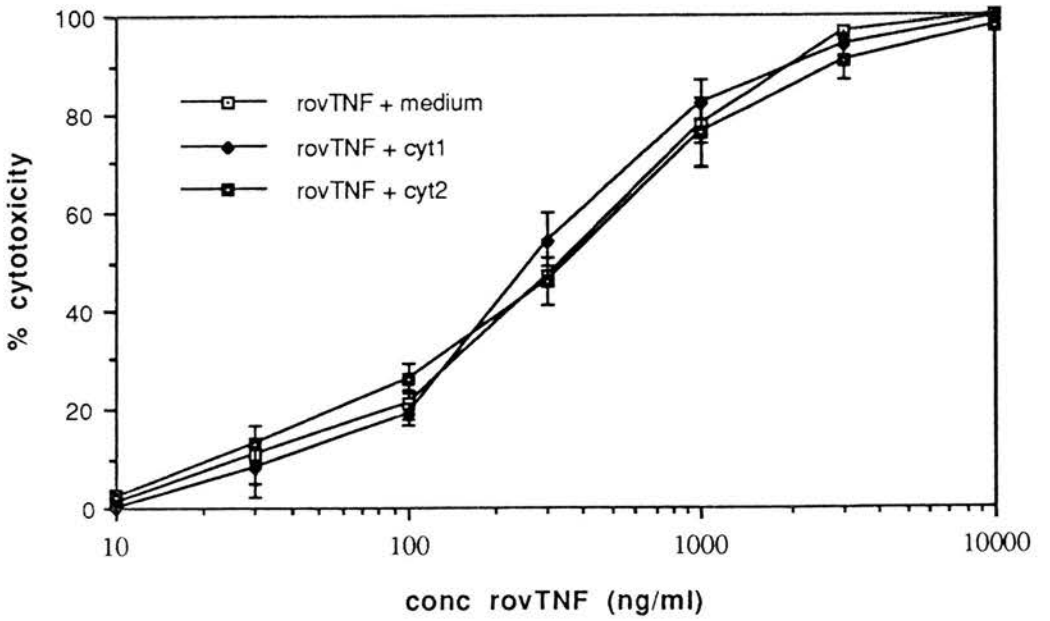


Figures 4.5a & (see over) b,c:- results of cytotoxicity assays performed on L929 cells exposed to samples of rovTNFα which.had been pre-incubated with 2% pre- or post-immune serum from rabbits 198 (4.5a) or 199 (4.5b), or 50% RPMI (with 15% FCS) or 50% saturated supernatants of **cyt 1** *or* **cyt 2** *(4.5c) (see symbol legends). Results are expressed as mean percentage cell death induced in quadruplicate wells. Concentrations of rovTNFα quoted are those in the samples which comprised 25% of the incubation volume.*

4.5b


100
80
60
40
20
0
% cytotoxicity
rovTNF + pre-immune
rovTNF + immune
10
100
1000
10000
conc rovTNF (ng/ml)

4.5c


100
80
60
40
20
0
% cytotoxicity
rovTNF + medium
rovTNF + cyt1
rovTNF + cyt2
10
100
1000
10000
conc rovTNF (ng/ml)

and post- immune sera from rabbit 199 with rovIL-1ß, which had been produced by the same expression system and which also has significant activity in this assay (Fiskerstrand et al., 1992) (1U of IL-1ß is defined as that amount of protein causing half maximal incorporation of label in this assay). Preincubation with immune serum did not cause the slightest reduction in the activity of this latter cytokine (fig.4.6d).

4.2.11.Initial use of **cyt1** to detect ovine TNFα:-

5 x 10^7 lung cells, freshly-isolated from a Scottish Blackface ewe, were cultured for 18 hours in a 125cm^2 tissue culture flask at 10^6 cells and 1µg of LPS /ml, before the SN was collected, clarified and stored frozen in aliquots. When the proteins in 1ml of SN were concentrated and analysed by Western blotting, using a 1:30 dilution of SSN of **cyt1** as the primary antibody, a c.25kDa protein was detected (fig.4.7a). This band stained more intensely than 1ng of rovTNFα detected on the same blot. No bands were seen when 1ml of the starting culture medium was similarly analysed, nor were any bands detected when a duplicate blot was incubated with a 1:30 dilution of **VPM53** SSN.

4.2.12.Ovine TNFα is glycosylated:-

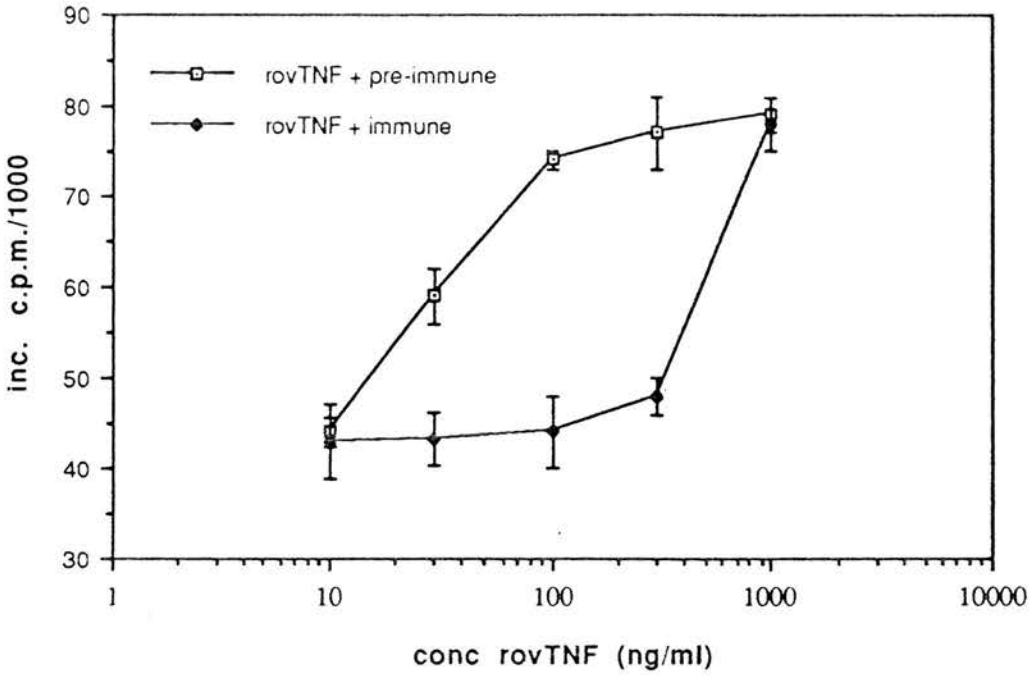
The factor detected in the above SN was clearly of greater molecular weight than rovTNFα. Since there is one potential Asn-linked glycosylation site predicted in the sequence of mature ovine TNFα (figs.2.8, 4.11), these two proteins were examined for differences in their glycosylation pattern. Incubation of rovTNFα with peptide-N-glycosidase-F (Plummer et al., 1984) made no detectable difference to its molecular weight, in contrast to the protein detected in ovine lung-cell SN, whose size was reduced following deglycosylation to more closely approximate that of rovTNFα (fig.4.7b).

4.2.13.Ovine lung-cell culture supernatant does contain biologically active TNFα:-

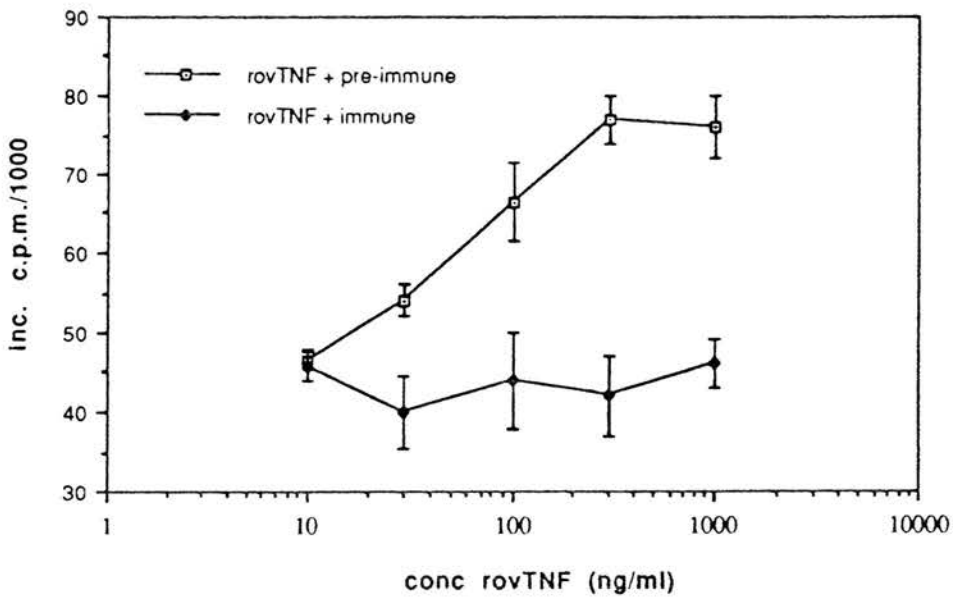
The SN described above (4.2.11) showed no detectable cytotoxicity to L929 cells in an assay capable of detecting 30pg/ml of rhTNFα (table 4.6). It did, however, show activity in a thymocyte co-mitogen proliferation assay, an activity which could be totally neutralised by

Figure 4.6:-

4.6a

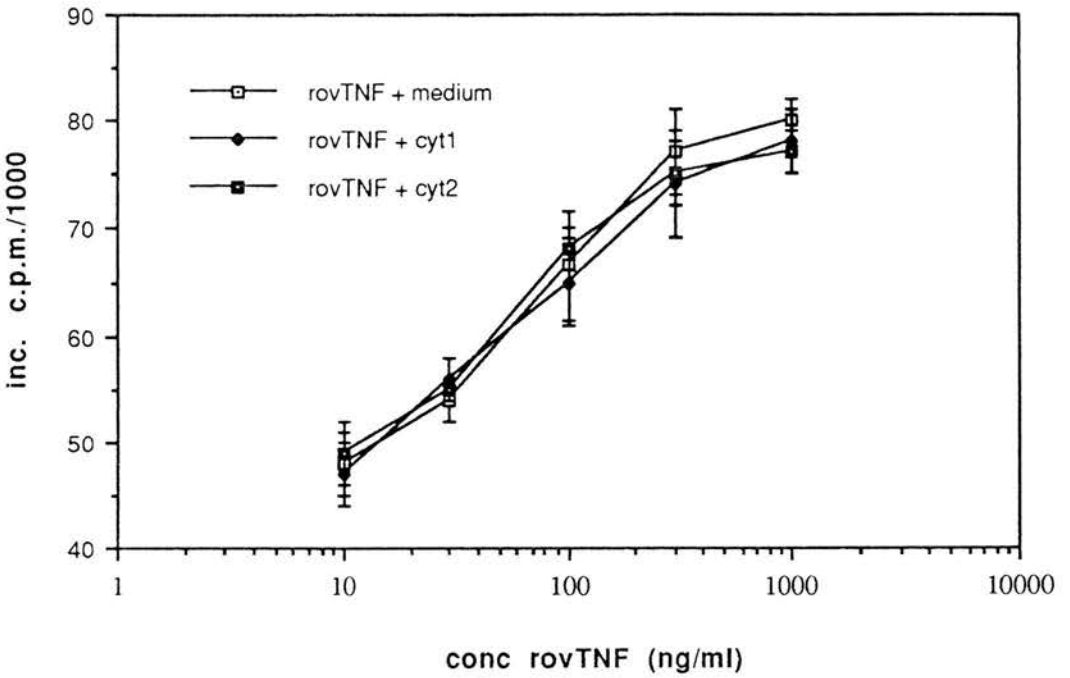


4.6b



Figures 4.6a, b & (see over) c &d:- results of co-mitogen proliferation assays, expressed as mean counts per minute per well (of quintuplicate wells: error bars indicate standard deviations) incorporated by ovine thymocytes, following incubation with samples of recombinant ovine cytokines which had been pre-incubated with 1% pre- or post- immune serum from rabbits 198 (4.6a) or 199 (4.6b,d), or 50% RPMI (with 15%FCS) or 50% saturated supernatants from ***cyt 1*** *or* ***cyt 2*** *(4.6c) (see symbol legends). Concentrations of cytokines quoted are those in samples, which comprised 50% of the incubation volume.*

4.6c


rovTNF + medium
rovTNF + cyt1
rovTNF + cyt2
inc. c.p.m./1000
90
80
70
60
50
40
1
10
100
1000
10000
conc rovTNF (ng/ml)

4.6d

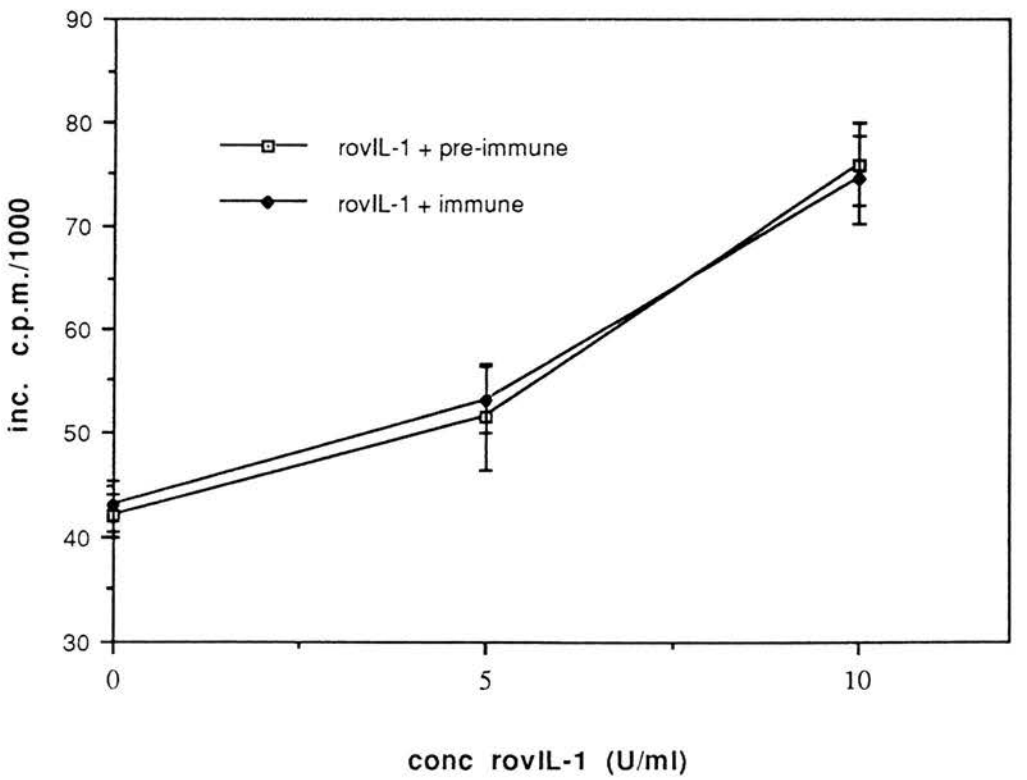
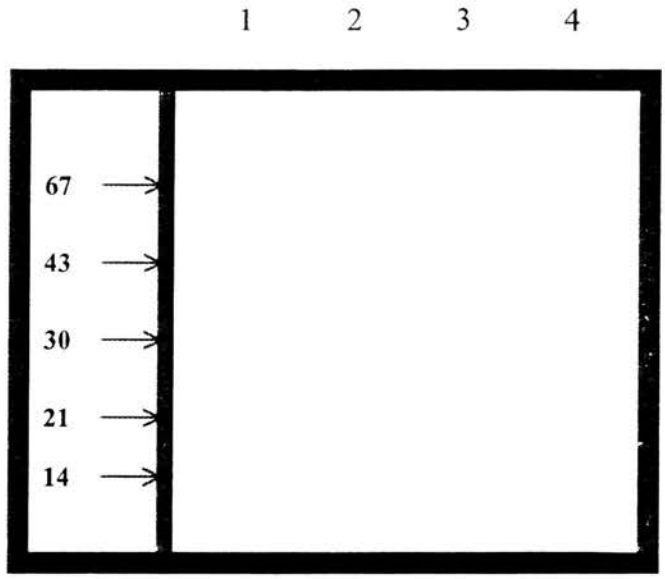
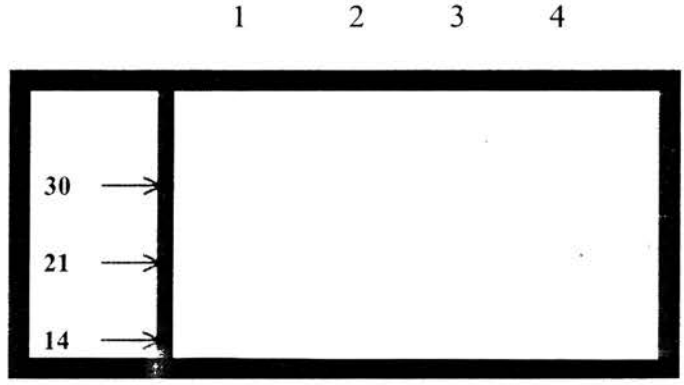

rovIL-1 + pre-immune
rovIL-1 + immune
inc. c.p.m./1000
90
80
70
60
50
40
30
0
5
10
conc rovIL-1 (U/ml)

Figure 4.7:-

4.7a



4.7b



Figures 4.7a & b:- Western blots of SDS- 5-20% gradient polyacrylamide gels, run under non-reducing conditions. Positions of molecular size markers are indicated (in kDa) for each. The primary detecting antibody was ***cyt 1****, used as the saturated supernatant diluted 1:30 in each case. Final detection in 4.7b was by enhanced chemiluminescence (see 6.3.14). Samples submitted to electrophoresis in 4.7a comprised:- 1ng rovTNFα (lane 3), 1μl of yeast extract/factor Xa negative control preparation (lane 4) & acetone precipitates derived from 1ml aliquots of Iscove's medium with 1μg LPS/ml (lane 1) or the supernatant of ovine lung-cells, cultured for 18 h in the same medium (lane 2). In 4.7b, samples comprised :- resuspended acetone precipitates, derived from 0.1ml aliquots of the latter supernatant, after incubation with (lane 3) or without (lane 4) peptide-N-glycosidase F, & 2ng rovTNFα incubated with (lane 2) or without (lane 1) the same enzyme.*

Table 4.6:-

Sample	Conc[a] or time	Mean OD_{540} (SD)	%cytotoxicity (SD)
rhTNFα (diluted in Iscove's medium with 1μg LPS/ml).	10ng/ml	_0.020 (0.005)	_100
	3ng/ml	_0.025 (0.011)	_98 (4.2)
	1ng/ml	_0.104 (0.004)	_68 (1.5)
	300pg/ml	_0.146 (0.021)	_52 (8.0)
	100pg/ml	_0.207 (0.013)	_29 (4.9)
	30pg/ml	_0.246 (0.012)	_14 (4.6)
	10pg/ml	_0.286 (0.019)	_-1 (7.2)
	0pg/ml	_0.283 (0.024)	_0
Ovine lung-cell supernatants (as in fig.4.9)	0 h	_0.280 (0.010)	_1 (3.8)
	2 h	_0.272 (0.013)	_4 (4.9)
	4 h	_0.268 (0.015)	_6 (5.7)
	6 h	_0.263 (0.015)	_7 (5.7)
	18 h	_0.280 (0.008)	_1 (3.0)
	25 h	_0.281 (0.010)	_1 (3.8)
	48 h	_0.278 (0.012)	_2 (4.6)
" (as in fig.4.7)	18 h	_0.285 (0.011)	_-1 (4.2)

Table 4.6:- results of an assay to assess the cytotoxic potential of the ovine lung-cell supernatants which have previously featured in figures 4.7 & 4.9. Results are expressed as mean percentage cytotoxicities, calculated from the displayed mean $OD's_{540}$ of quadruplicate wells, after residual cells, following the incubation of L929 cells with the stated samples, were stained with crystal violet. Standard deviations of the means are in brackets.

preincubation with antiserum from rabbit 199 (fig.4.8). (Relatively high 'background' counts were seen in this assay. However, because thymocytes for such assays were obtained from different breeds and ages of lambs at slaughter, a lot of variation normally exists in these assays [see fig.3.12].)

4.2.14.A time course of ovine TNFα production:-

The accumulation of the protein detected by **cyt1** in culture SN's was examined with respect to time. Lung cells were collected from a Scottish Blackface tup and plated at 1.5 x 10^7 cells/ flask in 7 x 25cm^2 tissue culture flasks, each with 7mls of medium containing LPS at 1μg/ml, before a flask SN was harvested at one of several time-points over the following 2 days. When the proteins in 1ml of each SN were analysed by Western blotting using **cyt1**, a c.25kDa protein was detected in all but the initial time-point (fig.4.9). The blot shows an early accumulation of this protein, whose concentration appears to 'plateau' from 6 to 25 hours before a subsequent decline. The intensity of staining of this protein in SN's collected between 2 and 48 hours after plating out was greater than that seen for 1ng of rovTNFα detected on the same blot. No proteins were detected when a duplicate blot was incubated with a similar dilution of **VPM53** SSN as the primary antibody. None of these SN's showed detectable cyto-toxicity to L929 cells in an assay where 30pg/ml rhTNFα was readily detectable (table 4.6)

4.2.15.The response of ovine lung cells to increasing doses of LPS:-

The accumulation of **cyt1**-binding protein in response to a range of LPS concentrations was also analysed. Freshly collected lung-cells from each of 2 Scottish Blackface ewes were plated out in identical conditions to those used for time course analysis (4.2.14), with the exception that flasks contained 0,1,10,100 or 1000 ng LPS/ ml of medium. In each case, analysis of SN's collected 20 hours later, revealed that higher concentrations of protein detected by **cyt1** accumulated when cells had been exposed to higher doses of LPS, although detectable amounts were still produced in the absence of added LPS (fig.4.10).

Figure 4.8:-

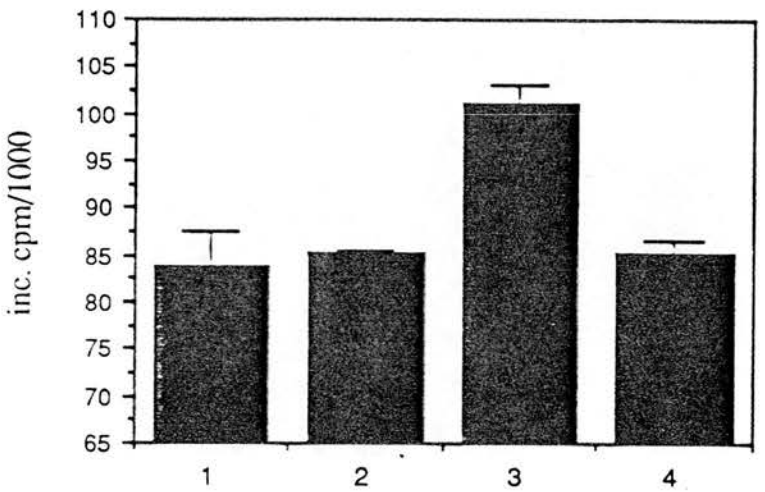


Figure 4.8:- the results of a co-mitogen proliferation assay, expressed as the mean counts per minute per well, incorporated by ovine thymocytes incubated in the presence of assorted samples in quintuplicate wells. Samples comprised Iscove's medium with 1μg LPS/ml (columns 1 & 2) and the supernatant of ovine lung-cells which had been incubated for 18 h in the same medium (columns 3 & 4). These samples had been preincubated with 0.5% pre-immune (columns 1 & 3) or immune (columns 2 & 4) serum from rabbit 199, immediately prior to incubation with the thymocytes. Error bars indicate standard deviations of the mean.

Figure 4.9:-

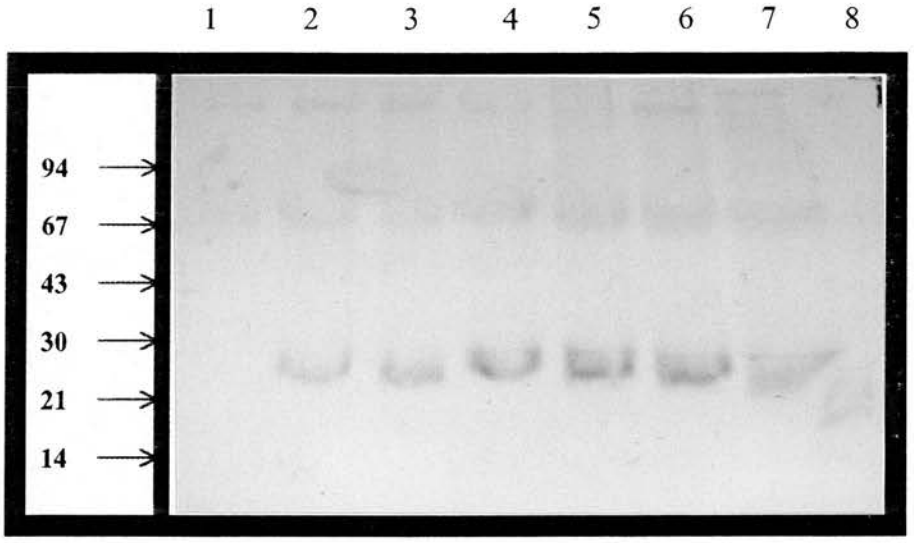
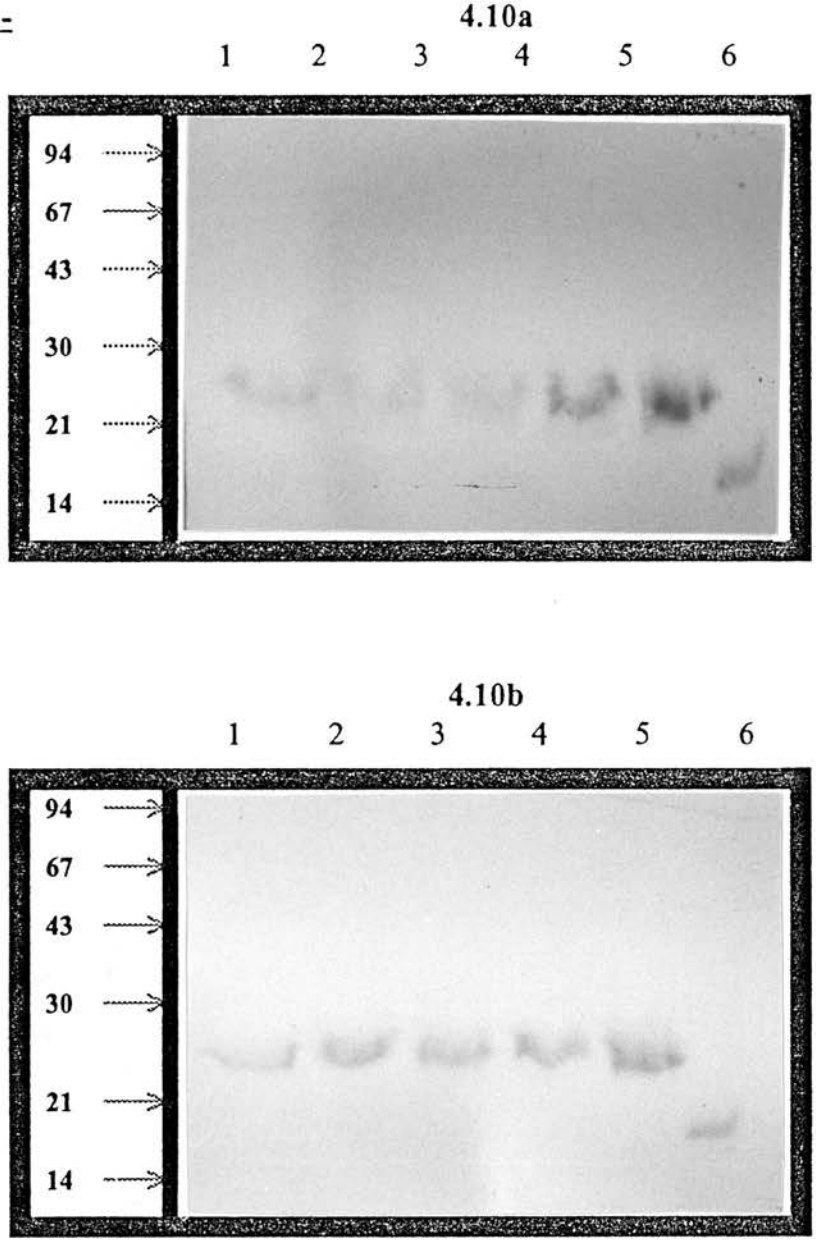


Figure 4.9:- a Western blot of an SDS- 5-20% gradient polyacrylamide gel, run under non-reducing conditions, using ***cyt 1*** *(as a 1:30 dilution of saturated supernatant) as the primary detecting antibody. The positions of molecular size markers are indicated (in kDa). Samples submitted to electrophoresis comprised 1ng of rovTNFα (lane 8) & the acetone precipitates derived from 1ml of supernatants collected 0, 2, 4, 6, 18, 25 & 48 h after ovine lung-cells were plated out in Iscove's medium with 1μg LPS/ml (lanes 1-7 respectively).*

Figure 4.10:-



Figures 4.10a & b:- Western blots of SDS- 5-20% gradient polyacrylamide gels, run under non-reducing conditions. The positions of molecular size markers are indicated (in kDa) for each. The primary detecting antibody was ***cyt 1****, used at a dilution of 1:30 of the saturated supernatant. Each blot is the result of an experiment performed on lung cells derived from a different sheep. Samples submitted to electrophoresis comprised 5ng rovTNFα (lanes 6) & acetone precipitates derived from 1ml of supernatant collected 20 h after cells were plated out in Iscove's medium containing 0 (lanes 1), 1 (lanes 2), 10 (lanes 3), 100 (lanes 4) or 1000 (lanes 5) ng of LPS/ml.*

4.3.Discussion:-

Several possibilities were originally considered for early failures to detect cytotoxic activity in the SN's of LPS-stimulated ovine lung cells using TNF-sensitive murine cells. These included considerations of whether (sufficient) TNFα was being induced, whether ovine TNFα would be active on murine cells and whether inhibitors of action might be present. A full appreciation of the reason(s) involved clearly required further analysis.

It was slightly surprising to find that the sensitivity of the WEHI 164: clone 13 cells used here was somewhat lower than that of the L929 cells (as confirmed in chapter 3, see fig.3.10), in contrast to the original description of these cells by Espevik and Nissen-Meyer (1986), although different protocols to these authors were used. Others have also found that these cells may be no more sensitive than L929 cells (e.g. Meager et al., 1989), blaming changes in phenotype of either cell line occurring with serial passage in culture. Unfortunately, little history was available for the cells used here.

The Northern blotting experiment described here (fig.4.2) confirmed some of the results of that presented in chapter 2 (see fig.2.4). In particular, the size of transcript detected and its induction in the absence of added LPS were as previously described. Western blotting (fig.4.10) later corroborated this latter finding, to an extent, by confirming that some ovine TNFα protein was also produced in the absence of added LPS. In the light of these results, no conclusion can be drawn regarding the induction of ovine TNFα mRNA by LPS. To what extent the induction seen was due to pre-existing LPS contamination of the media used, its possible presence in the sheep's trachea prior to lavage, or to some other procedure(s) adopted during lung-cell preparation, is unclear. Standiford et al. (1991) have noted the induction of TNFα in a human patient following broncho-alveolar lavage, whilst Schindler et al. (1990) have demonstrated the induction of IL-1 mRNA by the adherence of macrophages to plastic.

The kinetics of TNFα transcript accumulation, however, did appear to differ slightly from the first experiment. Whilst the time of achieving maximum transcript concentration in this experiment (between 0 and 120 minutes) overlaps with that seen previously (between 90 and 180 minutes), there was a noticeably slower subsequent decay, hinting

at a degree of individual variation. However, both experiments demonstrated undetectable levels in freshly-isolated cells, and an early accumulation of transcripts. These findings are in general agreement with another, subsequently-published, study of TNFα transcript accumulation in LPS-stimulated ovine alveolar macrophages (Nash et al., 1992). These authors demonstrated TNFα mRNA rising from undetectable levels in 'resting' macrophages, to a peak concentration within five hours following LPS stimulation and becoming undetectable by 24 hours. In their studies, however, the zero hour time-point, when LPS was added, was actually 1 hour after plating out (to allow for the selection of adherent cells), by which time very significant amounts of mRNA had been induced in my own study. Nor did they observe any subsequent mRNA induction in the absence of added LPS. Clearly, this may have been because they used different media and/or techniques in cell preparation. Alternatively, since they used a 100-fold higher concentration of LPS and an human TNFα cDNA probe, higher concentrations of transcripts may have been induced by LPS and the sensitivity of their Northern blot could have been less than my own.

Although the rovTNFα preparation contained many highly immunogenic impurities, it proved sufficiently pure for raising antibodies of use in the immunological detection of ovine TNFα. The initial failure of one rabbit to produce anti-rovTNFα antibodies, in response to a primary immunisation with hybrid VLP's, could simply reflect the poor response of an individual. Alternatively, the theory of improved antigenicity of a protein as a fusion partner with P1 because of its particulate nature (Adams et al., 1987a) may not hold true, particularly when significant homology with one of the host's own proteins exists. Under these circumstances, responses to the more foreign yeast element may dominate.

By comparison, the use of rovTNFα in cleaved and partially purified form always succeeded in producing a response to the appropriate component, in mice as well as rabbits, where two polyclonal antisera with significant avidity for rovTNFα were produced. Calculations based on the neutralisation assays of both type I and type II receptor-mediated TNFα activity (figs.4.5 & 4.6) suggest that approximately 6-7% of antibody binding sites in R199 serum could be directed against neutralising epitopes of rovTNFα (assuming M_r 1 antibody binding site

= 75,000 and 10mg Ig/ml of serum). However, Western blotting (figs.4.3a, 4.4b) clearly demonstrated that these antisera also recognise higher molecular weight contaminants in rovTNFα, some of which share immunological cross-reactivity with components of the yeast extract/Xa negative control preparation. Blotting also revealed quantitative and qualitative differences between the two sets of proteins, however, casting some doubt on the validity of the yeast extract/Xa preparation as a negative control for TNFα. It should be re-emphasised though that this preparation never displayed any activity in the assays described in chapter 3, at any concentration, and some of the qualitative differences noted on blots could be due to antibodies directed against TNFα dimers and/or residual, uncleaved P1-TNF fusion protein.

It was considered that the response of these antisera to contaminants in rovTNFα, including a possible response to bovine factor Xa and probably, therefore, ovine factor Xa, might limit their use in the detection of ovine TNFα. However, at least one very useful anti-rovTNFα monoclonal antibody, **cyt1**, was also produced.

Although it did not appear to neutralise rovTNFα activity (see below), the evidence that this monoclonal antibody recognises rovTNFα is considerable. Firstly, it specifically reacts with an appropriately-sized protein, not recognised by an irrelevant antibody of the same sub-class, in very small amounts of rovTNFα on Western blots. Secondly, it does not recognise any proteins in the yeast extract/Xa preparation either in a Western blot or (pre-second cloning) in an ELISA. Thirdly, it also reacts with proteins of appropriate size to be TNFα homo-dimers and trimers in cross-linked samples of rovTNFα (fig.3.16b), oligomerisation being a known phenomenon of TNF'sα (see 1.3.4 & chapter 3). Finally, it recognises, in ovine cell culture SN's, a glycoprotein of appropriate size and inducibility to be ovine TNFα (see below).

Cyt1 SSN, at a dilution of 1:2, failed to neutralise any rovTNFα activity in cytotoxicity or thymocyte co-mitogen proliferation assays, successful neutralisation by parallel pre-incubation of rovTNFα with R198 or R199 antisera serving as a positive control. Were **cyt1** a neutralising antibody and present at an average antibody concentration of 10μg/ml in saturated supernatant (Yokoyama, 1992) a 1:2 dilution

should have been capable of totally neutralising approximately 1.1µg rovTNFα/ml. No reduction of activity was noted, however, even though concentrations of rovTNFα as low as 30ng/ml were active in these assays. Given that **cyt1** SSN is active on Western blots at relatively high dilutions, it seems unlikely that its antibody concentration could be several-fold lower than the average quoted concentration. Thus one can conclude that **cyt1** is almost certainly a non-neutralising antibody.

Cyt1 consistently and specifically recognised a c.25KDa protein in the SN's of ovine lung-cell cultures, but not in starting media. Several lines of evidence suggest that this protein was mature ovine TNFα. Firstly, it was specifically recognised by **cyt1**. Secondly, deglycosylation studies confirmed that this is a glycoprotein, there being one potential Asn-linked glycosylation site in the predicted aa sequence of mature ovine TNFα (figs.2.8, 4.11). Thirdly, the size of the deglycosylated component was approximately the same as rovTNFα, rovTNFα being unglycosylated (fig.4.7b). Fourthly, this protein accumulated in stimulated-lung-cell SN's with kinetics in keeping with my own observations of the kinetics of ovine TNFα mRNA induction and with studies of TNFα accumulation in alveolar macrophage SN's in other species (e.g. Becker et al., 1989; MacKay et al., 1991a) (although comparing the results obtained using different types of assay may not be strictly valid [see 4.1]). Finally, as for TNF'sα from other species, the accumulation of this protein was LPS-responsive.

In spite of the fact that peptide-N-glycosidase-F removes all traces of the carbohydrate group (Tarentino et al., 1985), deglycosylated ovine TNFα still showed slightly slower mobility through a gel than rovTNFα. Whilst some retardation could have been caused by interference from the relatively large amounts of other SN proteins, it is also possible that, in spite of theoretical considerations (see chapter 3, 3.3) mature, native ovine TNFα may include a few more amino acids than rovTNFα. Knowledge of the true start site of mature ovine TNFα, however, awaits its purification and amino-terminal sequence analysis. 'Failure' of the Ty-VLP system to glycosylate rovTNFα was not surprising in the light of the experience of others expressing potential glycoproteins with this system (C.Cousens, Dr.N.Carey, personal communications).

Two other observations worthy of note were also made from Western blotting. Firstly, in a time-course experiment, **cyt1** also detected proteins of M_r c.70,000 and very high molecular weight (fig.4.9). These might be undissociated TNFα trimers or multimers, TNFα complexed with TNF-R's or other proteins, or simply other antigens with a shared epitope. However, it is noticeable that, in contrast to c.25kDa ovine TNFα, these proteins were also present at the zero time-point.

Secondly, in each of two dose-response blots (fig.4.10), more TNFα accumulated when 100ng LPS/ml were added than when none was added. Given that two Northern blotting experiments on cells from other individuals suggest similar levels of transcripts might be present in each case, this result hints that, as in other species (see 1.3.3 and 2.3), the translation of ovine TNFα transcripts is inducible by LPS.

In spite of proven ovine TNFα content, the underlying theme of total failure to detect cytotoxic activity in the SN's of ovine lung-cells, using murine cells, continued. Assuming complete protein transfer during blotting and equal affinity of **cyt1** for native and recombinant ovTNFα, the former protein was present at concentrations in excess of 1ng/ml in an 18 hour SN (see fig.4.7a) and in all but the initial SN of a time course series (fig.4.9), yet none of these samples were active in L929 cytotoxicity assays capable of detecting as little as 30pg of rhTNFα/ml.

The question of whether any of this TNFα was biologically active was answered using the 18 hour SN as a sample in a thymocyte co-mitogen proliferation assay. Some activity, completely neutralisable by R199 serum was clearly seen (fig.4.8). Complete neutralisation was surprising as this sample was likely to contain IL-1, another LPS-inducible monokine with activity in this assay (Gery et al., 1972). However, the activity of both α & β forms of ovine IL-1 is severely reduced by a single freeze-thaw cycle (C.Fiskerstrand, personal communication). Since R199 serum has no activity against rovIL-1β, and other proteins it may react with, such as P1 or factor Xa, have no activity in this assay, whether as part of the control yeast extract/Xa preparation (see chapter 3) or in purified form (C.Fiskerstrand, personal communication) one can conclude that the activity seen in this sample was almost certainly due to ovine TNFα.

Furthermore, although individual assays show a degree of variation, studies with rovTNFα (fig.3.12) suggest that the c.20% degree of proliferation enhancement seen in this experiment would have required a concentration of ovine TNFα in the range of 3-10ng/ml (assuming no influences from synergists or antagonists: synergy with IL-1 and IL-2 in this assay being known phenomena, [Ranges et al., 1988; Hurme, 1988]). Such a concentration would seem to be compatible with the intensity of ovine TNFα staining seen in a Western blot of this sample (fig.4.7a).

Unless inhibitors capable of blocking type I- but not type II-receptor- mediated activity were present in this sample, one can finally conclude that the L929 cells used are poorly sensitive to ovine TNFα in comparison to rhTNFα. Others have also failed to detect cytotoxic activity in the SN's of LPS-stimulated ovine macrophages using murine cells (Nash et al., 1992; Francey et al., 1992; Dr.G. Entrican, personal communication). These findings then are suggestive of a poor affinity of ovine TNFα for the murine TNF-RI, in agreement with the data presented in chapter 3. Why should this be, when murine cells have proved so amenable for the study of TNF'sα from many other species? Notwithstanding potential additional influences of glycosylation and/or a possibly-extended amino terminus in native ovine TNFα (see earlier), some potential answers can be gleaned from a comparison of the predicted aa sequences of several TNF'sα (fig.4.11), and knowledge gained from structure/function studies on human TNFα.

Ovine TNFα shares approximately 79 % homology with human TNFα over the entire pre-protein, (81 % homology for the mature protein) and 91% homology with a bovine TNFα sequence (presented in a review as un-published work, Goeddel et al., 1986). Some particular features are well conserved across all the species, including the sheep, helping to identify the ovine protein as a 'typical' TNFα. These include:- two cysteine residues in the mature part of the molecule, believed to form an intra-chain disulphide bridge (Davis et al., 1987); an arginine-serine doublet, of importance for the recognition by serine proteases of the cleavage site for generation of mature TNFα (Scuderi, 1989); 13 amino acids at the extreme carboxyl terminus; and a stretch of 20 hydrophobic residues in the pre-protein leader sequence (positions -27 to -46, fig.4.11), believed to be important for transmembrane

Figure 4.11:-

```
                        .           .           .■          .           .
OV -77 MSTKSMIRDVELAEEVLSNKAGGPQGSRSCWCLSLFSFLLVAGATTLFCL
BO     ..................E...........L.....................
HU     ...E...........A.PK.T.......R.LF.......I..........
MU     ...E...........E.PQ.M..F.N..R.L.....................
RA     ...E..........GP.PK........KR.L.....................
PO     ...E...........A.AK........R.L.....................

                    . ✠         .           1 .     ■   .
OV -27 LHFGVIGPQREEQSPAGPSFNRPLVQ--TLRSSSQASNNKPVAHVVA NIS
BO     .............V.S...IS.....--.........S.........D.N
HU     .............-F.RDL.LIS..A.--AV....RTPSD.........PQ
MU     .N........D.KF.N.LPLISSMA.TL.......N.SD.........HQ
RA     ...R.....E.....NNLHLVN.VA.MV....A.R.LSD..L......PQ
PO     ...E.....K..-F....LSIN..A.--G......T-SD.........VK

                .           .           .           .           .   ■
OV  22 APGQLRWGDSYANALMANGVELKDNQLVVPTDGLYLIYSQVLFRGHGCPS
BO     S......W............K.E.......AE.............Q....
HU     .E...Q.LNRR....L......R.......SE...........K.Q....
MU     VEE..E.LSQR....L...MD.........A.....V......K.Q...D
RA     VE...Q.LSQR....L...MK.T.......A............S.Q..R.
PO     .E...Q.QSG.....L....K.........................Q....

                .           .           .           . ■         .
OV  72 TPLFLTHTISRIAVSYQTKVNILSAIKSPCHRETLEGAEAKPWYEPIYQG
BO     P.PV..............................P.W.............
HU     .HVL..................L........Q...P............L.
MU     Y-VL....V..F.I...E...L...V....PKD.P....L........L.
RA     Y-VL....V..F....PN...L............P.E..PMA......L.
PO     .NV..................L........Q...P............L.

                .           .           .           .
OV 122 GVFQLEKGDRLSAEINLPEYLDYAESGQVYFGIIAL  157
BO     ..................D..................
HU     ................R.D...F..............
MU     .........Q....V...K...F.........V...
RA     ............T.V.Q.....L..............
PO     .......D..........D...F..............
```

Figure 4.11:- a comparison of the predicted amino acid sequences for TNF's α from assorted species (OV = ovine, BO = bovine, HU = human, MU= murine, RA = rabbit, PO = porcine). Sequences are taken from figure 2.8, Goeddel et al. (1986), Shirai et al.(1985), Pennica et al. (1985), Ito et al. (1986) & Pauli et al. (1989). The presumed first amino acid of the mature ovine molecule is marked by an overhead 1; its potential glycosylation site (NIS)is in bold print. Amino acids which are conserved in all other species illustrated, but differ in the sheep, are highlighted by an overhead ■*. The amino acid which does not appear in the ovine sequence of Young et al. (1991) is marked by an overhead* ✠*.*

anchoring of full-length TNFα (Kriegler et al., 1988). Unlike human TNFα, one potential Asn-linked glycosylation site is present in the ovine sequence, at a site 12 aa from the glycosylation site of murine TNFα.

Human TNFα has been the subject of a number of studies to attempt to define functional regions of the molecule. These have identified amino acids near the amino terminus (Socher et al., 1987), at the extreme carboxyl terminus (Gase et al., 1990) and a cluster of residues on either side of a groove formed between two sub-units of the trimer (Van Ostade et al., 1991) as being particularly important to function.

Comparison of the predicted TNFα sequences of 6 species reveal 3 positions (9, 67 & 106) in the mature TNFα molecule where amino acid residues differ solely in the sheep. Of these, position no.9 has previously been implicated as potentially having an important rôle in cytotoxic activity. In this study, Creasey et al. (1987) showed that deletion of the first 8 amino acids of human TNFα did not affect its activity on murine cells, whereas deletion of the first 10 led to a significant (c.3-fold) decline in cytotoxicity. Presumably, non-conservative substitution rather than deletion of amino acid no.9 could lead to an equal or even greater decline in activity. Amino acid no.10 also differs from the human sequence in the sheep (and the ox, see below). Interestingly, in three-dimensional models of TNFα (Eck & Sprang, 1989; Jones et al., 1989), amino acid no.9 lies immediately adjacent to the carboxy-terminal leucine, which has also been implicated as serving an important role in activity (Gase et al., 1990).

Although positions 67 and 106 have not been implicated as being important for function and appear to be somewhat remote from the putative receptor binding site(s), it is conceivable that a change in residue seen at any position could affect overall activity via a subtle conformational change. A change from the human, rabbit and murine sequences at position no.32 is also worthy of note, although the same tyrosine for arginine substitution is seen in the bovine and porcine sequences. This amino acid has also been implicated as important to function and forms part of the putative receptor binding site described by Van Ostade et al. (1991). Interestingly, although

Adams and Czuprynski (1990) described the use of WEHI 164: clone 13 cells to detect TNF-like activity in LPS-stimulated bovine alveolar macrophage SN's, these authors could not detect this activity using a somewhat less sensitive L929 assay. Furthermore, in assays on L929 cells, rboTNFα showed an activity which was intermediate, on a weight for weight basis, between rhTNFα and my own rovTNFα (Dr.G.Entrican, personal communication). Murine cells may also show relatively poor sensitivity to porcine TNFα (Dr.E.Peterhans, personal communication).

Whatever the molecular basis for the poor activity of ovine TNFα on murine cells, and whilst slight improvements to sensitivity could have been made by adjustments to protocol (e.g. sample volumes comprised only 25% of final volume) the inevitable consequence is that assays involving murine cells appear to be of no use for the routine detection of ovine TNFα, although it is conceivable that recent further improvements to the sensitivity of such assays by a log factor or more (Branch et al., 1991) may alter this situation.

In the light of all the above observations it was surprising to find 2 papers suggesting that L929 cells can be used to detect ovine TNFα. Unfortunately, in one paper (Wheeler et al., 1990) the authors merely claim to have detected cytotoxic activity, neutralisable by a monoclonal antibody to human TNFα, in the serum of LPS-treated sheep, without presenting any data. In the second, the authors demonstrate some cytotoxic activity in the SN's of some ovine alveolar macrophages (Ellis et al., 1991). However, in these experiments cell death never reaches 100%, in spite of using minimally-diluted samples and doses of LPS as high as 10μg/ml for TNFα induction. Furthermore, this activity was only partially neutralisable by a polyclonal antiserum raised to rboTNFα and the authors describe the effect as displaying considerable variability.

Such results are not incompatible with my own. If, as suggested by results in chapter 3, murine cells are 1000-fold less sensitive to ovine TNFα than human TNFα, it is conceivable that concentrations of ovine TNFα in excess of 30ng/ml might be detected in assays displaying an achievable sensitivity to rhTNFα of 30pg/ml. Given individual variability in production of TNFα (compare figs.4.10a & b) and the achievement here of concentrations in excess of 1ng/ml, as judged by Western blotting, it is likely that levels higher than 30ng/ml could

be achieved in some SN's, by using even higher concentrations of LPS in the presence of serum (known to enhance TNFα production [Matthews, 1981a]), particularly if using cells from a diseased individual, which may already be under the influence of a known enhancer of TNFα production such as IFN gamma (Beutler et al., 1986b). Unfortunately, judgement of the sensitivity of the assays used by Ellis et al. is impossible, since they used rboTNFα as a standard and the concentrations quoted in text and figure legends are contradictory. Furthermore, on some occasions, cytotoxic activity in similarly obtained ovine SN's can be demonstrated on TNF-resistant L929L/R cells (Dr.G.Entrican, personal communication), whilst 'Tumour Necrosis Serum' is known to be more potent at inducing cytotoxicity than supernatant (Matthews, 1978), presumably because of synergistic effects.

One final possible contributory factor which might occasionally allow detectable activity of ovine TNFα on murine cells could be polymorphic variation between individual sheep. Although different alleles would seem to be present in the 3' UTR of the ovine TNFα gene (see 2.3), it is entirely possible that they may also occur within the translated region. Given that the change of a single residue of TNFα can drastically alter its activity (Van Ostade et al., 1991) and/or presumably its immunoreactivity, such polymorphism in any species could play havoc with more than one form of assay - another potential problem in the detection and quantification of TNFα to add to those described earlier (4.1).

CHAPTER 5, part i:- A PRELIMINARY APPRECIATION OF THE POTENTIAL ROLE OF TNFα IN MAEDI-VISNA DISEASE.

5.1. Introduction:-

Maedi (dyspnoea) and Visna (paralysis/wasting) are descriptive terms for two of the commonest clinical syndromes (now known to be caused by the same virus) seen in slowly-progressive diseases with lengthy incubation periods, which occurred as epidemics in Iceland earlier this century (Sigurdsson, 1954; Sigurdsson et al., 1957). Similar syndromes have now been described in many other countries (Marsh, 1923; Ressang et al., 1968; de Boer, 1975), including the UK (Jones et al., 1982). The aetiological agent, Maedi-Visna (MV) virus (MVV) has been characterised (see below) and is the prototype of a sub-family of retroviruses, the lentivirinae, which includes the Human Immunodeficiency Viruses (Gonda et al., 1986).

MV virions display typical lentiviral structure comprising a positive-stranded c.10kb RNA genome and reverse transcriptase (RT) enzyme in an electron-dense, bar-shaped core of non-glycosylated proteins, surrounded by a lipid envelope from which glycoprotein spikes project (Thormar, 1961; Pautrat et al., 1971; Brahic et al., 1973; Haase & Baringer, 1974; Clements & Narayan, 1981; Gonda et al., 1985). Several isolates of this virus from different countries have now been cloned and sequenced (Sonigo et al., 1985; Braun et al., 1987; Quérat et al., 1990; Sargan et al., 1991a; Staskus et al., 1991). These studies reveal that, as in other lentiviruses, the genome comprises three large ORF's, gag, pol, and env, which encode the structural core, replicative and envelope proteins respectively, as well as several small ORF's, which may be used to produce regulatory proteins, following multiple splicing events (see below). The genome is flanked at each end by a 'long terminal repeat' (LTR). Sequence variation between strains, particularly in parts of the env gene, is a prominent feature (Braun et al., 1987) and, during the course of infection, antigenic variants may appear in an individual animal (Lutley et al., 1983; Thormar et al., 1983). Under conditions of intensive housing, MVV is spread laterally via respiratory and/or possibly oral routes (Sigurdsson et al., 1953), although in other

farming systems lateral transmission occurs very slowly and lactogenic passage from ewe to lamb may be the main method of viral dissemination (de Boer et al., 1979).

The clinico-pathological features of MV disease (MVD), as they occur in one British flock, have recently been described (Watt et al., 1992). Sick sheep frequently become cachectic. Other clinical signs result from chronic, active inflammatory lesions in one or more of the following organs:- lung, central nervous system (CNS), joints, mammary gland, spleen and lymph nodes. In the lung, the commonest organ to be affected, massive infiltration of predominantly mononuclear cells, proliferation of lymphocytes to form germinal centre-like structures in the interstitium, and smooth muscle hyperplasia, all combine to cause the progressive respiratory embarassment seen in many cases. In a smaller number of animals, the CNS is affected. Here, lesions comprise a mononuclear cell infiltration of the choroid plexus, and perivascular cuffing, gliosis, astrocytic hypertrophy and demyelination in multiple focal sites within the brain and spinal cord. Such lesions seem to be responsible for the progressive paresis and ataxia seen in some sheep. In a few cases, lameness also results from joint pathology. Thickened synovial membranes, due to proliferation of the lining layer and inflammatory cell infiltration with occasional formation of germinal centres, may be seen, particularly in the carpal and/or tarsal joints, sometimes in association with erosions of the articular cartilage. Induration of the mammary glands can also occur as a result of similar infiltration, combined with fibrosis and loss of acinar tissue. Lympho-proliferation may also be seen in the spleen and assorted lymph nodes. Consequently, MVD shares many clinical features with HIV infection and has been proposed as an experimental model for some aspects of AIDS (Perk, 1988; Pétursson et al., 1989).

In contrast to the latter disease, however, secondary infections do not appear to be a major feature of MVD, possibly because MVV, unlike HIV, does not seem to infect $CD4^+$ lymphocytes (Gorrell et al., 1992). Like HIV, however, MVV is macrophage-trophic (Narayan et al., 1982). Macrophages, at sites of pathology, are the predominant cell type in which viral replication can be readily detected in vivo (Kennedy et al., 1985; Gendelman et al., 1985), though viral antigens and/or

nucleic acids can be found in association with other cell types such as oligodendrocytes and astrocytes, as well as epithelial cells of the choroid plexus after direct experimental infection of the CNS (Brahic et al., 1981; Stowring et al., 1985). Infected, multinucleate giant cells (possibly a consequence of virally-induced cell fusion [see below]) may also be seen (Peluso et al., 1985). Following infection by the respiratory route, some bone marrow monocyte/macrophage precursors harbour virus, forming a reservoir of infection (Gendelman et al., 1985), and virus may be spread to other tissues within monocytes or possibly dendritic cells (Peluso et al., 1985; Gorrell et al., 1992). What governs the cell and tissue tropism of MVV is unclear, though factors in the viral LTR may be involved (Small et al., 1989). Maturation of monocytic 'Trojan horses' to tissue macrophages can then be associated with increased viral replication (Gendelman et al., 1986). However, infected cells are present in only very small numbers in lesions of MVD and only a small percentage of these may actually be expressing viral protein at any one time (Haase et al., 1977). Different workers have concluded that this restriction involves transcriptional (Brahic et al., 1981; Geballe et al., 1985) or post-transcriptional blocks (Gendelman et al., 1985). Whatever the mechanism, sheltering of viral nucleic acid within cells which do not express viral antigen would seem to be one mechanism by which MVV persists in sheep in the face of both cellular and humoral immune responses (Pétursson et al., 1976; Larsen et al., 1982).

The development of the dramatic pathology of MVD in the presence of such a small amount of antigen has been a puzzle. Since immuno-suppression greatly diminishes the severity of lesions seen in experimentally-induced Visna (Nathanson et al., 1976) and hyper-immunisation can exacerbate this disease (Nathanson et al., 1981), lesions may have an immuno-pathological basis. One conceivable pathogenetic mechanism for a macrophage-trophic virus could be via dysregulating the activity of one or more monokines, including TNFα. A chronic increase in TNFα activity could clearly contribute to the cachexia, inflammatory cell accumulation, lymphocyte proliferation, demyelination, astrocytosis, fibrosis and cartilage destruction seen in MVD (see 1.4,5).

Little is known about the involvement of cytokines in this disease, though a unique form of IFN may be produced by ovine lymphocytes following specific interaction with MVV-infected macrophages (Narayan et al., 1985). This 'lentiferon' can markedly restrict the replication of MVV in macrophages in vitro (Kennedy et al., 1985), though it may have a less marked effect on other infected cell types (Lairmore et al., 1988). In spite of restricting viral replication, lentiferon would seem to be one potential amplifier of the inflammatory lesions of MVD, working in part via enhancing the expression of MHC class II proteins by local macrophages (Kennedy et al., 1985). Alveolar macrophages lavaged from Maedi lungs also appear to be 'activated' by other criteria and spontaneously secrete 'neutrophil chemotactic activity,' though the factors involved have not been characterised (Cordier et al., 1990).

Having established that ovine TNFα exists and has many of the same properties as TNF'sα in other species, it was of interest to examine its potential role in MVD, beginning with some preliminary experiments to determine whether MVV could induce ovine TNFα and/or rovTNFα could influence the life-cycle of MVV in vitro.

In contrast to the in vivo situation, MVV replicates productively in ovine or caprine macrophages and fibroblast-like cells in vitro (Sigurdsson et al., 1960; Thormar, 1963; Narayan et al., 1982), following binding to a cell surface receptor (Crane et al., 1991a; Dalziel et al., 1991). Exposure of cells to a high moi of MVV can cause cell-fusion 'from without' (Harter & Choppin, 1967), an effect which may be mediated via the transmembrane component of its envelope glycoproteins (Crane et al., 1991b). Once the viral core has entered the cell, RT activity produces proviral dsDNA (Haase et al., 1982), most of which, though nuclear in location, does not appear to integrate into host cell DNA, in contrast to the oncogenic retroviruses (Harris et al., 1984).

Expression of viral mRNA then proceeds and can be divisible into an early phase, when small, multiply-spliced, viral transcripts appear, and a later phase when larger transcripts, including full length genomic RNA's, accumulate (Vigne et al., 1987). Hess et al. (1985) showed that enhancement of MVV gene expression by viral products occurs. This effect can be accounted for by the accumulation of

regulatory proteins, such as tat and rev, largely translated from the small ORF's located within the early transcripts (Mazarin et al., 1988; Gordou et al., 1989; Sargan & Bennet, 1989). These proteins have similar functions to their homologs in HIV. Tat transactivates viral expression via interaction with an element in the LTR (Davis & Clements, 1989), whilst sufficient rev must accumulate before larger transcripts, needed for the translation of structural proteins, appear in the cytoplasm (Tiley et al., 1990).

Viral core proteins are produced from these unspliced transcripts as a polyprotein precursor joined to replicative enzymes, which include RT and, by sequence analogy to other retroviruses, viral proteases responsible for processing this large protein (Vigne et al., 1982; McClure et al., 1987). MVV envelope proteins may be produced from singly-spliced or unspliced transcripts. These structural components, including unspliced transcripts which form new genomes, are assembled at cell membranes (Coward et al., 1970), where they may then induce fusion from within or be secreted. Eventually viral replication may lead to cell lysis, especially in fibroblasts. This entire cycle can be completed within a few days. However, infection of macrophages, which seem to be more resistant to fusion from without, may involve fewer cytopathic effects (CPE). Furthermore, many virions appear to bud from the internal membranes of these cells and accumulate in cytoplasmic vacuoles rather than cell supernatants, resulting in lower final titres of virus (Narayan et al., 1982).

Different strains of MVV appear to have varying abilities to replicate productively in culture (Narayan et al., 1982; Quérat et al., 1984). Those strains which replicate most productively and cause the most CPE in macrophages in vitro tend to be the most pathogenic in vivo (Lairmore et al., 1987). The strain of MVV used in the following experiments was EV-1, a British isolate, which has been cloned and sequenced, and which can replicate productively to induce syncitial formation in fibroblasts (Sargan et al., 1991a).

5.2.Results:-

5.2.1.RovTNFα has an antiviral effect on MVV-infected ovine fibroblasts:-

When WSCP cells were infected with MVV at a low moi (0.17 $TCID_{50}$/cell) the level of viral proteins (as assayed by the level of RT activity) had risen by the end of 9 days of incubation. However, a dose-dependent reduction in the total (SN and cell-associated) viral yield was caused by continuous incubation with rovTNFα, from 2 days post-infection (PI) onwards (fig.5.1). The yeast extract/Xa negative control preparation had no effect. Since some ovine cultures may harbour latent retrovirus which can be induced by various stimuli (Barban et al., 1984), uninfected cells were also analysed. No spontaneous RT activity appeared in these cells nor was any induced by rovTNFα. When a duplicate plate was stained and analysed, it was apparent that rovTNFα had increased the overall density of uninfected wells, in agreement with findings in chapter 3, and viral infection had reduced the numbers of remaining cells at the end of the experiment, though no clear, dose-dependent effect was discernible for rovTNFα-treated infected wells (table 5.1).

Since the antiviral effect of TNFα, as mediated via 2'-5' oligoadenylate synthetase and/or IFN induction, may be even greater following pretreatment of aged fibroblasts (Van Damme et al., 1987; Mestan et al., 1988), the above experiment was repeated on WSCP's which had been maintained as a monolayer for several days and treated with rovTNFα, or controls, for 24 hours prior to infection, when incubation with the same sample concentration continued. Whilst the same amount of virus was inoculated into each well as in the previous experiment, the moi here was impossible to assess owing to cell replication post-plating. Once again rovTNFα caused a reduction in total viral yield in duplicate plates at both 7 and 10 days PI, though the maximum effect seen (c.50% reduction in RT activity) was similar to that in the previous experiment (fig.5.2). On the 10th day, refractile, necrotic-looking cells were seen detaching from infected wells treated with concentrations of rovTNFα in excess of 10ng/ml, but from no other wells (fig.5.3).

Figure 5.1:-

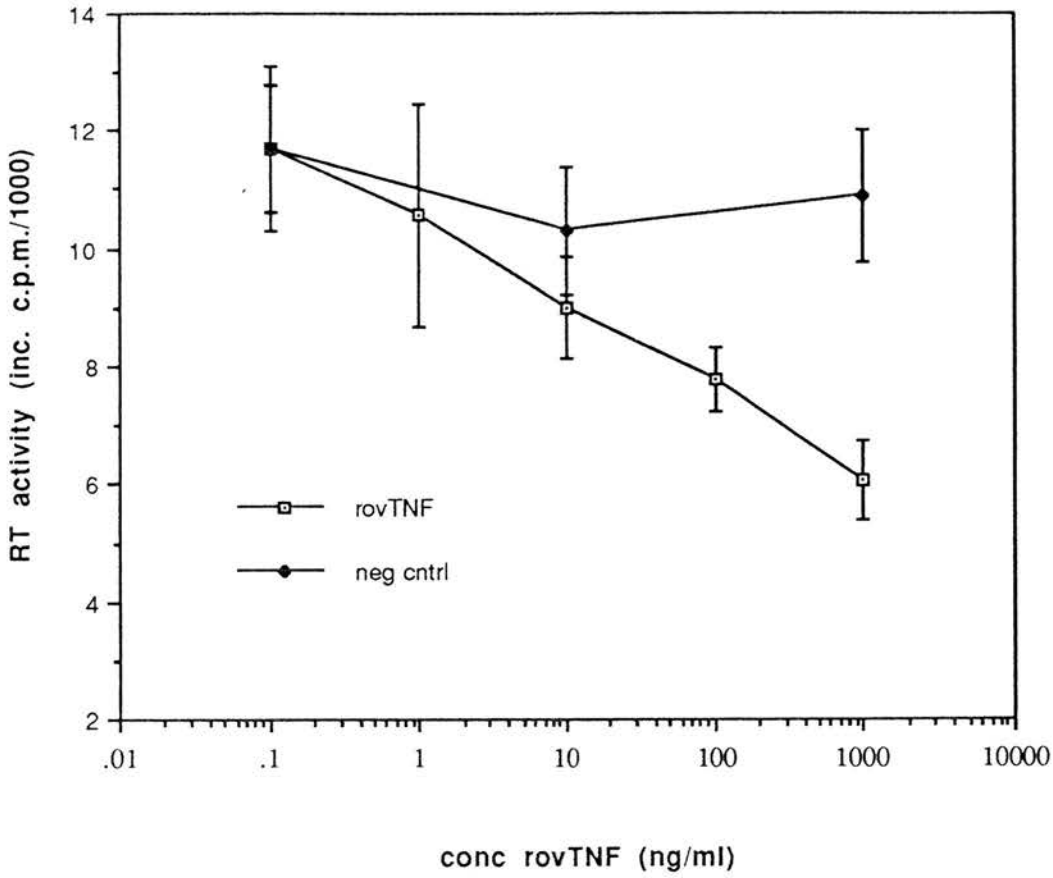


Figure 5.1:- results of an assay to analyse the effect of incubating EV-1- exposed WSCP cells with different concentrations of rovTNFα, or yeast extract/factor Xa negative control preparation (plotted at equivalent dilutions;see symbol legends), on the accumulation of total (supernatant & cell-associated) reverse transcriptase (RT) activity, 9 days post-infection. Results are expressed as the mean RT activities of sextuplicate wells (error bars indicate standard deviations). The RT activities associated with similarly-incubated uninfected wells, whether or not 1μg rovTNFα/ml was added, and with the initial viral inoculum (diluted to the final concentration assayed), were <300 and 605 c.p.m., respectively.

Table 5.1:-

EV-1	Conc^{n} rovTNFα (ng/ml)	OD_{540}	SD
-	0	_0.664	_0.041
-	1,000	_1.117	_0.033
+	0	_0.497	_0.029
+	1	_0.503	_0.043
+	10	_0.538	_0.045
+	100	_0.425	_0.021
+	1,000	_0.447	_0.046

Table 5.1:- the effects of incubation with rovTNFα and/or EV-1 infection on the eventual density of WSCP cells in an experiment which duplicated, and was run in parallel with, that described in fig.5.1. Results are expressed as the mean OD's$_{540}$ of sextuplicate wells (with standard deviations [SD's]) after residual cells at the end of a 9d incubation had been stained with crystal violet.

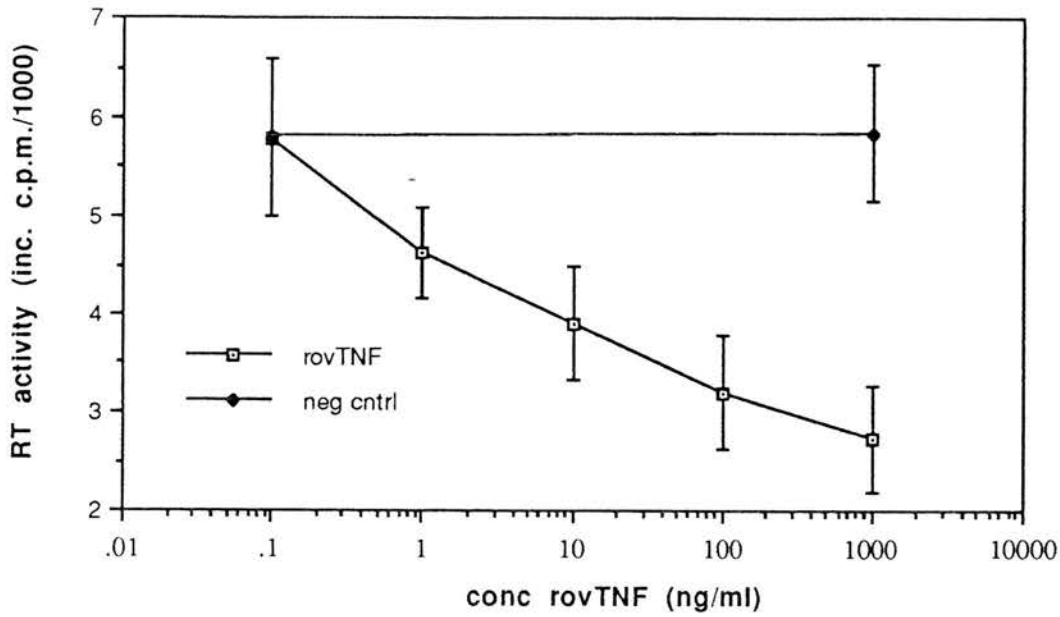
Table 5.2:-

EV-1	Conc^{n} rovTNFα (ng/ml)	Mean RT activity (c.p.m.: SD's in brackets)		
		Exp^{t} 1	Exp^{t} 2	Exp^{t} 3
-	0	_ 625 (198)	_ 811 (171)	_ 532 (95)
-	500	_ 716 (233)	_ 754 (154)	_ 498 (127)
+	0	_10,822 (1240)	_ 8,892 (981)	_1,987 (482)
+	5	_ 9,830 (1117)	_ 8,079 (1173)	_2,676 (604)
+	50	_11,406 (1349)	_ 8,205 (892)	_3,278 (852)
+	500	_13,054 (1205)	_11,120 (903)	_3,287 (495)
	Conc^{n} neg. cntrl (eq.dil^{n}).			
+	500	_11,074 (1221)	_ 9,088 (1073)	_2,270 (488)

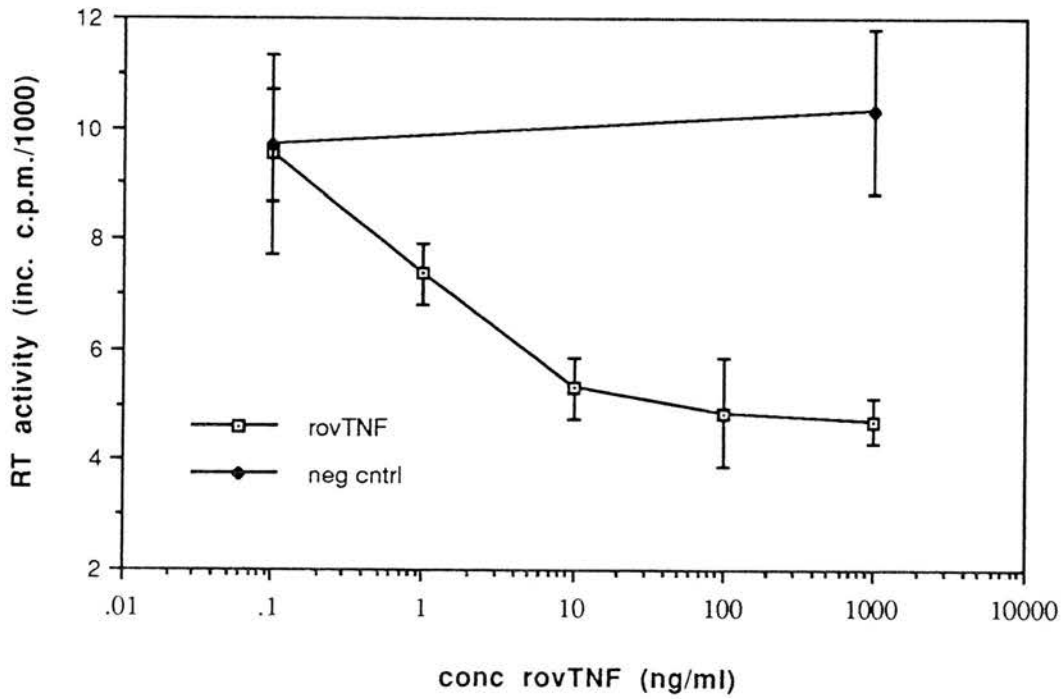
Table 5.2:- results of assays to determine the total accumulated RT activity in cultures of 3 different preparations of adherent, ovine lung-cells 13, 12 and 9 d post-infection with EV-1(experiments 1,2 & 3, respectively), following incubation with rovTNFα or a yeast extract/factor Xa negative control (whose concentration is expressed as an equivalent dilution to rovTNFα [ng/ml]). Results are expressed as mean c's.p.m. (& standard deviations) of sextuplicate wells. The RT activity associated with the initial viral inoculum (diluted to the final concentration assayed) was <300 c.p.m. in each experiment.

Figure 5.2:-

5.2a



5.2b



Figures 5.2a & b:- the effects on total, accumulated (at 7 & 10d post-infection, respectively) RT activity in cultures of EV-1-infected WSCP cells, following pre-infection treatment (& continued post-infection exposure) to rovTNFα or a yeast extract/factor Xa negative control preparation (plotted at equivalent dilutions; see symbol legends). Results are expressed as the mean c's.p.m. (with error bars indicating standard deviations) of sextuplicate wells.

Figure 5.3:-

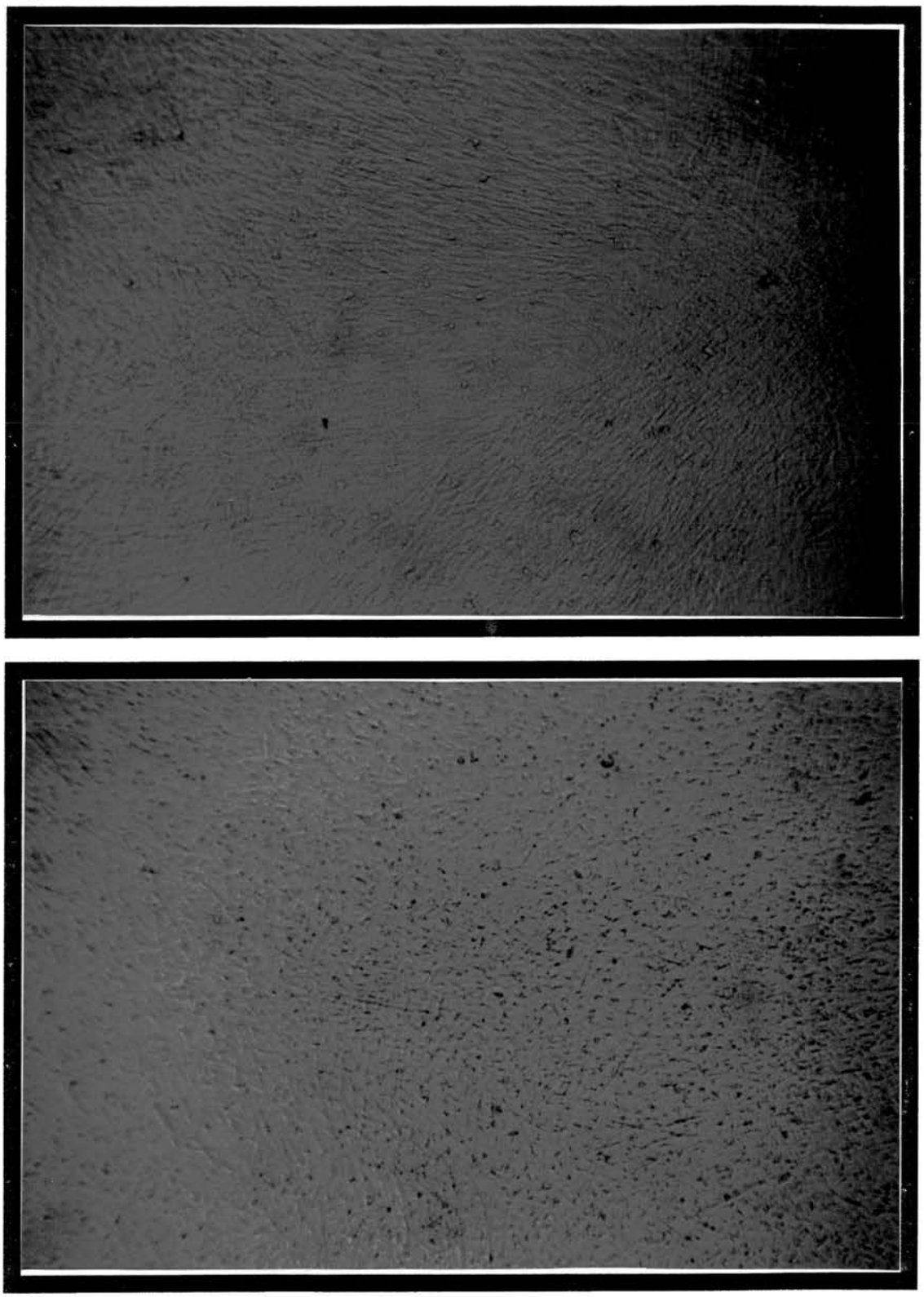


Figure 5.3:- representative fields of unstained, EV-1-infected, WSCP cells from the experiment represented in fig.5.2b, when examined by light microscopy prior to the measurement of RT activity. Cells had been incubated with 0 (above) or 1,000 (below) ng rov TNFα /ml.

5.2.2.RovTNFα does not have an antiviral effect on MVV-infected ovine lung-cells:-

3 similar experiments, using an moi of 0.1 $TCID_{50}$/cell, with rovTNFα or control samples being added at (experiments 1 & 2) or 24 hours after (experiment 3) the time of infection, were repeated on the adherent fraction of an ovine lung-cell preparation, which had been allowed to settle for several days after preparation. Wells were incubated with the same sample concentration throughout and remained free from bacterial, yeast or fungal infection (as judged by light microscopy). No statistically-significant difference in the eventual total viral yield (9-13 days later) was caused by incubation with rovTNFα, though a slight increase in yield was consistently caused by the highest concentration of rovTNFα (table 5.2). RovTNFα caused no increase in the low level of spontaneous RT activity seen in uninfected wells.

It was apparent, however, that within 24 hours of treatment, rovTNFα consistently caused both infected and uninfected cells to aggregate into large clusters which persisted for several days (fig.5.4). It was also noted that when giant cells, presumably syncitia, began to form in infected wells from 6 days PI, these were, on each occasion, larger and more numerous in wells treated with rovTNFα than in those subjected to control preparations (fig.5.5). No giant cells were noted in un-infected wells at any stage of culture.

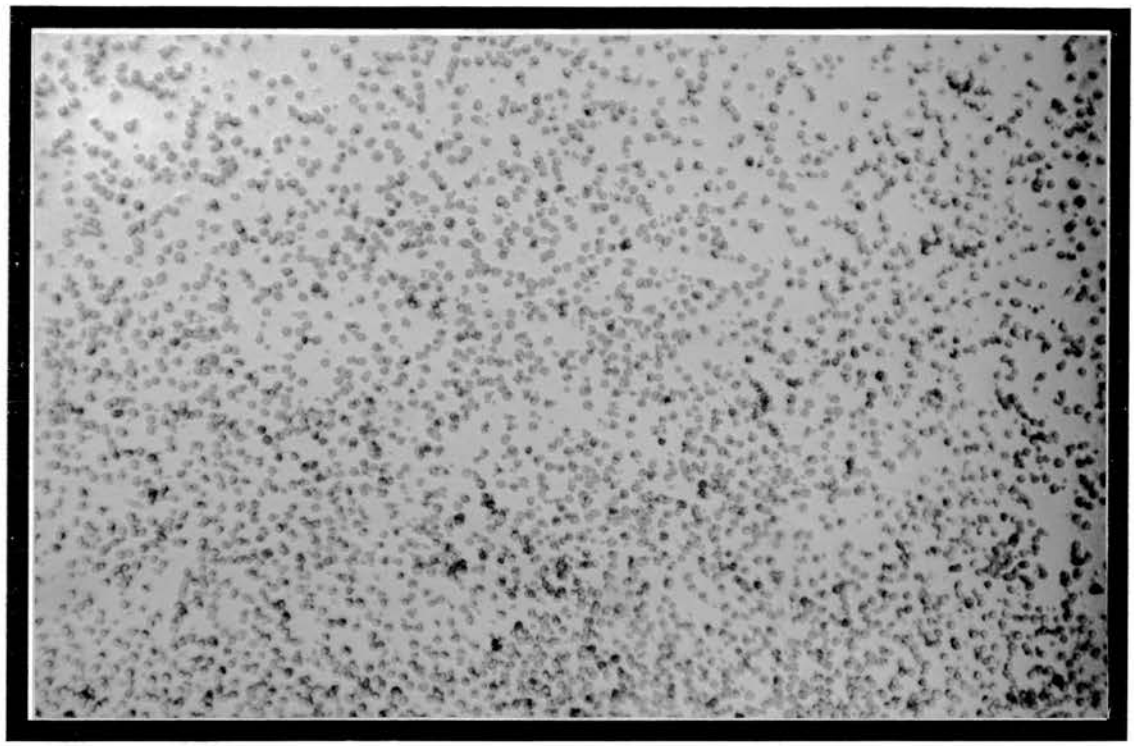
5.2.3.MVV induces ovine TNFα:-

When SN's from wells treated with PBS/BSA only in experiment 3 (table 5.2) were analysed by acetone precipitation and Western blotting, using **cyt1** as the primary antibody, ovine TNFα was found to have accumulated in the infected but not the uninfected wells (fig.5.6).

Similar cell preparations were infected with MVV at a higher moi (1 $TCID_{50}$/cell) for 2 hours, before the inoculum was removed and the cells refed. This treatment seemed to induce a change in morphology (mainly elongation) of a large percentage of the cells over the following 24 hours (fig.5.7). When the SN's, which were collected at assorted timepoints after refeeding were analysed as above, ovine TNFα could be seen to have accumulated in those wells which had been

Figure 5.4:-

5.4a



5.4b

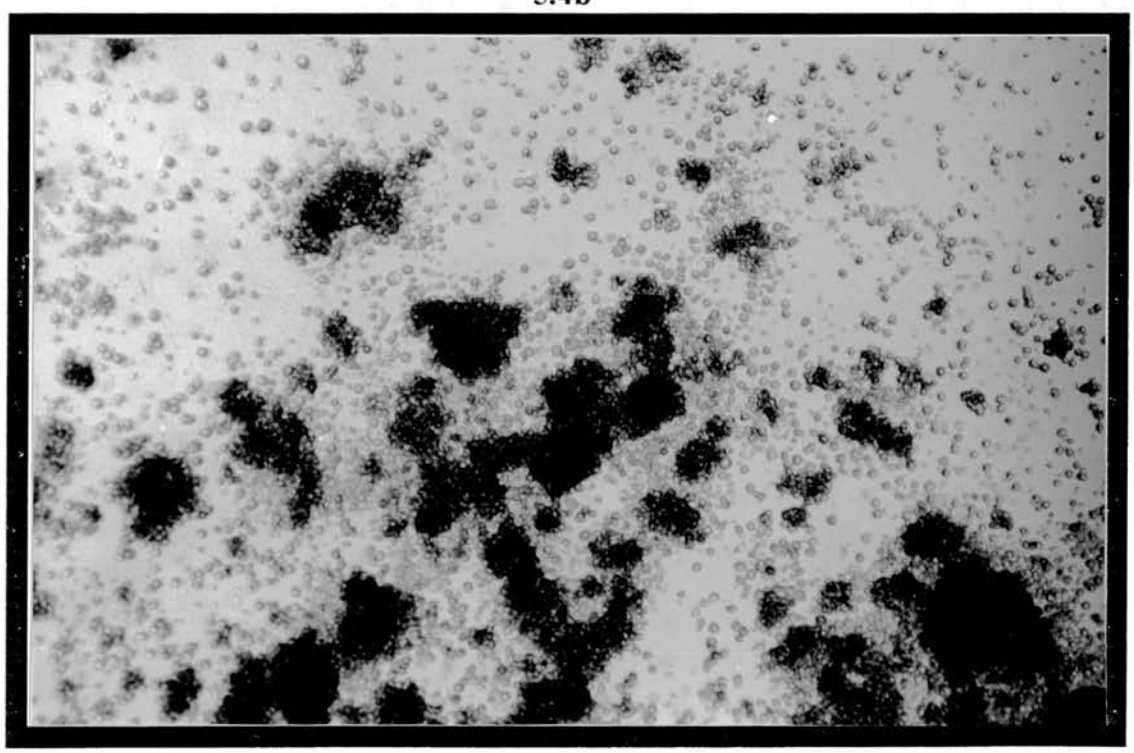
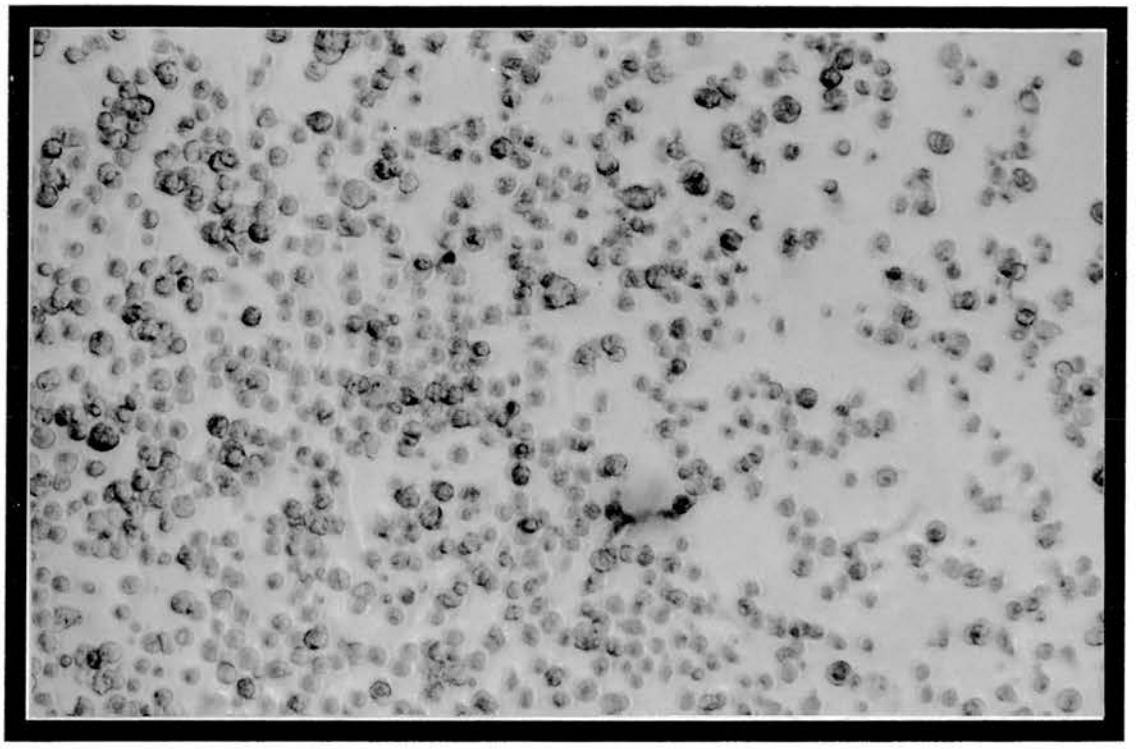


Figure 5.4a,b & (see over) c,d:- representative, light microscope fields at low (5.4a & b) and high (5.4c & d) powers of uninfected ovine lung-cells, 4 d after adding rovTNFα to 0 (5.4a & c) or 500 (5.4b & d) ng/ml.

5.4c



5.4d

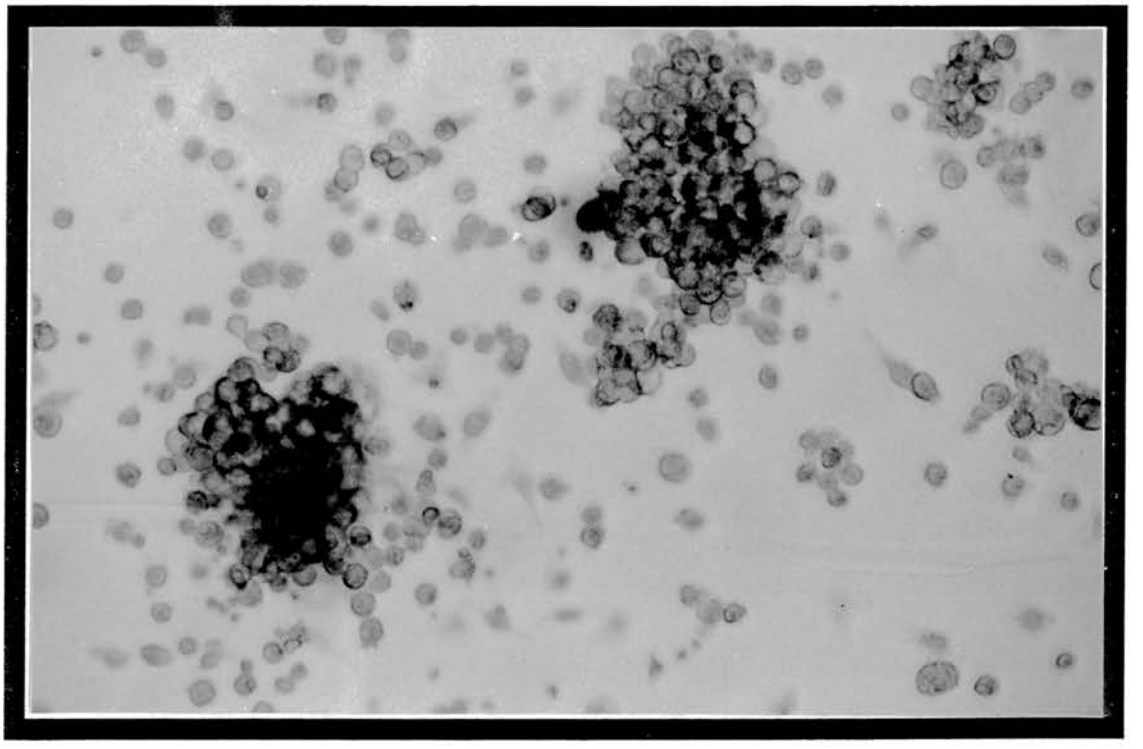


Figure 5.5:-

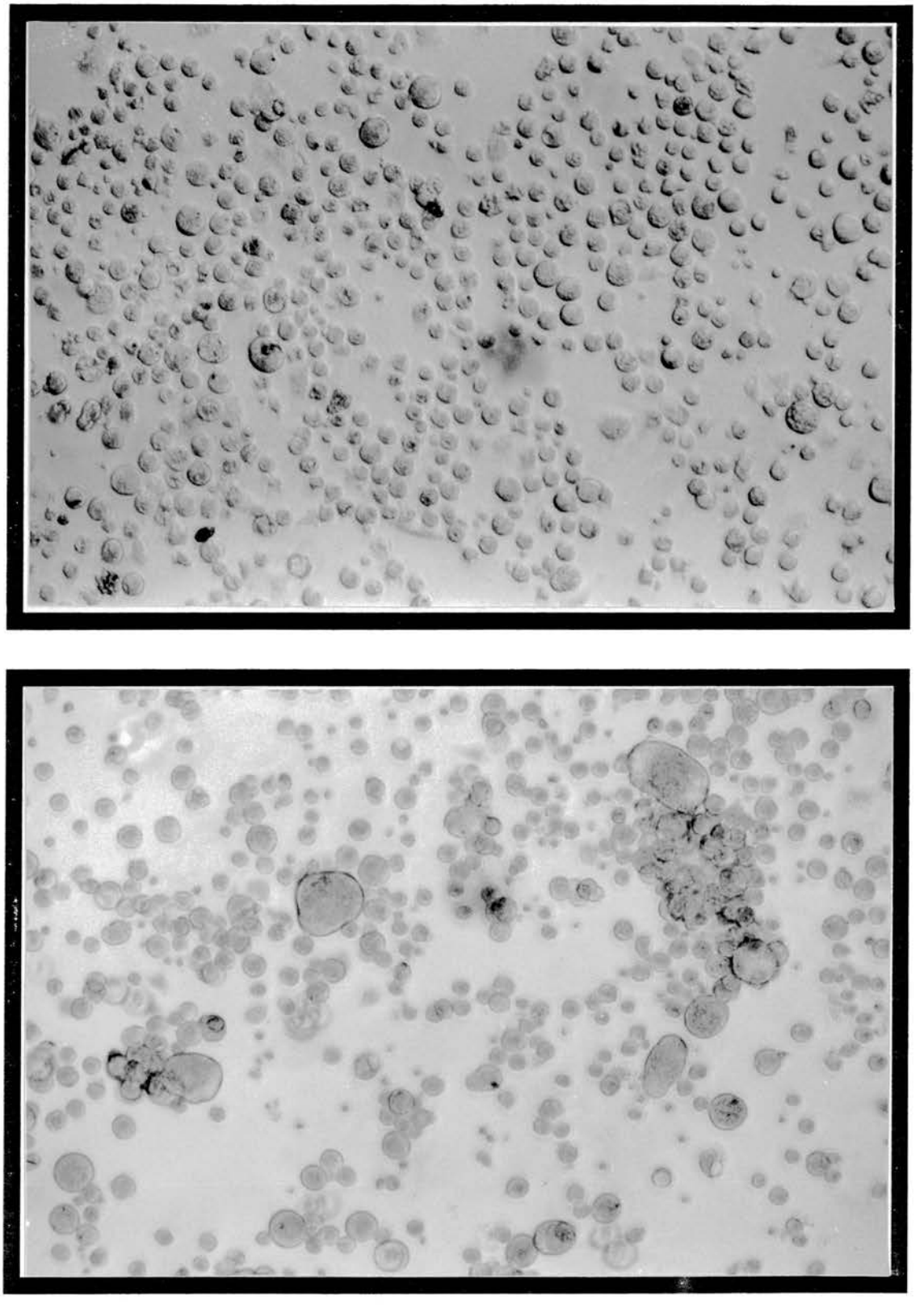


Figure 5.5:- representative, light-microscope fields of ovine lung-cells, 7 d post-infection with EV-1. Cells had been incubated with 0 (above) or 500 (below) ng rovTNFα / ml.

Figure 5.6:-

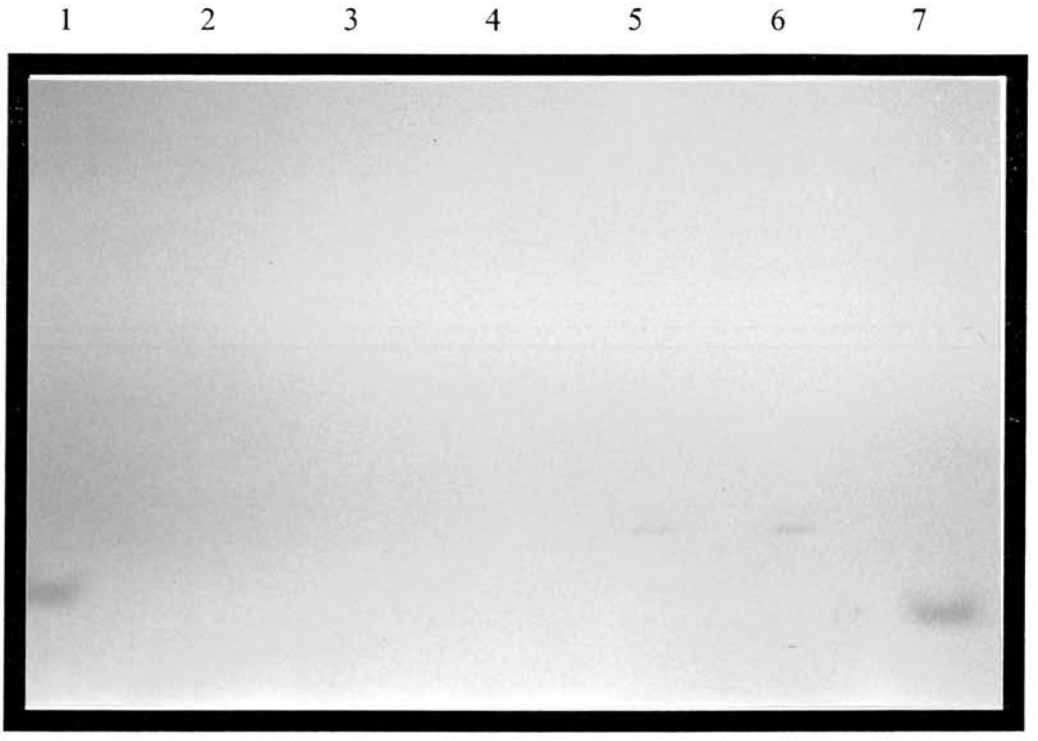


Figure 5.6:- a Western blot of an SDS- 5-20% gradient- polyacrylamide gel, run under non-reducing conditions. The primary detecting antibody was ***cyt1****, used at a 1:30 dilution of the saturated supernatant. Samples submitted to electrophoresis comprised 3ng rovTNFα (lanes 1 & 7) and acetone precipitates collected from 0.33 ml of medium (lane 2) or from 0.33ml of contents pooled from 6 uninfected (lanes 3 & 4) or 6 EV-1-infected (lanes 5 & 6) wells of cultured ovine lung-cells. Duplicate plates of the lung-cell preparation represented in table 5.2, experiment 3, were frozen 5 (lanes 3 & 5) or 9 (lanes 4 & 6) d post-infection, prior to collection of samples.*

Figure 5.7:-

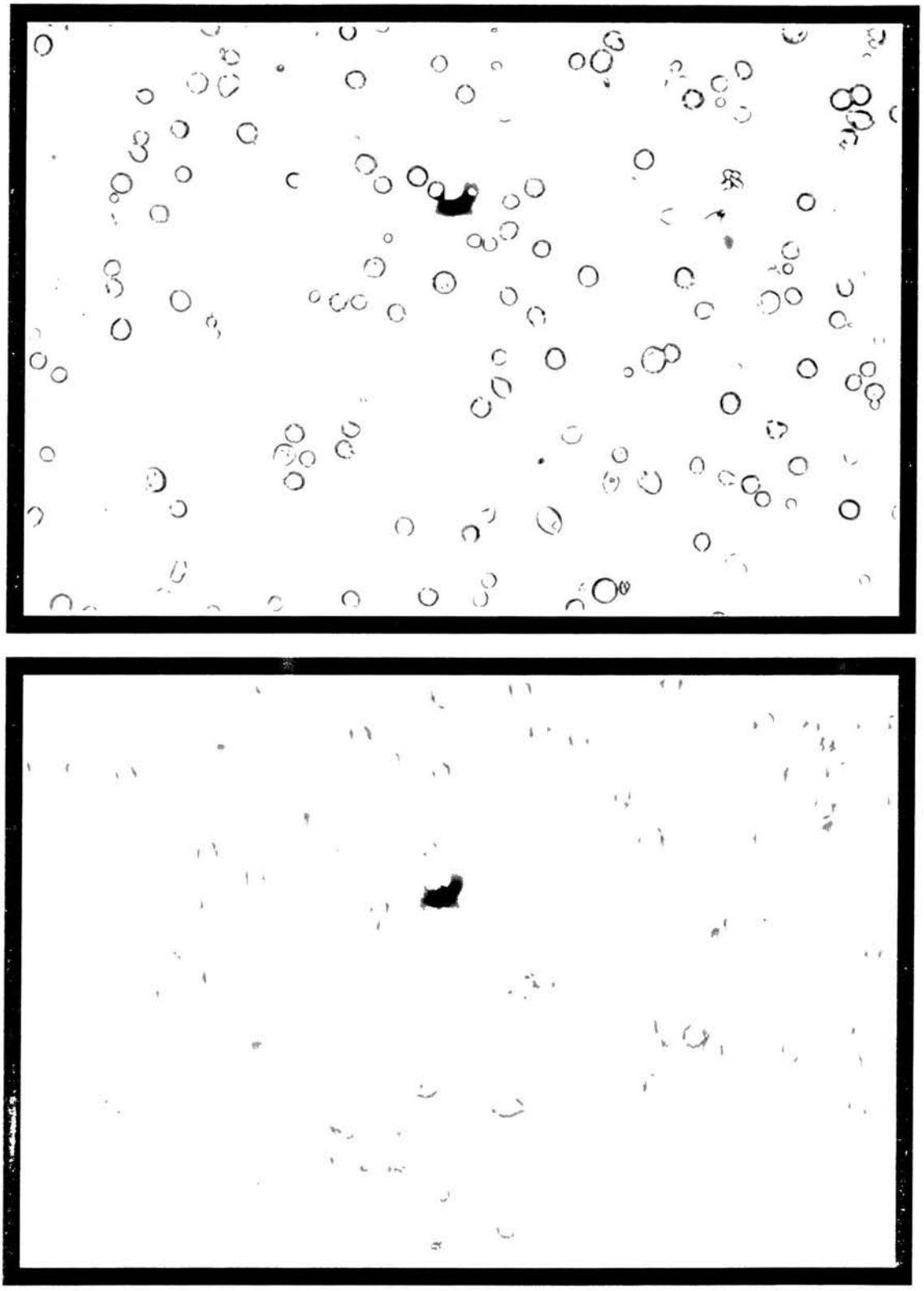


Figure 5.7:- representative light microscope fields of unstained, ovine lung-cells 24h after the addition of conditioned medium (above) or EV-1-containing medium (below).

infected with MVV, reaching a peak concentration between 2 and 24 hours. No ovine TNFα was detected in wells treated with conditioned medium (fig.5.8). When tested by a limulus amoebocyte lysate assay, the viral inoculum contained <50pg/ml of LPS (the detection limit of this assay). No ovine TNFα was detected when conditioned medium which had been deliberately doctored with 500pg/ml of LPS (derived from Salmonella abortus equi, a pathogenic source) was similarly used on these cells (fig.5.8).

5.3.Discussion:-

RovTNFα clearly had a negative net effect on the accumulation of RT activity in cultures of infected fibroblasts (figs.5.1,2), in spite of enhancing the density of potential host cells (table 5.1). Although different protocols and cell-types were involved, the concentrations of rovTNFα displaying anti-lentiviral activity in these assays are similar to the range of concentrations (c.10-1000ng/ml) previously described as having activity against HIV-1-infected cells (Wong et al., 1988, Matsuyama et al., 1989a). How the downregulation of viral expression was achieved in this case is unclear, however. Whilst more than one mechanism has been described for TNFα's antiviral actions (see 1.4.4,6), the appearance of apparently dead cells in rovTNFα-treated wells (fig.5.3) suggests that a proportion of the activity seen here might be mediated via the killing of infected cells. Alternatively, rovTNFα may have protected the majority of cells from infection, but enhanced viral replication in a small subpopulation, leading to their earlier death. Cytotoxicity, cellular protection and enhancement of viral replication by TNFα have all been observed in HIV infections of lymphocytes (see 1.8.5) and therefore one or more actions could operate here, though further work is clearly required to determine which mechanisms are actually involved. Subsequent studies have failed to detect any IFN in rovTNFα-treated, MVV-infected WSCP cell extracts or SN's, using a Vesicular Stomatitis virus growth retardation assay (Dr.G.Entrican, personal communication), nor do these cells seem to die by apoptosis, as judged by microscopic appearance and the absence of 'nucleosomal ladders' (Dr.D.Sargan, personal communication).

Figure 5.8:-

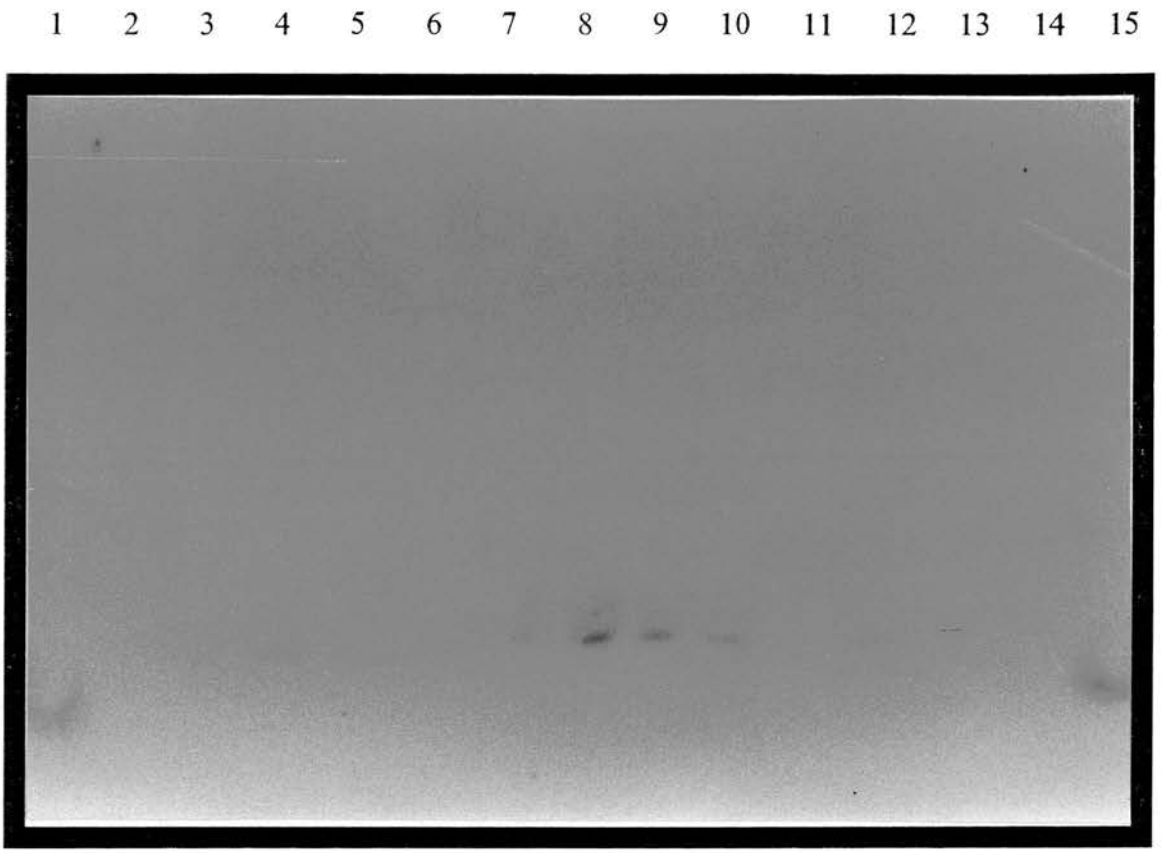


Figure 5.8:- A Western blot of an SDS- 5-20% gradient-polyacrylamide gel run under non-reducing conditions. The primary detecting antibody was ***cyt1****, used as a 1:30 dilution of the saturated supernatant. Samples submitted to electrophoresis comprised 3ng rovTNFα (lanes 1 & 15) or the acetone precipitates collected from 0.33ml of pooled supernatant removed from triplicate wells exposed to conditioned medium (lanes 2-6), EV-1-containing medium (lanes 7-10) or conditioned medium contaminated with 500pg LPS/ml (lanes 11-14). These supernatants had been removed 0 (lane 2), 2 (lanes 3, 7 & 11), 5 (lanes 4, 8 & 12), 24 (lanes 5, 9 & 13) or 48 (lanes 6, 10 & 14) h after a 2h exposure to the above media.*

Analysis of the net effect of rovTNFα on a population of infected lung cells, rich in macrophages, was complicated by several factors, including the induction of endogenous ovine TNFα (fig.5.6). This might account for the fact that any changes noticed were slight and only seen at the highest concentration of rovTNFα added. Though not statistically-significant in individual experiments, an enhancement of the final accumulation of RT activity was consistently seen at this concentration in each of three preparations. Notwithstanding the fact that blocking studies are required to ascertain the part played by endogenous TNFα, one might conclude, therefore, that the net effect of rovTNFα on this population was not antiviral but possibly even the opposite. Cell-type restriction of TNFα's antiviral activity is certainly a feature in other species and infections (Schijns et al., 1991) and, furthermore, Ohmann et al. (1989a) found that rboTNFα was unable to induce 2'-5' oligoadenylate synthetase in bovine alveolar macrophages and leukocytes, in contrast to its effects in non-lymphoid cells.

TNFα is known to enhance the replication of HIV via the induction of NF-kappaB proteins binding to sites in the viral LTR (see 1.8.5). Strains of MVV so far sequenced, including EV-1, do not contain any NF-kappaB sites in their LTR's (Sonigo et al., 1985; Braun et al., 1987; Quérat et al., 1990; Sargan et al., 1991a; Staskus et al., 1991). However, they do contain more than one site for another TNFα-inducible transcriptional factor, AP-1, and these sites appear to be important in controlling basal MVV transcriptional activity (Hess et al., 1989). Furthermore, transcriptional activation of another retrovirus, Human Foamy Virus, also appears to be modulated via AP-1 sites in its LTR (Maurer et al., 1991) and the binding of AP-1 to an intragenic enhancer in the HIV provirus has been linked to upregulation of lentiviral transcription (van Lint et al., 1991). 'Activation' of ovine macrophages by phorbol esters can certainly increase transcription directed by the MVV LTR (Small et al., 1989) and an AP-1 site appears to be essential for such inducibility (Gabuzda et al., 1989). Therefore enhancement of MVV replication in macrophages by ovine TNFα remains a possibility. In agreement with

this, Ellis et al. (1991) claim to have increased the replication of another strain of MVV in ovine alveolar macrophages, using rboTNFα, though they give no details.

RovTNFα may have influenced viral replication by other mechanisms, however. Although TNFα can upregulate the expression of several cell adhesion molecules (see 1.4.1), the induction of macrophage aggregation by rovTNFα was an unexpected finding, as this does not appear to be a generally-recognised phenomenon in other species. It has been observed for one human promonocytic cell line however (Kharbanda et al., 1990), whilst macrophage aggregation in response to lymphocyte-derived factors has been well documented (Lolekha et al., 1970; Garcia-Moreno & Myrvik, 1977). Whether the aggregation seen here represents a species-specific response to TNFα by ovine cells, was influenced by contaminating cell-types, or was perhaps due to some combined activity of rovTNFα and impurities such as factor Xa, remains to be seen. However, such aggregation could certainly have helped increase viral replication by aiding the inter-cellular spread of virus.

Several workers have observed that rhTNFα can enhance the CPE (in the form of syncitial size and frequency) seen in HIV-infected cell cultures (Matsuyama et al., 1989b; Vyakarnam et al., 1990). Though this might be attributable to the upregulation of viral replication, others have found that viral replication and CPE do not always correlate, suggesting the involvement of other factors (Pantaleo et al., 1991). Whilst similar enhancement of CPE was seen here (fig.5.5), and enhanced viral replication at the highest concentration of TNFα might have contributed, pre-existing cellular intimacy, as a result of aggregation, would seem to be a more likely cause of the phenomenon in this model. Furthermore, the resulting changes in cell numbers and sizes clearly add to the factors complicating the net effect of rovTNFα on viral replication.

Addition of viral samples to these cells appeared to activate them, as judged by their change in morphology (fig.5.7), and was associated with the production of ovine TNFα, following either the acute administration of large quantities of virus (fig.5.8) or infection with a smaller inoculum given time for further replication (fig.5.6). There did not appear to be any 'spontaneous' ovine TNFα production

from these alveolar macrophages, which had been cultured for several days, in contrast to the situation with freshly-isolated cells (see chapter 4). Considerable controversy over the ability of other lentiviruses to induce TNFα has clearly existed (see 1.8.5). Some have blamed observed inductions on LPS, or possibly components of the cells on which the virus was grown, contaminating viral inocula (Molina et al., 1990a), though Merrill et al. (1989) and Clouse et al. (1991) have been able to prevent induction using a soluble form of CD4, the HIV receptor. Since the addition to conditioned medium of more than ten times the amount of LPS known to contaminate the preparation of EV-1 failed to induce detectable ovine TNFα, it seems unlikely that a higher level of LPS in the viral sample than the conditioned medium negative control was to blame per se. In addition, the virus isolate used here was free of mycoplasmal contamination (D.Sargan, personal communication) and was grown on normal, not transformed, cells, further suggesting that the accumulation of ovine TNFα was a direct consequence of the presence of MVV. Ellis et al. (1991) have also looked for induction of TNFα by MVV. Though they failed to detect accumulation of TNFα in the SN's of MVV-infected ovine alveolar macrophages, their assay involved L929 cells and, almost certainly, therefore, a poor level of sensitivity (see chapter 4).

Given that the presence of MVV can lead to TNFα accumulation, the mechanism of induction becomes an important consideration in trying to ascertain whether sufficient TNFα might be produced in vivo to account for some of the pathology of MVD, since acute exposure to a large number of viral particles in vitro may bear little similarity to the situation in vivo where few virions are to be found (see 5.1). Merrill et al. (1989) proposed that the binding of gp120 to CD4 was responsible for the induction of TNFα by HIV. Though CD4 is not a receptor for MVV, this virus interacts with members of the same immunoglobulin superfamily to which CD4 belongs, MHC class II molecules (Dalziel et al., 1991), as well as other cell surface proteins (Crane et al., 1991a). The production of ovine TNFα within a few hours of exposure to MVV (fig.5.8) is certainly consistent with a receptor-mediated mechanism of induction. Since the structure of the envelope glycoprotein includes highly-variable sites in both HIV (Starcich et al., 1986) and MVV (Braun et al., 1987), one might also

speculate that differences in this molecule between viral strains could mediate differential abilities to induce TNFα, particularly since more than one epitope appears to be involved in contact with cell surface antigens (Crane et al., 1988). This might also help to account for the failure of some workers using different strains to observe the induction of TNFα by lentiviridae (see 1.8.5 and above).

Alternative and/or additional mechanisms leading to the induction of TNFα by MVV also exist, however. Infection with HIV seems to be associated with a TNFα-independent increased availability of some forms of NF-kappaB (Bachelerie et al., 1991; Neuvert et al., 1991; Roulston et al., 1992) and this may be related in part to an ability of viral protease to process a precursor of one of the subunits (Riviére et al., 1991). HIV can also upregulate the intracellular levels of cyclic nucleotides (Nokta & Pollard, 1991). Clearly, such effects could enhance TNFα gene transcription (see 1.3.2). More direct viral transactivation of the ovine TNFα gene might also occur. Tax, the viral transactivator of another retro-virus, HTLV-1, can transactivate the TNFβ gene, possibly via its single NF-kappaB site (Paul et al., 1990; Lindholm et al., 1992) and HIV-tat has also been shown to transactivate this gene in one cell line (Sastry et al., 1990). Furthermore, HIV-tat can also be secreted and influence cell functions in a paracrine fashion (Ensoli et al., 1990; Viscidi et al., 1989). MVV-tat displays some similarities to HIV-tat (Davis & Clements, 1989; Gourdou et al., 1989) though whether it can transactivate any cellular genes remains to be demonstrated. Extremely heightened sensitivity to low levels of contaminating LPS, as has been postulated for HIV (see 1.8.5), and/or induction by factors produced by MVV-infected fibroblasts when preparing the EV-1 stocks, remain two other possible causes of the accumulation of ovine TNFα seen in these studies. One must also be aware that macrophages may not be the only source of TNFα in MVD. Proliferating lymphocytes, which abound in this disease (see 5.1), can also produce TNFα (see 1.2). Once again, further studies, to elucidate the mechanisms involved and to ascertain whether ovine TNFα is actually chronically overproduced during MVD in vivo, are in order.

Given that ovine TNFα might be produced during MVD and the well-documented synergy (and/or occasionally antagonism) between TNFα and IFN's (see 1.7), interesting possible interactions with 'lentiferon' exist. Not only might the latter further enhance the production of TNFα in response to assorted stimuli, but the two could synergise in causing the observed upregulation of class II MHC expression in MVD lesions (Kennedy et al., 1985), as well as in their antiviral activity, including cytotoxicity, on some cell types.

Two intriguing aspects of infection with MVV have been the restricted nature of its replication in macrophages and an apparent failure to infect most fibroblasts in vivo, in spite of its ability to replicate productively in both cell types in vitro (see earlier). Whilst increasing cellular maturation may be one mechanism allowing productive replication in macrophages (Gendelman et al., 1986) and cell-culture adaptation could also be involved (Narayan et al., 1982), it seems likely that antiviral activities exerted by factors derived from other cell types could contribute to the in vivo restriction. A powerful antiviral combination of lentiferon and TNFα might therefore be one mechanism of removing infected fibroblasts and/or preventing the infection of this cell type in vivo. Ligand for Fas antigen has also been shown to kill HIV-infected cells (Kobayashi et al., 1990b) and clearly other factors could therefore also contribute. In this regard, TNFα is but one of several monokines whose activity is potentially dysregulated by MVV.

In contrast to the situation in fibroblasts, lentiferon and TNFα would seem to have antagonistic effects on viral replication in macrophages. Consequently, one might speculate that viral latency or progression could be influenced, at least in part, by the balance between lentiferon and TNFα or other activating factors. Removal from the restriction of lymphocyte-derived lentiferon and possibly some autocrine stimulation of viral transcription by ovine TNFα might then assist the conversion to productive viral replication in purified macrophages in vitro.

Notwithstanding the fact that further work is clearly required to more fully assess the part that ovine TNFα plays in the pathogenesis of MVD, these preliminary findings suggest that the rôle TNFα plays in this disease is, not surprisingly, likely to be complex, as in many

other diseases (see 1.8). Furthermore, given the induction of ovine TNFα by MVV and its anti- and probable pro-lentiviral activities, the rôle that this cytokine plays in MVD would seem to share similarities with the rôle that human TNFα plays in AIDS. Since TNFα has been considered one of the most influential factors in the pathogenesis of the latter disease (Matsuyama et al., 1991), this adds to the case for considering MVD as a model for some aspects of HIV infection.

CHAPTER 5, part ii:- CONCLUSIONS.

Whereas little was previously known about ovine TNFα, it is now possible to make several conclusions about this cytokine and the work described in this thesis.

In summary:-

1) Cloning and sequencing of amplified ovine cDNA demonstrates the existence in sheep of a mRNA with a high degree of sequence homology to the mRNA's encoding TNFα in other species.

2) As in other species this mRNA is rapidly and greatly inducible from a cell population rich in macrophages.

3) Comparison of this sequence with those of of other published TNFα cDNA sequences confirms the conservation of several structurally and functionally important regions and suggests the probability of allelic polymorphisms in other regions of the ovine molecule.

4) Amplification of cDNA molecules using inverse PCR may not be as 'straightforward' as using this technique on uniform length genomic segments of DNA.

5) An unglycosylated, recombinant TNFα has been translated from ovine cDNA, starting at a presumed (by analogy with mature human TNFα) first codon of the mature protein, via a yeast Ty VLP expression system.

6) This expression system works well for the production and simple partial purification of rovTNFα.

7) RovTNFα shows a wide range of biological activities similar to TNF'sα from other species. These include cytotoxicity, enhancement of thymocyte and fibroblast proliferation and cartilage-degrading and anti-viral activities.

8) RovTNFα also shows similar multimeric structure to other TNF'sα, with an ability to self-associate into trimers and/or dimers.

9) RovTNFα shows activity in assays on ovine cells at concentrations comparable to those described for other TNF'sα in similar syngeneic systems.

10) However, it is 1000-fold less active than rhTNFα in cytotoxicity assays on TNF-sensitive murine cells on a weight-for-weight basis.

11) 2 monoclonal antibodies have been raised which specifically recognise rovTNFα.

12) At least one of these, **cyt1**, specifically recognises a glycoprotein of appropriate size to be mature ovine TNFα in the supernatants of stimulated ovine cell cultures.

13) Since all such ovine supernatants failed to induce detectable cytotoxicity when tested in assays on L929 cells (sensitive to 30pg rhTNFα/ml) in spite of the fact that many contained >1ng ovine TNFα/ml and at least one of these contained biological activity attributable to ovine TNFα through its neutralisation by a polyclonal antiserum to rovTNFα, TNF-sensitive murine cells are insufficiently sensitive to ovine TNFα to be of use in its routine detection.

14) Such results suggest that there may be a higher degree of species specificity in the interaction of murine TNF-RI's with TNF'sα from some species than had previously been recognised.

15) As in other species ovine TNFα is inducible and its induction is enhanced by LPS.

16) TNFα can be induced from a population of adherent ovine lung cells (predominantly alveolar macrophages) <u>in vitro</u> by media containing MVV but not conditioned media.

17) RovTNFα has an antiviral activity on MVV-infected ovine fibroblasts but not on adherent lung cells. Indeed its effect on these cells may even be pro-viral.

18) Consequently, ovine TNFα may play a complex rôle in the pathology of MVD.

CHAPTER 6:- MATERIALS AND METHODS.

6.1. Materials and methods - general:-

6.1.1. General considerations:-

During the course of this work different methods or conditions have been used for similar procedures. If methods are incompletely defined in this chapter, or alternatives are described, precise conditions pertaining to individual experiments will be found in the appropriate results sections.

Some other, general features are worthy of note. Aseptic techniques are implied throughout for handling bacterial, yeast and established mammalian cell cultures, hence all media used for culture were autoclaved prior to use, as were, where possible, concentrated stock solutions of pure chemicals from which buffers or other complex solutions were generally derived. Where autoclaving was inappropriate, solutions were filter-sterilised. In deriving primary tissue cell cultures, 'clean' rather than aseptic techniques were used. Disposable sterile plasticware or baked/autoclaved containers were used throughout to limit contamination of samples.

Unless otherwise stated, all incubations of bacteria and yeast were carried out in air at 37°C and 30°C respectively, with those cultures involving liquid media being placed in shaking incubators. Mammalian cells were incubated at 37°C in air with 5% added CO_2. All other procedures where no temperature (T°) is given were performed at room T°. Manipulations involving virions were performed in class II safety cabinets. All other tissue culture manipulations and preparations for PCR's were performed in laminar flow hoods. L-glutamine was added, to 2mM, to all media used for mammalian cell culture and, when used, penicillin, streptomycin and fungizone were added to 100U, 100µg and 2.5µg/ml respectively.

In this chapter, water means distilled water and 'microfuging' of samples means spinning them at 13,000 rpm in an MSE 'microcentaur' centrifuge for the stated period. Unless details to the contrary, or G forces, are given, 'centrifugation' means spinning at the stated speed in a bench centrifuge (Beckman TJ6, MSE centaur 2 or Wifug 500E).

All dialyses were performed at 4°C over a minimum of 16h with at least 3 changes of the stated buffer. Each change of buffer was >100 x the sample volume. All dialysis tubing was prepared by boiling for 5min in each of two changes of 5% $NaHCO_3$, 0.1% NaEDTA.

Finally, all animal inoculations and blood sampling were actually performed by suitably-licensed colleagues.

6.1.2. Materials:-

Unless otherwise stated, the materials used in this work were from the following sources:- all radionucleotides and hybridisation membranes were supplied by Amersham (UK) Ltd. and all enzymes by Northumbria Biologicals Ltd. (NBL), whilst tissue culture media were by Gibco/BRL; all other 'general' consumables were purchased from Sigma. Reagents were of analytical or tissue culture grade as appropriate. RovIL-1ß and P1-P25 fusion proteins were kind gifts from C.Fiskerstrand and Dr.H.Reyburn, respectively. A polyclonal anti-P1 antiserum was supplied by Dr.S.Adams (British Biotechnology Ltd.). All 'control' monoclonal antibodies and normal murine splenocyte SN's as well as plasmids, 'phages, bacteria, yeast and a P1 preparation were from departmental stocks. Sources of primers, a human TNFα cDNA probe, rhTNFα and cell lines are described elsewhere (6.2.3,12, 6.3.9 & 6.4.4).

6.1.3. Formulae:-

The formulae of all buffers, media and other complex solutions referred to in the rest of this chapter are listed in alphabetical order below.

BamHI buffer:- 10mM Tris/HCl pH8.0, 100mM NaCl, 5mM $MgCl_2$, 1mM 2-ME.
Carbonate coating-buffer:- 7.63g/l Na_2CO_3, 4.6g/l $NaHCO_3$, pH9.6.
Circularisation buffer:- 50mM Tris/HClpH 7.4, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, 10µg/ml gelatin.
DNA denaturing solution:- 1.5M NaCl, 0.5M NaOH.
DNA loading buffer:- 0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll (w/v), 5xTAE (see below).

DNA pre-hybridisation mix:- 20mM $NaPO_4$ pH7.0 (see below), 3xSSC (see below), 0.5% milk powder (w/v), 0.1% SDS (w/v), 1mM EDTA, 100µg/ml yeast RNA.

Formate buffer:- 2.1ml formic acid, 2g Na formate, 5ml ethanol/l.

Freezing mix:- 90% v/v FCS, 10% v/v DMSO.

GTC:- 5M Guanidine thiocyanate, 25mM Na citrate, 0.5% Na laurylsarcosinate (w/v), 100mM 2-ME, 1mM EDTA.

GTE:- 50mM glucose, 25mM Tris/HCl pH8.0, 10mM EDTA.

High-salt digestion buffer:- 10mM Tris/HCl pH7.5, 100mM NaCl, 7mM $MgCl_2$, 7mM 2-ME.

High-salt elution buffer:- 580g NH_4OAc/l, 0.01% bromophenol blue (w/v).

Kinase buffer:- 10mM Tris/HCl pH7.5, 7mM $MgCl_2$, 0.1mM EDTA, 5mM DTT, 10µM ATP.

5M KOAc pH4.8:- 60mls 5M KOAc, 11.5mls glacial acetic acid /100ml.

LB:- 50mM Tris/HCl pH7.5, 10mM $MgCl_2$, 5% PEG 6000 (w/v), 1mM ATP, 1mM DTT.

Ligase buffer:- 50mM Tris/HCl pH7.5, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 100µg/ml BSA.

Low-salt digestion buffer:- 10mM Tris/HCl pH7.5, 60mM NaCl, 7mM $MgCl_2$, 7mM 2-ME.

Low-salt running buffer:- 200mM Tris/HCl pH8.0, 2mM EDTA, 50mM NaCl.

20mM $NaPO_4$ pH7.0:-1.7g Na_2HPO_4, 1.24g $NaH_2PO_4.2H_2O$/l.

Neutralising solution:- 2M NaCl, 1M Tris/HCl pH7.0.

Ohara buffer:-10mM Tris/HCl pH8.8, 1.5mM $MgCl_2$, 3mM DTT, 50mM KCl.

OLB:- a 100:250:150 mix of solutions A,B & C, where:- solution A = 1000 parts 1.25M Tris/HCl pH8.0, 0.125M $MgCl_2$ to 18 parts 14.33M 2-ME, 5 parts 0.1M dATP, 5 parts 0.1M dTTP, and 5 parts 0.1M dGTP (each dNTP being dissolved in 3mM Tris/HCl pH7.0, 0.2mM EDTA); solution B = 2M Hepes pH6.6, 4M NaOH; and solution C = random hexadeoxyribo-nucleotides at 90 OD_{260} U/ml in 10mM Tris/HCl pH7.5, 1mM EDTA.

PBS:- 170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4 pH7.2.

Peroxide substrate solution:- 0.04% w/v OPD, 0.008% v/v H_2O_2 in a buffer made by adding 0.1M citric acid to 0.2M Na_2HPO_4 until pH=5.0.

Pretreatment buffer:-0.1M 2-ME, 20mM EDTA, 200mM Tris/HCl pH8.0.

Protein loading buffer:- 100mM Tris/HCl pH6.8, 30% glycerol v/v, 1% SDS w/v, 2.9M 2-ME and 0.01% w/v bromophenol blue. For non-reducing conditions, the 2-ME was omitted.

Protein running buffer:- 25mM Tris base, 50mM EDTA, 0.1% w/v SDS, 0.1% w/v glycine.

Regeneration agar:- 3% w/v bacto-agar, 0.67% w/v yeast nitrogen base without amino acids (Difco), 1% w/v glucose, in 1M sorbitol.

RNA denaturing buffer:- 60% deionised formamide (v/v), 7.2% formaldehyde (w/v), 20mM $NaPO_4$ pH7.0 (see above), 0.1mM EDTA.

RNA loading buffer:- 30% ficoll, 0.5% bromophenol blue (w/v), 250µg/ml EtBr, 20mM $NaPO_4$ pH7.0 (see above).

RNA prehybridisation mix:- 5xSSC (see below), 50% formamide (v/v), 0.5% dried milk powder (w/v), 0.5% SDS (w/v), 100µg/ml yeast RNA.

RNA resuspension buffer:- 0.3 M NaOAc pH5.2, 10mM EDTA, 0.1% SDS(w/v).

$RPMI_{10}ME$:- RPMI with 10% v/v FCS, 10^{-5}M 2-ME, penicillin and streptomycin.

$RPMI_{15}$:- RPMI with 15% v/v FCS, 1mM Na pyruvate.

$RPMI_{15}HAT$:- RPMI with 15% v/v FCS, 0.1mM hypoxanthine, 0.4µM aminopterin, 16µM thymidine, 1mM Na pyruvate.

$RPMI_{15}HT$:- RPMI with 15% v/v FCS, 0.1mM hypoxanthine, 16µM thymidine, 1mM Na pyruvate.

$RPMI_{20}$:- RPMI with 20% v/v FCS.

$RPMI_{20}HAT$:- RPMI with 20% v/v FCS, 0.1mM hypoxanthine, 0.4µM aminopterin, 16µM thymidine, 1mM Na pyruvate.

2.5x RT reaction mix:- 375mM KCl, 25mM $MgCl_2$, 25mM Tris HCl pH8.0, 5mM DTT, 1.25mM EGTA, 0.25% w/v Triton X-100, 62.5µg/ml BSA, 75 µg/ml poly (rA).oligo(dT 12-18), 5% v/v deionised ethane diol and 100µCi/ml ^{3}H-TTP (20 Ci/mMol).

Sc-glc agar:- a filtered, 1% w/v solution of L-tryptophan added, to 2ml/l, to an autoclaved preparation of 2% w/v bacto-agar, 0.67% w/v yeast nitrogen base without amino acids (Difco), 1% w/v glucose.

Sc-glc medium:- as Sc-glc agar (above) but without agar.

Sc-glc/gal medium:- 0.67% w/v yeast nitrogen base without amino acids (Difco), 0.3% w/v glucose, 1% w/v galactose with L-tryptophan added as for Sc-glc above.

SSC:- 0.15M NaCl, 0.015M Na Citrate, pH7.0.

STC:- 1M sorbitol, 10 mM Tris/HCl pH7.5, 10mM $CaCl_2$.

TAE:- 40mM Tris/OAc pH7.8, 1mM EDTA.

TBE:- 89mM Tris/HCl pH8.0, 8.9mM boric acid, 2mM EDTA.

TC buffer:- 100mM Tris/HCl pH7.4, 10mM $CaCl_2$.

TCM buffer:- 10mM Tris/HCl pH7.5, 10mM $MgCl_2$, 10mM$CaCl_2$.

TE 10:1:- 10mM Tris/HCl pH8.0, 1mM EDTA.

TEN buffer:- 10mM Tris/HCl pH7.4, 2mM EDTA, 140 mM NaCl.

TE RNAse A:- 10mM Tris/HCl pH7.5, 1mM EDTA , 50µg/ml RNAse A.

TNE 20:20:1:- 20mM Tris/HCl pH7.5, 20mM NaCl, 1mM EDTA.

TNE 10:100:1:- 10mM Tris/HCl pH8.0, 100mM NaCl, 1mM EDTA.

2xYT broth:- 10g NaCl, 10g yeast extract and 16g bactotryptone/l.

6.2. Manipulation and analysis of nucleic acids; culture of bacteria:-

6.2.1. Purification of nucleic acids by phenol/chloroform extraction and ethanol precipitation:-

Nucleic acids (NA) in complex solutions were purified by phenol/ chloroform extraction and ethanol precipitation. Phenol/chloroform extraction involved successive additions to an aqueous, NA-containing solution of an equal volume of:- phenol; a phenol/ chloroform/ iso-amyl alcohol mix (25:24:1 v/v/v); and finally, a chloroform/ iso-amyl alcohol mix (24:1 v/v). (Here, phenol means phenol with 0.1% hydroxy-quinoline and equilibrated with an appropriate buffer:- 0.3M NaOAc pH5.2 for purification of RNA or 0.1M Tris/HCl pH8.0 for DNA.) After each addition, samples were vortexed to form an emulsion before separation of the aqueous and organic phases by briefly microfuging. The aqueous phase was then removed and transferred to a fresh tube ready for the next addition. When purifying DNA, NaOAc was added to the aqueous phase (to 0.3M) after the final separation. NA's were then precipitated by adding 2.5 volumes of absolute ethanol and storing the tubes in dry ice for a minimum of 30min. Precipitates were collected by microfuging for 15min at 4°C then washed in 70% v/v ethanol and recentrifuged as above. SN's were discarded and the final pellet desiccated before resuspension in water or an appropriate buffer.

6.2.2. Collection and purification of RNA from cell cultures:-

Total RNA was prepared by the method of Sargan et al. (1986). Cell culture SN's were decanted and clarified by centrifuging for 10min at 2500 rpm and 4°C, whilst the adherent cells were dissolved in 5ml/ flask of GTC buffer (6.1.3). The cell pellet formed from any non-adherent cells was dissolved in the GTC buffer used to dissolve cells from the corresponding flask. Thus, RNA was always collected from the total starting cell population.

These 'GTC' solutions were then diluted with GTC buffer (to an appropriate volume for the polyallomer ultracentrifuge tubes subsequently used) and layered over 1.6ml of a solution containing 5.7M CsCl (previously baked at 180°C for 2h), 25mM NaOAc pH5.2, 2mM EDTA before being centrifuged for 16h at 76,000 x g and 20°C. Resulting pellets were resuspended in 300µl of ice-cold RNA re-suspension buffer (6.1.3). The RNA in these solutions was then further purified (6.2.1) with particular attention being paid to keeping them ice-cold throughout. RNA pellets were finally resuspended in 30µl of water.

The concentrations of RNA obtained were estimated by measuring the OD_{260} of aliquots diluted 1:500 with water and applying the equation:- conc^n of RNA = OD x 500 x 40 µg/ml. OD's$_{280}$ were also measured. Any solutions with a ratio of OD_{260} : OD_{280} of <1.8 were considered insufficiently pure and submitted to a further round of purification (6.2.1), resuspension and OD measurement.

6.2.3. Oligonucleotide primers:-

All oligodeoxyribonucleotide primers, with the exception of poly-dT primer (Amersham) and reverse sequencing primer (Pharmacia), were synthesised by the Oswel DNA service, University of Edinburgh.

6.2.4. Synthesis of cDNA:-

Ss and ds cDNA's were synthesised by the method of Gubler and Hoffman (1983), from total RNA (6.2.2), using a commercial kit (cDNA Synthesis System Plus, Amersham). Oligo dT-primed 1st strand cDNA synthesis was generally performed by incubating 12µg of RNA with 48U of reverse transcriptase and 500µM each dNTP for 1.5h at 42°C, in a total volume of 70µl with other reagents as recommended by the

manufacturer. To monitor the efficiency of 1st strand synthesis, 10µCi of ^{35}S dCTP (>600Ci/mmol, 10µCi/µl) were incubated with a separate 20µl aliquot of the above mix. In the synthesis of ss cDNA, the above reactions were stopped by adding EDTA to 10mM.

To synthesise ds cDNA, 1st strand reactions were diluted 5-fold, as recommended, with the addition of 0.8U of E.coli ribonuclease H and 23U of E.coli DNA polymerase I / 100µl of diluted mix. To monitor the efficiency of 2nd strand synthesis, 10µCi ^{35}S dCTP (as above) were added to a separate 20% aliquot of previously unlabelled 1st strand reaction, before successive incubations of the tubes at 12°C for 60min, then 22°C for 60min and lastly 70°C for 10min. After cooling, 1U of T4 DNA polymerase was added to each 50µl of reaction mix and incubation was continued at 37°C for a further 10min. Reactions were finally stopped by the addition of EDTA to 10mM.

To analyse the efficiency of synthesis of either strand, paired aliquots of a labelled reaction mix, after completion of the appropriate incubation, were spotted onto each of 2 Whatman glass microfibre filter discs. 1 disc from a pair was then flushed through thoroughly with 5% TCA (w/v) before both were dried and counted in a proprietary scintillant (Optisafe, LKB) using a 'rack' β-scintillation counter. The mass of cDNA synthesised was estimated from the percentage of dCTP incorporated into acid-precipitable DNA as determined above, assuming equal usage of the four nucleotides.

cDNA from stopped reactions was then purified (6.2.1). The sizes of cDNA synthesised were analysed by agarose gel electrophoresis (6.2.7) of an aliquot of purified DNA from a labelled reaction followed by autoradiography of the gel (6.2.14).

6.2.5. Circularisation of cDNA:-

Ds cDNA was circularised by the method of Ochman et al. (1988). cDNA was diluted to 0.1µg/ml in circularisation buffer (6.1.3). T4 DNA ligase was added to 1 Weiss unit /60µl and reaction mixes incubated for 16h at 12°C. After stopping reactions by a 10min incubation at 70°C, the DNA was repurified (6.2.1).

6.2.6. Polymerase chain reactions:-

PCR's were performed on an automated thermocycler, in reaction mix volumes of 100µl, overlaid with paraffin oil. Buffer conditions were those used by Ohara et al. (1989) (6.1.3). 170µg/ml BSA, 1U Taq polymerase and 100µM of each dNTP were also included in every reaction. Unless otherwise stated, indicated primers were used at 0.1µM on the DNA templates described.

Typically, PCR's involved 35 cycles of denaturation at 95°C for 0.5min, 0.5min at an indicated, lower, annealing T°, and 1.5min at an extension T° of 70°C with a final 7min extension at 70°C. DNA produced by a PCR was examined by analysing (6.2.7,9) 10% of the mix.

6.2.7. Agarose gel electrophoresis of DNA:-

DNA samples were analysed by electrophoresis through 1-2% (w/v) agarose gels containing 1xTAE (6.1.3) and 0.5µg/ml EtBr, followed by UV transillumination (6.2.9). Samples were mixed with 0.25 volumes of DNA loading buffer (6.1.3) before loading in individual wells cast in horizontal slab gels. Running buffer was 1xTAE and an electrophoretic gradient of 5 V/cm was generally used. Some gels used low gel T° (LGT) agarose and in these cases electrophoresis was performed at reduced voltage (3 V/cm) at 4°C. Samples undergoing 'preparative' electrophoresis were also submitted to the same reduced voltage. Electrophoresis was allowed to proceed until the leading dye front had migrated a suitable distance (3-10cm).

6.2.8. Agarose gel electrophoresis of RNA :-

RNA samples were analysed by electrophoresis on 25cm-long, horizontal, slab, 1.2% (w/v) agarose gels containing 7.2% formaldehyde and 20mM $NaPO_4$ pH7.0 (6.1.3), using a circulating running buffer of 20mM $NaPO_4$ pH7.0, followed by UV transillumination (6.2.9). Before electrophoresis, 1 volume of RNA sample was incubated at 52°C for 15min with 3 volumes of RNA denaturing buffer (6.1.3), then mixed with 1 volume of RNA loading buffer (6.1.3) before samples were submitted to electrophoresis at 2 V/cm for 16h.

6.2.9. Visualisation of nucleic acids in gels. Estimating size and mass:-

NA bands in agarose gels were identified by their fluorescence on transillumination at 302nm. Estimates of molecular size and mass of DNA bands were made by comparing their mobility, and intensity of fluorescence, with those of known standards (1µg EcoRI, HindIII-cut lambda phage DNA [NBL]) run in parallel. All transilluminated gels were photographed alongside rulers in order to identify band positions on blots and autoradiographs.

6.2.10. Transfer of nucleic acids from gels to nitrocellulose membranes:-

NA's contained within agarose gels were transferred to, and immobilised on, nitrocellulose membranes (Hybond-C) using techniques based on the methods of Southern (1975) for DNA ('Southern blots') and Thomas (1980) for RNA ('Northern blots').

After electrophoresis and visualisation of NA's, agarose gels were suitably trimmed. Before transfer of NA's to membranes, DNA-containing gels were bathed in DNA denaturing solution (6.1.3) for 15min then soaked in 3 changes of neutralising solution (6.1.3) over 1h, whilst RNA gels were simply soaked in 10xSSC (6.1.3) for 10min to wash out formalin.

Treated gels were laid on a wick of Whatmann 3mm filter paper (connected to a reservoir of 10xSSC) and overlaid with a nitrocellulose membrane, prewetted in 10xSSC. Transfer of NA's to the membrane was effected by a continual upward migration of buffer for 16h, induced by overlaying the membrane with 2 sheets of 3mm paper and a weighted wad of paper towels. After then air-drying the membranes, NA's were cross-linked to them by baking for 2h at 80°C under vacuum.

6.2.11. Radio-labelling of DNA:-

The technique of random prime labelling (Feinberg & Vogelstein, 1983, 1984) was used to label DNA to a high specific activity for use as probes. 50ng of appropriate DNA was added to 6µl of OLB (6.1.3) and diluted with water to a total volume of 25µl, then boiled for 3min and allowed to cool. After adding 45µCi of ^{32}P dCTP (>400Ci/mmol, 10µCi/µl) and 2U Klenow fragment of DNA polymerase, reaction mixes

were incubated for 3h at 37°C. Reactions were stopped by adding 170µl of 1M Tris/HCl pH7.4, 10mM EDTA. The percentage of radioactivity incorporated into acid precipitable DNA was measured using paired aliquots of stopped reaction mixes and glass microfibre filter discs in an identical manner to the assessment of the efficiency of cDNA synthesis (6.2.4). This allowed calculation of the degree to which the probe had been labelled. All probes used were labelled to > 4 x 10^7 counts/minute/µg.

Labelled DNA was stored at -20°C until use, which was always within two weeks of its preparation.

6.2.12. A cDNA Probe for Human TNFα :-

A cloned fragment of the cDNA for human TNFα, corresponding to positions 402-854 (numbering according to Pennica et al., 1984) was a gift from Dr.K. Murray.

6.2.13. Hybridisation of labelled probes to immobilised nucleic acids:-

After transfer and cross-linking of NA's from gels to nitrocellulose (6.2.10) or from bacterial colonies to nylon (6.2.19), membranes were prewetted in 5xSSC (6.1.3) before being incubated, with shaking, in a DNA or RNA pre-hybridisation mix (6.1.3) as appropriate (typically 40ml/membrane). Incubations were performed at 52°C for hybridisation of DNA probes to RNA, or at 65°C for DNA-DNA hybridisations.

After 4-20h, prehybridisation mixes were removed and replaced with similar fresh mixes (typically 10ml/membrane) to which a freshly-boiled (for 2min) and quickly-cooled DNA probe had been added (at $2x10^6$ incorporated counts/membrane). Incubations were continued for a further 4-16h.

Membranes were then washed. 3 successive initial washes in 2xSSC, 0.1% SDS (w/v), each for 15min, were followed by 2 further 15min washes, also containing 0.1%SDS (w/v) with other conditions defined by 'final stringency' parameters in the appropriate results section. After the final wash, blots were air dried before being submitted to autoradiography (6.2.14).

6.2.14. Autoradiography of blots and sequencing gels:-

Hybridised membranes (6.2.13) were autoradiographed by exposure to X ray film (XAR or Xomat S, Kodak) at -70°C within light-proof cassettes fitted with intensifying screens. Dried sequencing gels (6.2.23) or cling film-wrapped agarose gels were similarly radiographed, though here no intensifying screens and room temperatures were used.

Initial exposure times varied from 1h to 1 week. For sequencing gels, initial exposures were generally made overnight. Lengths of subsequent exposures, where required, were based on the appearance of the initial radiograph.

6.2.15. Preparation of PCR product for:- ligation; labelling as a probe; or direct sequencing:-

After verifying the synthesis by a PCR of an appropriate product ([6.2.7,9] +/- [6.2.10,13,14]), DNA contained within the remainder of a PCR mix was purified (6.2.1).

PCR product destined for 'blunt-ended' ligation was diluted to <1nM in kinase buffer (6.1.3). After incubation with 600U/ml of T4 polynucleotide kinase for 30min at 37°C to phosphorylate the 5' ends of the DNA molecules, 0.2 volumes of the above buffer with 0.6mM of each dNTP were added. This was followed by a further 30min incubation at room T° with 2.5 U of Klenow fragment of DNA polymerase/60µl to 'end-fill' DNA termini. Reactions were stopped by the addition of EDTA to 20mM and the DNA was repurified (6.2.1).

PCR product destined for 'cohesive-ended' ligation was resuspended in 20µl of BamHI buffer (6.1.3) with 20U BamHI and incubated at 37°C for 6h. Reactions were stopped by a 10min incubation at 70°C, before the DNA was repurified (6.2.1).

For PCR product destined for use as a probe, or in direct sequencing, no such preliminary procedures were required.

Specific DNA bands of interest were then separated from contaminating bands and identified by preparative agarose gel electrophoresis (6.2.7,9) and slices of gel containing appropriate bands of DNA were excised.

During the course of this work, 3 different techniques were applied to make use of DNA within gel slices. For some separations LGT agarose had been used and subsequent procedures took place in its presence. In other cases purification was achieved using a commercial kit (Gene clean II, Bio 101), to the manufacturer's instructions. In later experiments, use was made of an electro-elution apparatus (Uni-directional electroelutor analytical, International Biotechnologies inc.). Elution of DNA from a gel slice into 80µl of a trapped high-salt elution buffer (6.1.3) was effected by electrophoresis at 100V for 30min in a low-salt running buffer (6.1.3). DNA recovered by electroelution was repurified by protocol 6.2.1 with a slight modification. Due to the presence of a high concentration of salt, no NaOAc needed to be added and DNA was precipitated with 2 volumes of isopropanol instead of ethanol. The final DNA precipitate was resuspended in 10µl or less of water. Yield and purity of DNA recovered by 'gene-cleaning' or electroelution was checked by analysis (6.2.7,9) of an aliquot of the final preparation.

6.2.16. Preparation of plasmids for ligation:-

Phagemids pTZ18R and pTZ19R (Mead et al., 1986) were originally obtained by the department from Pharmacia and the plasmid pOGS40 (Kingsman et al., 1990) from Dr.S.Adams, British Biotechnology Ltd.

SmaI-digested, calf intestinal phosphatase (CIP)-treated, gel-purified and gene-cleaned pTZ19R (at 50ng/µl in water), used for blunt ended ligations with PCR products, and EcoRI-, HindIII-digested, CIP-treated, gel-purified pTZ18R (at 50ng/µl in LGT agar), used to accept inserts excised from pTZ19R, were obtained from departmental stocks.

3µg of pTZ18R or pOGS40 were linearised by incubation with 30U of BamHI in total volumes of 30µl of BamHI buffer (6.1.3) for 6h at 37°C. The termini of these digested plasmids were dephosphorylated by including 20U of CIP for the final 30min of incubation and a further 20U for the final 5min. Reactions were then stopped by a 10min incubation at 70°C. Plasmid DNA was then purified (6.2.1), after checking for complete digestion of circular forms by analysis (6.2.7,9) of an aliquot.

6.2.17. Ligation of DNA into plasmids:-

2 different methods were employed for inserting DNA into plasmids. The choice of method was dependent on whether the DNA fragment contained 'blunt' or 'protruding' termini.

Blunt-end ligations were performed in LB (6.1.3), in total reaction volumes of 20µl containing 2 Weiss U of T4 DNA ligase. 50ng of SmaI-digested, CIP-treated pTZ19R (6.2.16) were included per reaction as well as PCR product (see below) suitably prepared for insertion (6.2.15). These reaction mixes were incubated at 15°C for 16h.

Ligations between the cohesive ends of digested PCR product (6.2.15), or purified DNA fragments excised from plasmids (6.2.21), and appropriately digested plasmids (6.2.16) took place in total volumes of 20µl of ligase buffer (6.1.3) with 2 Weiss U of T4 DNA ligase. As well as DNA for insertion (see below) each reaction mix contained 50ng of BamHI-digested, CIP-treated pTZ18R, 50ng of EcoRI-, HindIII-digested, CIP-treated pTZ18R, or 200ng of BamHI-digested, CIP-treated pOGS40. These mixes were incubated at 25°C for 16h.

For most DNA fragments to be inserted several reaction mixes, containing a range of concentrations of that fragment, were incubated. Control mixes, with either no ligase or no DNA for insertion, were also included in each set of ligation reactions. Ligations involving DNA contained in LGT agarose were set up at 37°C, with a maximum LGT agarose concentration of 0.3% (having first melted the agarose by a brief spell at 60°C), before cooling to the incubation T°. Estimates of the DNA concentration in LGT agarose were based on analysis of the DNA (6.2.9) and the weight of the gel slice.

After incubation, all ligation reactions were stopped by a 10min incubation at 70°C before the DNA was repurified (6.2.1).

6.2.18. Transformation and growth of E.coli:-

Transformation-competent E.coli, strains JM83 or JM101 (Messing, 1979), prepared according to the '$CaCl_2$' method of Maniatis et al. (1982), were obtained from departmental stocks, which were stored at -70°C in 200µl aliquots, at an estimated concentration of $5x10^8$ bacteria/ml. Strain JM83 was used for transformation by pOGS40-derived plasmids. All other transformations involved strain JM101.

Purified DNA from a ligation reaction (6.2.17) was resuspended in 200µl of TCM buffer (6.1.3), added to a thawed aliquot of E.coli and left on ice for 45min. After a 42°C 'heat shock' for 1.5min followed by cooling on ice, 1ml of L-broth was added and suspensions were incubated for 1h at 37°C. The bacteria were then pelleted by a brief microfugation, before being resuspended in 200µl of L-broth. 20, 50 or 130 µl portions of this suspension were then spread over 3 previously prepared agar plates.

Each plate contained L-broth, with ampicillin at 150µg/ml, set in 1.5% w/v bacterial agar. For growth of bacteria transformed by pTZ-derived plasmids these plates also contained a thinner, 'top (0.8%) agar' layer which included X-gal at 400µg/ml and IPTG at 170µg/ml.

Control plates, included with each set of transformations, were coated with a 50µl portion of an E.coli aliquot, transformed with 1ng of undigested plasmid vector, or left untransformed.

After allowing the added fluid to be absorbed, plates were inverted and incubated for 16h before being inspected for the growth of ampicillin-resistant (i.e. transformed) bacteria.

6.2.19. Screening of transformed bacteria. Glycerol stocks:-

After growth of transformed E.coli (6.2.18), bacteria transformed by 'phagemids pTZ18R or pTZ19R containing inserted DNA were identified by their colony colour (white/pale blue = recombinant; dark blue = non-recombinant). No such colour selection exists to distinguish recombinant and non-recombinant pOGS40 plasmids.

Colonies with recombinant, pTZ-derived plasmids and all colonies of bacteria transformed by pOGS40-derived plasmids were screened for the presence of a desired insert by hybridisation (6.2.13,14) of an appropriate probe to bacterial NA's immobilised by the following method. Selected colonies, as well as a negative- (non-recombinant plasmid containing) and, where possible, a positive- control colony were individually transferred to identifiable positions on each of two duplicate bacterial agar plates (6.2.18). These plates were incubated for a further 20h. One plate from each pair was then stored at 4°C, whilst colonies from the second were transferred to a nylon membrane (Hybond-N), which was laid on the plate in a marked orientation for 30s. Membranes were then laid colony side up on Whatman 3MM filter

paper soaked in 2xSSC (6.1.3), 5% w/v SDS for 2min, before being incubated in a microwave oven at 650W for 2min. After hybridisation (6.2.13) these membranes were washed to a final stringency of 0.2xSSC, 60°C, regardless of the probe used. Autoradiography (6.2.14) then allowed identification of positively-hybridising colonies. Corresponding colonies on the duplicate plate were used to inoculate 5ml aliquots of L-broth with ampicillin (150µg/ml), which were then incubated for 16h.

Following transformations with pTZ-derived plasmids where <12 colonies containing recombinant plasmids were obtained, this screening procedure was bypassed and all such colonies were used for these inoculations.

A portion of each of these 5ml cultures was then used to make a 'mini' preparation of plasmid DNA as follows. 1.5ml of each culture was centrifuged for 3min at 2,500 rpm (the remainder of the culture was meanwhile stored at 4°C) and cell pellets were resuspended in 50µl of TNE 10:100:1 (6.1.3). Suspensions were vortexed with 50µl of phenol pH8.0/chloroform/isoamyl alcohol (25:24:1 v/v/v), then microfuged for 5min to separate aqueous and organic phases. After recovery, the aqueous phases were incubated at 70°C for 10min to inactivate any endogenous nucleases, before plasmid DNA was further purified by a modification of procedure 6.2.1. (NH_4OAc being added, instead of NaOAc, to 2M, immediately prior to ethanol precipitation). NA pellets were then resuspended in 8µl of TE RNAse A (6.1.3). These preparations of plasmid DNA were analysed (6.2.7,9) either directly after a 0.5h incubation at room T° to digest contaminating RNA and/or after being digested with appropriate restriction endonucleases (6.2.21).

The remains of selected 5ml cultures were then used to inoculate further cultures (6.2.20,22) and to make glycerol stocks in case of future requirement. These were prepared by adding glycerol to 20% (v/v) and stored at -70°C.

6.2.20. Large-scale preparations of plasmid DNA:-

These were used for ds sequencing of plasmids, transforming yeast and producing larger amounts of insert DNA for use, following excision (6.2.21), as probes.

1ml of an appropriate culture (6.2.19) was used to inoculate 500ml of L-broth, with ampicillin (150µg/ml). After 16h incubation, bacteria were collected by centrifugation (2,500 x g, 10min) and resuspended in 10ml of GTE (6.1.3) with 5mg/ml lysozyme in a 50ml tube. After 5min, 20ml of 0.2M NaOH with 1% w/v SDS were added and the tube left on ice for 10min. Next, 15ml of 5M KOAc pH4.8 (6.1.3) were mixed in before a further 10min incubation on ice. This suspension was then centrifuged for 20min at 2,500 rpm, 4°C, after which the SN was collected and mixed with 0.6 volumes of isopropanol. The precipitate formed after 15min at room T° was collected by centrifugation for 30min at 2,500 rpm, washed in 10ml of 70% ethanol, repelleted by a 10min spin at 2,500 rpm and desiccated. Next, it was dissolved in 30ml of TE 10:1 (6.1.3), to which 30g baked (6.2.2) CsCl and 2.4ml of EtBr (at 10mg/ml) were then added. This solution was centrifuged in a sealed tube in a Beckman L8-60M ultracentrifuge at 40,000 rpm, for 18h in a vertical rotor (VTi 50). The plasmid band, separated in the density gradient so formed, was visualised by transillumination at 302nm and withdrawn by side puncture of the tube. This sample was shaken with several changes of an equal volume of isoamyl alcohol, with the 2 phases being separated by 5min of centrifugation at 1,500 rpm on each occasion. The aqueous phase was retrieved and dialysed against TNE 10:100:1 (6.1.3), before the DNA was precipitated by adding 2 volumes of ethanol and leaving for 30min on dry ice, then collected by centrifugation at 2,500 rpm for 30min. It was washed in 70% v/v ethanol, repelleted by a 10min spin at 2,500 rpm and dried. This plasmid DNA was finally resuspended in 40µl of water and an aliquot analysed (6.2.7,9) for purity and yield.

6.2.21. Excising inserts from plasmids:-

Inserts were excised, both from small- (6.2.19) and large- (6.2.20) scale preparations of plasmid, for analysis, transfer to another plasmid or separation of DNA suitable for labelling and use as a probe. Similar protocols were followed in each case.

Recombinant plasmids derived by ligating DNA to BamHI-digested plasmids were digested with BamHI. All other recombinant plasmids were sequentially digested by HindIII then ECoRI.

BamHI digestion of plasmid preparations (whether purified pellets, or dissolved in TE RNAse A) was performed by diluting DNA to <3µg/µl, in reaction mixes, which finally contained 1µl of 10x BamHI buffer (6.1.3) and 10U BamHI /10µl, then incubating for 16h at 37°C.

Sequential digestions were commenced by making similar dilutions, but with the final inclusion of 1µl of 10x low-salt digestion buffer (6.1.3) and 10U of HindIII/ 10µl (instead of BamHI enzyme and buffer), followed by a 2h incubation at 37°C. Samples were then diluted 2-fold by adding a solution containing 1µl of 10x high-salt digestion buffer (6.1.3) and 10U ECoRI/ 10µl and incubated for a further 1h. All digestions were stopped by a 10min exposure to 70°C.

Samples were then analysed (6.2.7,9) to identify excised inserts, which were recovered, when required, by previously described methods (6.2.15).

6.2.22. Preparing single-stranded DNA in E.coli strain JM101:-

500µl of a culture of a selected transformant (6.2.19) were used to inoculate 10ml of 2xYT broth (6.1.3) and the bacteria grown to an OD_{600} of 0.6. (approximately 2h incubation). 400µl were then transferred to a 50ml tube with 30µl M13KO7 phage (Pharmacia) at $7x10^{10}$/ml in 2xYT (departmental stocks) and incubated for 1h. 10ml of 2xYT and 29µl kanamycin at 25mg/ml were then added before continuing incubation.

After 16h, this culture was centrifuged at 2,000 rpm for 10min. 2.5ml of 20% w/v PEG 6000 in 3M NaCl were added to the clarified SN, which was then left on ice for 30min. The resulting precipitate was collected by centrifugation at 40,000 rpm in a Beckman L8-60M ultracentrifuge, Ti 70.1 rotor, for 40min at 4°C and dissolved in 400µl of TNE 20:20:1 (6.1.3). This DNA was then purified (6.2.1 - modified by an additional phenol /chloroform /isoamyl alcohol extraction immediately after the first), resuspended in 20µl of water and heated to 70°C for 10min before a 5% aliquot was analysed (6.2.7,9) to check the yield.

6.2.23. Sequencing:-

All sequencing was performed with the aid of a commercial kit (Sequenase version 2.0, USB) based on the chain termination method of Sanger et al. (1977). Sequencing reactions for ssDNA preparations were performed exactly to the manufacturer's protocol, using 1µg of ssDNA (6.2.21) annealed to an equimolar concentration of an indicated primer with polymerisation by T7 DNA polymerase, chain termination by one of a set of 4 dideoxynucleotides and the inclusion of 0.5µl [α-^{35}S]dATP (10mCi/ml, 400Ci/mmol) for labelling.

Alterations to the recommended protocol were made for ds sequencing. For the direct sequencing of PCR product, only 300ng of DNA template (6.2.15) were used/ reaction, whilst for the ds sequencing of inserts in pOGS40, 3µg of plasmid DNA (6.2.20) were used. These templates were boiled for 2min with equimolar concentrations of primer in the manufacturer's buffer with DMSO added to 30% v/v, then snap-cooled on ice for the annealing step. Additions for the labelling reactions were as recommended but the latter were performed by a 5min incubation on ice. Termination reactions were at 50°C for 2min.

All sequencing reactions were repeated with the substitution of dITP for dGTP in the labelling step to clarify sequence in GC-rich regions (Mizusawa et al., 1986).

All sequencing reactions were analysed by electrophoresis through 45cm-long x 1mm-thick, vertical, slab gels containing 6% w/v (degassed) acrylamide, 0.3% w/v bis-acrylamide, 7.67M urea and 0.5xTBE (6.1.3), set by the addition of APS to 0.025% and TEMED to 0.1% (w/v). 3.5µl aliquots of each of the 4 terminated reactions from a set were heated to 70°C for 2min before being submitted to electrophoresis, at 2500V and 50°C in a running buffer of 0.5xTBE, for periods of 1.5, 4 and 7 h. Afterwards, gels were dried at 80°C for 2h under vacuum before being autoradiographed (6.2.14) then 'read.' When bands appeared in more than one lane at a given position on the radiograph (as happened on occasion, particularly when directly sequencing PCR product) the densest band was interpreted as the 'true' result.

6.3.Yeast cell culture; manipulation and analysis of protein samples:-

6.3.1. Transformation of yeast:-

The host strain of yeast (sp. Saccharomyces cerevisiae) used in this work, BJ 2168 (Jones, 1991), was transformed with plasmid DNA by the method of Hinnen et al. (1978).

A freshly-grown (from departmental stock), 1.5l culture of untransformed yeast, at 1.5 x 10^7 cells /ml, was centrifuged at 3,500 x g for 15min. Harvested cells were washed in 20ml of 1M sorbitol, recentrifuged as above, then suspended in 10ml of 1M sorbitol. After adding 200µl of glusulase (DuPont [>10,000U sulphatase, >90,000U glucuronidase/ml]) the cells were incubated, with continual rotation, at 30°C for 2h. Having verified that this procedure had produced a suitably high percentage (>90%) of spheroplasts competent for transformation (see below), the cells were washed twice with 10ml of 1M sorbitol and once with 10ml of STC solution (6.1.3) with intervening centrifugation steps as above. Verification of the formation of spheroplasts was by light microscopy of an aliquot of cell suspension diluted in 10 volumes of water (lysed cells = spheroplasts). The cells were finally resuspended in 1ml of STC.

Various amounts of DNA (5 or 10µg pOGS40-derived plasmid [6.2.20] and 5 or 10µg pUG41S plasmid [originally supplied by BBL], +/- 20µg of carrier:- boiled, salmon sperm DNA), in total volumes <15µl, were mixed with 100µl aliquots of the spheroplast suspension and left for 15min. Then, 1ml of sterile 44% w/v PEG 4000 was added to each of the aliquots, which were left for a further 10min. A 45s microfugation was then used to pellet the cells, which were gently resuspended in 1ml of 1M sorbitol. A quarter of this suspension was added, along with 40µl of a filtered, 1% w/v solution of L-tryptophan, to each of 4 tubes containing 20ml of warm (but <48°C), molten, regeneration agar (6.1.3). 40µl of 1% w/v solutions of uracil and leucine were also added to one tube of agar, which was mixed with an aliquot of untransformed cells and used as a positive control for yeast regeneration. Each spheroplast/agar mix was poured into a petri dish and allowed to set. These plates were then inverted and incubated for several days, with a daily inspection for colony growth.

Once identified, a few transformed colonies, selected on the basis of faster growth, were sub-cloned by streaking onto Sc-glc agar plates (6.1.3) and incubating for a further 3d.

6.3.2. Small-scale culture of transformed yeast. Preparation and storage of glycerol stocks:-

One fast-growing sub-clone derived from each of four original transformants (6.3.1) was selected for further culture. Each sub-clone was used to inoculate 5ml of Sc-glc medium (6.1.3). This was incubated for 24h, before a 1ml aliquot was used to initiate a culture in 100ml of Sc-glc which was incubated for 1d. An aliquot was then checked by light microscopy for healthy yeast (presence of multiple budding forms) and freedom from bacterial contamination. A further 12ml of culture were also removed at this point and mixed with an equal volume of 40% v/v glycerol. 2ml aliquots of this mix were then stored at -70° C forming parent stocks used for subsequent initiation of larger scale cultures. The remainder of the culture was pelleted by centrifugation at 3,500 x g for 10min and resuspended in 100ml of Sc-glc/gal medium (6.1.3). The yeast were then incubated for a further 24h.

6.3.3. Verifying the presence of fusion protein in initial yeast cultures:-

After growth in Sc-glc/gal (6.3.2), yeast cells were pelleted by centrifugation at 3,500 x g for 5min and resuspended in 1ml of TEN buffer (6.1.3). They were then broken by vortexing with an equal volume of acid-washed (0.2M HCl for 16h), water-rinsed, glass beads for 3 x 1min spells with 1min on ice between. Aliquots of each crude extract were electrophoresed through SDS-polyacrylamide gels (6.3.12), alongside similar extracts (taken from departmental stocks) of other yeasts. Gels were then stained with Coomassie blue (6.3.13) or analysed by Western blotting (6.3.14).

6.3.4. Large-scale yeast culture:-

A glycerol stock (6.3.2) of yeast was thawed and used to inoculate 100ml of Sc-glc medium (6.1.3), then incubated for 48h. Next, this culture was equally divided between 4 flasks, each of which contained a further 100ml of Sc-glc medium, before another 24h of incubation.

Each of these cultures was then added to 1l of Sc-glc/gal (6.1.3) and incubated for a final 24h. Aliquots of cultures were removed and assessed for cell density (by measuring OD_{600}), cell viability and freedom from bacterial contamination (by light microscopy) after each incubation step. On occasion, a 10ml aliquot was also removed at the end for analysis by electron microscopy (6.3.6), before cells were harvested by centrifugation at 3,500 x g for 15min and subjected to 3 washes, each in 40ml of water, with repelleting by 5min centrifugations at 3,500 x g in between. Finally, cells were resuspended in TEN (6.1.3), repelleted as above and, after removing the SN, frozen at -20°C until required.

6.3.5. Purification of hybrid TNF-Ty, virus-like particles:-

Hybrid VLP's were purified by a modification of the method of Adams et al. (1987b). Frozen cells (6.3.4) were thawed and resuspended in 20ml of TEN (6.1.3). All subsequent procedures in the purification of VLP's were then performed at 4°C. Cells were broken by vortexing with 20ml of acid-washed glass beads (6.3.3) for 10 x 1min spells, with 1min on ice between. The suspension was then clarified by centrifugation at 2000 x g for 5min and the VLP-containing SN retained. A further 8ml of TEN were added to the cell pellet. Rounds of vortexing, centrifugation and resuspension were repeated as above until the integrity of >85% of cells was destroyed, as assessed by phase contrast microscopy of an aliquot.

SN's from each round were pooled and centrifuged at 13,000 x g for 20min. The clarified SN obtained was then centrifuged at 100,000 x g for 1h in tubes containing a 2ml cushion of 60% w/v sucrose in TEN at the bottom. These cushions, together with cushion interfaces, were then collected and dialysed against TEN. The dialysate was microfuged for 20min before being divided and loaded on the top of four, 15-45% sucrose gradients. These gradients had been prepared by layering 8ml aliquots of solutions of 45, 35, 25 and 15 % w/v sucrose in TEN, in Beckman SW28 tubes, 20h earlier. A bottom 2ml cushion of 60% w/v sucrose in TEN was added immediately prior to use. These tubes were then centrifuged at 53,000 x g for 3h, before the gradients were separated into 2ml fractions. These fractions were analysed (6.3.12,13) and those containing high concentrations of protein of

appropriate size for a P1-TNF fusion protein were pooled and dialysed against TC buffer (6.1.3). Removal of precipitates formed during dialysis was by microfuging for 10min. Aliquots of this VLP preparation were removed for analysis (6.3.6,10,12,13) whilst the remainder was stored at 4°C prior to cleavage of the fusion protein (6.3.7).

6.3.6. Electron microscopy of yeast cells and purified virus-like particles:-

An aliquot of yeast cell culture (6.3.4) was prepared for electron microscopy by the method of Byers and Goetsch, (1975). Cells were pelleted by centrifugation at 2,500 rpm for 5min and suspended in 10ml of 3% w/v glutaraldehyde (in 0.1M cacodylate pH6.8, 5mM $CaCl_2$). After a 30min incubation, with rotation, at room T°, cells were pelleted as before, then resuspended in 10ml of glutaraldehyde as above, prior to a further 16h incubation at 4°C.

Cells were then harvested as before and washed 3 times in 10ml of pretreatment buffer (6.1.3) before resuspension in 5ml of 0.2M citrate /PO_4 pH5.8. After adding 200µl of glusulase (6.3.1) the cells were incubated for 1h at 30°C, with rotation, then repelleted. Finally, the cells were washed twice in 10ml of 0.2M citrate/PO_4 pH5.8 and resuspended in 5ml of the same.'Post-fixing,' mounting and sectioning of the cells was performed by staff in the 'EM suite' by standard methods.

Purified VLP's (6.3.5) were prepared for electron microscopy by allowing a drop of VLP suspension to dry on an EM grid, which was then washed by successively adding and removing 3 drops of water. VLP's were negatively stained by adding 1 drop of 2% w/v uranyl OAc. Surplus stain was removed with filter paper and the grid allowed to dry.

Stained VLP's and fixed, sectioned yeasts were examined by transmission electron microscopy.

6.3.7. Cleavage of P1-TNF fusion protein. Final purification and storage of recombinant ovine TNFα:-

Cleavage of P1-TNF fusion protein was achieved by the addition of chaotropic agents and bovine factor Xa (BCL) to purified VLP's (6.3.5) followed by an 18h incubation, with occasional agitation, at 25°C.

After assessing (6.3.12,13) the cleavage obtained in aliquots of the VLP suspension using different chaotropic agents and concentrations of factor Xa, subsequent large-scale cleavage of VLP preparations was performed by adding the detergents CHAPS and NaDOC, each to 0.05% (w/v), and using bovine factor Xa at 2% (w/w) of the total protein content of the suspension.

Having checked for 100% cleavage by analysing (6.3.12,13) an aliquot, cleavage mixes were clarified by centrifugation at 100,000 x g for 1h and resulting SN's dialysed against PBS (6.1.3). This dialysate, the final rovTNFα preparation, was aliquoted, either with the addition of BSA to 0.1% (w/v), for subsequent use in bioassays, or without, for use in analysis (6.3.10,12,13,14,15,16) and immunisations and stored, until needed, at -70°C.

6.3.8. A control preparation of yeast extract with factor Xa:-

A preparation of yeast extract with added bovine factor Xa was made for use as a control as follows:- a glycerol stock of yeast strain BJ2168 transformed with pMA 5620 (which constitutively produces yeast protein P1 [Adams et al., 1987a]), was used to initiate cultures grown in identical conditions (except for the extra addition of 2ml/l of 1% w/v uracil) as yeast transformed with recombinant-pOGS40 + pUG41S (6.3.4). These yeast were harvested and submitted to further procedures identical to those used for preparing, and storing, rovTNFα (6.3.5,7). This included collecting gradient fractions from the same positions as those taken to collect hybrid VLP's and using factor Xa at the same final concentration (w/v) used to cleave P1-TNF.

6.3.9 Recombinant human TNFα:-

E.coli-derived, rhTNFα was purchased from BBL in lyophilised form, reconstituted (in PBS with 0.1% w/v BSA) to 1µg/ml and stored in aliquots at -70°C till needed.

6.3.10. Measurement of protein concentration:-

The concentration of protein in a sample was measured by the method of Bradford (1976). A linear range of concentrations (from 5 to 30µg/ml) of BSA in PBS (6.1.3) and assorted dilutions (in PBS) of sample were prepared. 0.4ml of diluted sample or BSA standard was

mixed with 0.1ml of Bradford dye (Biorad). After 15min the OD_{595} of each was measured. Protein concentrations of suitably diluted samples were estimated by fitting their OD results to a standard curve constructed from the results of known concentrations of BSA.

6.3.11. Acetone precipitation of protein:-

Dilute proteins were concentrated, for analysis (6.3.12,14,16), by acetone precipitation. After the addition of 4 volumes of cold acetone, samples were left at -20°C for at least 1h. Precipitates were then collected by centrifugation at 2,500 rpm for 10min and resuspended as described.

6.3.12. SDS-Polyacrylamide gel electrophoresis of protein samples:-

Proteins were separated according to size, by electrophoresis using the method of Laemmli (1970). Samples were generally run through 1mm thick, vertical slab gels using the Biorad mini protean II system apparatus. The larger amounts of protein in acetone precipitates of conditioned $RPMI_{20}$ (6.4.2,3), however, were separated in 1.5mm-thick gels using the Biorad protean II slab cell system.

Separating gels contained either a single percentage, or concentration gradient (from 5-20% w/v), of polyacrylamide, as stated (bis-acrylamide was included at 1 part to 37.5 parts acrylamide w/w). Separating gels also included 250mM Tris HCl pH 8.7, 1.3mM EDTA and 0.1% w/v SDS, with APS and TEMED being added (each to 0.05%) to set the gel.

Separating gels were overlaid by 3-4mm of stacking gel, comprising 3.5% w/v acrylamide with bis-acrylamide as above, 145 mM Tris/HCl pH 6.8, 0.1% w/v SDS, set using APS and TEMED at 0.05% and 0.1% w/v respectively.

Samples for analysis were mixed with an equal volume of protein loading buffer (6.1.3) and boiled for 2min before being loaded into wells set in the stacking gel. (Precipitates were resuspended in suitably small volumes of 0.5 x loading buffer before boiling.) A set of proteins of known molecular weight (Sigma) were used as size markers.

Electrophoresis conditions were 100-200V for mini gels, or 50V for larger gels in protein running buffer (6.1.3) until the dye front reached the bottom of the gel.

6.3.13. Staining of SDS Polyacrylamide gels:-

Protein gels (6.3.12) were stained by Coomassie blue. This involved immersion in 0.25% w/v Coomassie brilliant blue G, 20% v/v methanol, 5% v/v glacial acetic acid for 30min. Gel backgrounds were then destained in several changes of 20% v/v methanol, 5% v/v glacial acetic acid over 24h. After staining, gels were dried at 80°C under vacuum.

6.3.14. Western blotting:-

As an alternative to staining (6,3,13), separated proteins were also analysed by transfer to membranes and immunodetection (Western blotting).

After electrophoresis (6.3.12), transfer was effected as follows:- gels, laid in marked orientation on nitrocellulose membranes (Hybond-C), were sandwiched between 6 sheets of 3mm paper (Whatman) (membranes and paper had been prewetted in 25mM Tris/HCl pH9.5, 20% v/v methanol) in a semi-dry electroblotting apparatus (Ancos, Denmark), through which 120 mA were passed for 1h.

After protein transfer, membrane portions corresponding to size marker tracks were cut off and stained in 0.5% w/v amido black, 50% v/v methanol, 5% v/v glacial acetic acid, for 30min, then destained in several changes of 50% v/v methanol, 5% v/v glacial acetic acid over 2h. Such portions were later realigned with the rest of the membrane to determine the size of detected proteins.

The rest of the membrane was incubated in PBS (6.1.3) with 5% w/v milk powder for 1.5h at room T° to block remaining binding sites and then incubated, for 16h at 4°C, with an indicated, primary antibody, diluted, as described, in PBS with 1% w/v milk powder.

After washing for 30min in at least 5 changes of 1% w/v milk powder in PBS, blots were next incubated for 1h at room T° with a secondary antibody, also diluted in 1% w/v milk powder in PBS. All secondary antibodies were:- polyclonal; affinity-purified; commercially prepared; and used at recommended dilutions. They comprised:- an

alkaline phosphatase-conjugated goat anti-[rabbit immunoglobulin G] preparation, used, at a dilution of 1:8000, when the primary antibody was derived from rabbits; and an alkaline phosphatase-conjugated rabbit anti-[mouse immunoglobulin G] preparation, used, at 1:1000, when the primary antibody was murine, unless the detection system used was enhanced chemiluminescence (ECL- see below), when a horseradish peroxidase-conjugated rabbit anti-[mouse immunoglobulin G] preparation was used at 1:2000.

After incubation with secondary antibody, blots were again washed in PBS/milk powder as above. Then, for alkaline phosphatase-based detection, there were two further washes, over 10min, in 0.1M Tris/HCl pH9.5. These blots were developed in 10ml of 0.1M Tris/HCl pH9.5, 0.2mg/ml NBT, 0.1mg/ml BCIP, 20mM $MgCl_2$. Colour development was stopped by rinsing in water.

Detection by ECL, which was used once only (see fig.4.7b, 6.3.16), was performed using a commercial kit (Amersham). A washed blot was covered, in the dark, with 2ml of a 50:50 mix of supplied reagents A & B. After 1min, the fluid was drained off, the blots were wrapped in cling film and then autoradiographed using hyperfilm ECL (Amersham) in a cassette with intensifying screens and an exposure time of 4h.

6.3.15. Cross-linking of recombinant ovine TNFα:-

RovTNFα was cross-linked by the method used by Van Ostade et al. (1991) on rhTNFα. 6µl of BSOCOES (Pierce) at 30mg/ml in DMSO (or, for control, 6µl DMSO) were added to 40µl of sample in PBS (6.3.7) and left for 30min before proteins were analysed (6.3.12,13,14).

6.3.16. Deglycosylation of ovine TNFα:-

Ovine TNFα was submitted to procedures recommended by Haselbeck & Hösel (1988), to test for the presence of glycosylation. Proteins in 1ml aliquots of SN from ovine lung cells stimulated with 1µg/ml LPS for 18h (6.4.3), or 20ng aliquots of rovTNFα (6.3.7), were precipitated (6.3.11), resuspended in 20µl 1% w/v SDS, boiled for 2min, and diluted by the addition of 180µl of 100mM $NaPO_4$, pH7.0 (6.1.3), 25mM EDTA, 1% w/v octyl glucoside. An aliquot of each type

was then incubated with 0 or 2 U peptide-N-glycosidase F (BCL) for 18h at 37°C. Afterwards, 10% of each mix was analysed by Western blotting (6.3.12,14).

6.4. Mammalian cell culture; bioassays; antibody production:-

6.4.1. Cell Counting:-

The concentration of viable cells in any suspension was estimated by counting the number of unstained cells in 4 x 10^{-4}mls on an haemocytometer slide after applying an aliquot of recently-mixed suspension, diluted 100 fold with 1% w/v trypan blue.

6.4.2. Ovine lung-cell cultures:-

Populations of ovine lung cells were derived by broncho-alveolar lavage performed on lungs dissected from a freshly-slaughtered sheep. 2l of cold Hank's balanced saline solution (HBSS) were poured into a pair of lungs via the trachea. Lavage fluid was retrieved by the same route and spun at 1500 rpm, in an MSE coolspin 2, for 15min at 4°C. Resulting cell pellets were washed in 50ml HBSS and centrifuged at 1500 rpm for a further 15min at 4°C before resuspension in 9ml HBSS. Erythrocytes were lysed by the addition of 13.5ml of water to induce a hypotonic shock. Normal osmolarity was returned by adding 1.5ml of 10xPBS (6.1.3) 5s later. After adding 25ml of the medium to be used in incubation (see below), the cells were repelleted by a 15min centrifugation at 1500 rpm and 4°C. Finally, the cells were re-suspended in incubation medium, counted (6.4.1) and diluted as required, before plating out as described. Incubation media used depended on the duration of the experiment. Short term incubations (<3d) were conducted in Iscove's medium with penicillin and streptomycin. For incubations lasting >3d, cells were cultured throughout in $RPMI_{20}$ (6.1.3) with penicillin, streptomycin and fungizone. In experiments not involving immediate stimulation of the cells, only cells which were adherent after 24h were selected, by discarding SN's, washing with PBS and refeeding with fresh medium. Cells cultured for >5d were totally refed with fresh medium twice weekly or as described (6.5.4).

6.4.3. Lipopolysaccharide stimulation of lung cells:-

Preparations of ovine lung cells (6.4.2) were stimulated by the addition of LPS derived from Salmonella abortus equi, at the final concentrations, and times after plating out, described. Unless cells were stimulated immediately after cell preparation, LPS addition also involved complete replacement of the old incubation medium with an equal volume of fresh medium. SN's were later harvested, clarified by centrifugation for 10min at 2,500 rpm and 4°C, then aliquoted and stored at -70°C until analysed.

6.4.4. Sources and maintenance of established cell lines:-

L929 and L929L/R cells (Matthews & Watkins, 1978) in culture were donated by Dr.G.Entrican. Frozen stocks of these cells were made as follows:- cells were harvested by trypsinisation and centrifugation (see below) and resuspended in freezing mix (6.1.3); aliquots were then frozen slowly to -70°C and transferred to liquid nitrogen for storage until required. WEHI 164: clone 13 (Espevik & Nissen-Meyer, 1986) and ST6 cells (derived from an ovine intestinal adenocarcinoma) were obtained from aliquots of departmental stocks stored in liquid nitrogen. When used to initiate a culture, a frozen aliquot of cells was rapidly thawed, in a 37°C water bath, before being added to culture medium and transferred to an appropriate flask.

Weybridge sheep choroid plexus cells (WSCP's, a fibroblastoid cell line) were obtained, in culture, from departmental stocks. A line of sheep cells derived by infecting peripheral blood mononuclear cells with sporozoites of Theileria annulata (line 5, Entrican et al., 1991) was obtained, in culture, from Dr G.Entrican.

WEHI 164: clone 13, ST6 and WSCP cells were cultured in DME medium with 5% v/v FCS. L929 and L929L/R cells were cultured in Iscove's medium with 5% v/v FCS, and line 5 cells in RPMI with 10% v/v FCS (media these cells were supplied in). All media also included penicillin and streptomycin.

Passage and maintenance of cells in culture was as follows:- suspensions of line 5 cells, which are non-adherent, were divided twice weekly, by a simple 4-fold dilution in fresh medium; cultures of the remaining, adherent cell lines were divided, when cells approached confluency, by trypsinisation (see below) and dilution; typically,

L929 and L929L/R cell cultures were divided 7-fold, twice weekly, WEHI 164: clone 13 cultures 4-fold, twice weekly and ST6 and WSCP cultures 3-fold every 5-7 days; WSCP cells were cultured in 75cm^2 flasks containing 25ml of medium; all other cells were grown in 25cm^2 flasks with 8ml of medium. L929, L929L/R and WEHI 164: clone 13 cells were passaged no more than 6 times before use in bioassays.

Trypsinisation of a cell monolayer involved rinsing the cells, first in PBS (6.1.3) then in 0.02% w/v versene (in PBS), before they were just covered in 0.05% w/v trypsin, 0.02% w/v versene (in PBS) and incubated until the majority had detached. Digestion was then stopped by adding an equal volume of culture medium. Detached cells were harvested by centrifugation at 1500 rpm for 10min. Cells were then resuspended in freezing mix (when destined for freezing, see above) or culture medium. Cells destined for immediate use in bioassays were counted (6.4.1) before being plated out as described for each assay, whilst those used for maintaining a culture were diluted and returned to tissue flasks as above.

6.4.5. Cytotoxicity assays:-

Cytotoxicity assays were performed on L929, L929L/R, WEHI 164: clone 13 and ST6 cells using methods based on those described by Flick & Gifford (1984). Harvested cells (6.4.4) were diluted to 5 x 10^4 cells/ml in their respective media and plated out, at 100µl/well, in 96-well, flat-bottomed plates. After 20h incubation, additions were made as follows:- in assays involving L929 and L929L/R cells, 50µl of medium with 4µg/ml of actinomycin D and 50µl of sample were added; in all other assays 100µl of sample only were added. A set of samples in any assay included:- a range of concentrations of recombinant proteins (6.3.7,8,9) in PBS with 0.1% BSA; or undiluted, conditioned media (6.4.3) and rhTNFα (6.3.9) at various dilutions in unconditioned medium (+/- LPS at the concentration used for lung-cell stimulation); or, for neutralisation assays, rovTNFα (6.3.7), which had been diluted to 2x sample concentration in PBS, 0.1% w/v BSA, then incubated for 1.5h with an equal volume of indicated antibodies similarly at 2 x sample concentration. All samples were assayed in quadruplicate.

After these additions, L929 and L929L/R cells were incubated for 18h, whilst all other cells were incubated for 3d. After incubation, wells were examined by light microscopy, then the SN's were decanted and remaining cells rinsed once in PBS before being fixed and stained using 0.25% w/v crystal violet, 20% v/v methanol, at 40µl/well, for 2min. Afterwards, cells were gently, but thoroughly, rinsed in water and dried. The absorbance of each well at 540nm was then measured using an ELISA plate reader (Titertek). Figures for cytotoxicity are derived as follows:-

$$\%\text{age cytotoxicity} = \frac{\text{mean abs. neg. control*} - \text{mean abs. sample} \times 100}{\text{mean abs. neg. control*} - \text{mean abs. pos.control**}}$$

* = PBS/0.1% BSA, or unconditioned culture medium + LPS.
** = 10ng/ml rhTNFα (or, where appropriate, 100ng/ml rhTNFα or 10µg/ml rovTNFα) giving 100% cytotoxicity as detected by light microscopy.

An assay on non-adherent T.annulata transformed cells was performed slightly differently and was designed to be able to detect induction of cytotoxicity /cytostasis or increased cytoproliferation. Line 5 cells (6.4.4) were plated out and initially incubated as described for the other cell lines above. After the addition of 100µl samples (recombinant proteins in PBS, 0.1% w/v BSA) in quadruplicate, cells were incubated for 3 more days with the inclusion of 1µCi of ^{3}H thymidine/well for the final 18h. After light microscopy, cells from a well were transferred onto individual discs in glass fibre mats (Skatron) and rinsed, using a semi-automatic cell harvester (Titertek), before incorporated radioactivity was measured by placing dried discs in vials with 1ml of scintillation fluid (Optisafe, LKB) and counting with a rack β-scintillation counter.

6.4.6. Thymocyte proliferation assays:-

Assessment of the proliferation of sheep thymocytes in the presence of samples, using phytohaemagglutinin (PHA) as a co-mitogen, was based on the method of Harkiss et al. (1989). A normal thymus was obtained from a freshly-slaughtered lamb for each assay. Thymocytes were teased from small pieces of tissue into RPMI with 10% v/v FCS, pelleted by

centrifugation at 1500 rpm for 15min at 4°C and resuspended in 50ml of $RPMI_{10}ME$ (6.1.3). After waiting 5min to allow large pieces of tissue to settle, the top 40ml was transferred to a fresh tube and spun as above, before resuspending the pellet in 10ml of $RPMI_{10}ME$. These cells were then counted (6.4.1) and diluted, in the same medium, to 4 x 10^6/ml, before being plated out into the central 60 wells of round-bottomed, 96-well plates at 50µl/well (the outer wells were filled with PBS). 50µl of $RPMI_{10}ME$ with PHA at 36µg/ml and 100µl of sample were also added to each well. All samples were assayed in quintuplicate. For assessing the activity of recombinant proteins, samples comprised recombinant proteins (6.3.7,8,9) diluted in PBS, 0.1% w/v BSA. For assessing the neutralising capability of antibodies, samples comprised rovTNFα (6.3.7) (or rovIL-1ß) diluted and preincubated with antibodies as for cytotoxicity assays (6.4.5); for assessing thymocyte proliferating potential of conditioned media (6.4.3), samples comprised 99.5µl of conditioned or unconditioned (with LPS at the concentration used for stimulation) media, preincubated for 1.5h with 0.5µl of pre-immune, or post-final immunisation, rabbit serum (6.4.9).

After 2d incubation, wells were pulsed with 1µCi of ^{3}H-thymidine each and incubated for a further 18h. All wells were checked by light microscopy to confirm freedom from bacterial contamination before the radioactivity incorporated into cells in a given well was measured as in the cytostasis assay on line 5 cells (6.4.5).

6.4.7. Cartilage degradation assays:-

Cartilage degradation assays were performed by modifications of the method of Harkiss et al. (1989). A xiphoid cartilage was obtained from a freshly-slaughtered sheep for each assay and stripped of adherent adipose and fibrous tissue. >60 2mm diameter discs of cartilage were punched from the flattest region of the cartilage and cultured in petri dishes containing DME medium with 5% v/v FCS, penicillin, streptomycin and fungizone. After 2d, discs were transferred to the central 60 wells of flat-bottomed, 96-well tissue culture plates (1 disc/well) with 150µl of fresh medium as above and 50µl sample/well (outer wells were filled with PBS). Samples were recombinant proteins (6.3.7,8,9) diluted in PBS with 0.1% w/v BSA. All samples were assayed

in quintuplicate. Some wells were filled with medium and sample but no disc. After a further 2d incubation, all discs were removed and plates were frozen at -20°C. Analysis of the relative concentrations of chondroitin sulphate in the SN's was performed as follows:- 10µl of thawed SN from each well were transferred to individual wells of an ELISA plate in duplicate; 250µl of a 0.0018% w/v solution of dimethylmethylene blue (Pierce) in a formate buffer (6.1.3) were added to each well and the absorbance of each well at 540nm was then measured using an ELISA plate reader (Titertek).

6.4.8 Fibroblast proliferation assay:-

A fibroblast proliferation assay was perfomed, based on the method of Vilcek et al. (1986). WSCP cells (6.4.4) were plated out at 6 x 10^3 cells/well into the central 60 wells of a flat-bottomed, 96-well plate in 100µl of DME medium with 5% v/v FCS, penicillin and streptomycin (the outer wells were filled with PBS), then incubated for 18h before the addition of 50µl sample to each well. Samples were recombinant proteins (6.3.7,8) in PBS with 0.1% w/v BSA. All samples were assayed in quintuplicate. Plates were then incubated for a further 3d, with 1µCi of ^{3}H-thymidine/well being included for the final 18h. Wells were checked by light microscopy before cells were detached from the plate by trypsinisation (6.4.4). Cellular incorporation of radioisotope was then measured for each well as before (6.4.5).

6.4.9. Immunisation of rabbits. Collection and treatment of sera:-

3 rabbits were immunised during the course of this work. 15ml of blood were withdrawn from each rabbit, to act as a control, immediately prior to its first immunisation. The first rabbit (R175) was immunised with 250µg of hybrid VLP's in 0.5ml of TC (6.3.5) emulsified in 0.5ml CFA. This was injected SC via several sites. 3 weeks later it was boosted, SC, with 50µg of rovTNFα in 0.5ml PBS (6.3.7), emulsified with an equal volume of IFA. 10ml of blood were withdrawn 2 weeks after the 2nd injection.

Two subsequently immunised rabbits (R198 & R199), were immunised only with purified rovTNFα (6.3.7). Their first immunisations consisted of 150µg of protein/ rabbit in 1.5ml of PBS emulsified with 1.5ml of CFA, injected SC via several sites. These were followed by

2nd immunisations 3 weeks later of 50µg in 0.5ml PBS, emulsified with 0.5ml IFA. 5ml of blood were withdrawn from each 2 weeks later, and a third immunisation performed 1 week after that by injecting 20µg in 0.2ml PBS IV. 20ml of blood were removed from each rabbit 2 weeks after the final injection.

After collection, blood was allowed to clot overnight, before the serum was removed and clarified by centrifugation at 2500 rpm for 10min. All sera were then 'heat-inactivated' by incubation at 56°C for 30min before being aliquoted and stored at -20°C until use.

6.4.10. Immunisation of mice:-

3 Balb/c mice were immunised with rovTNFα (6.3.7) at a concentration of 100µg/ml. Each mouse was initially immunised SC with 20µg protein in PBS emulsified with an equal volume of CFA. They were sequentially boosted with 10µg (emulsified with 100µl IFA) SC, 13µg IP and 10µg IP, 3, 7 and 10 weeks later respectively. One mouse, selected for use in a fusion (6.4.11), was immunised 3 weeks after the final boost with 18µg IP and 2µg IV.

Blood samples (10µl) were taken from the tail veins 2 weeks after the 3rd and 4th injections. Serum, collected from these samples after overnight clotting, was used fresh, as a primary antibody in Western blots (6.3.14) or after aliquoting, storage at -20°C and thawing, as a positive control in ELISA's (6.4.12).

6.4.11. Fusion of mouse splenocytes to myeloma cells. Early hybridoma culture:-

3d after a mouse's final IV/IP boost (6.4.10), a frozen aliquot (departmental stock) of mixed thymocyte medium (MTM), previously prepared according to Reading (1982), was thawed, plated out at 100µl/well in the central 60 wells of each of 6, 96-well, flat-bottomed tissue culture plates and pre-incubated (mammalian cell culture conditions).

Fusion of murine splenocytes to NSO myeloma cells was then performed by the method of Galfre & Milstein (1981). 4d after its final boost, the mouse was killed by cervical dislocation and its spleen was removed and homogenised in 10ml of RPMI medium. The freed splenocytes were then pelleted. This and all subsequent

centrifugations in this procedure were performed at 1000 rpm for 5min. Cells were then resuspended in 10ml of RPMI. After allowing 2min for large pieces of tissue to settle, the suspension was transferred to a fresh tube and the number of cells counted (6.4.1). These were diluted to 9 x 10^6/ml. A 1ml aliquot was removed and added at 100µl/well to 10 of the wells containing MTM for control purposes (see below).

NSO myeloma cells were cultured (in RPMI medium with 10% v/v FCS, 1mM Na pyruvate), harvested by centrifugation, resuspended in RPMI, counted (6.4.1) and diluted to 3 x 10^6 cells /ml. 20ml of splenocyte suspension were then mixed with 20ml of NSO suspension and centrifuged. The cell pellet and a 50% w/v solution of PEG 1500 (Boehringer) in 75mM Hepes were simultaneously warmed at 37°C for 2min. 1ml of PEG solution was then slowly added to the cell pellet, with gentle rotation, over the course of 1min, then left at 37°C for a further 4min. 25ml of RPMI were next added, dropwise, over the course of 6min, again with gentle rotation of the suspension. The cells were then repelleted before suspension in 35ml of $RPMI_{20}HAT$ (6.1.3), and plated out at 100µl/well in the 350 wells containing MTM only. All 6 plates were then incubated for several weeks. Cells were refed by adding 50µl of fresh medium to each well twice weekly. After the first week, subsequent refeeds required prior removal of an equivalent volume of old medium. After 7d, each well was examined daily, under the light microscope, for growth of a colony of hybridoma cells. Any such colonies growing in a tightly clustered formation were dispersed by gentle agitation of the medium.

Once the hybridoma colony/colonies in any given well had grown to nearly cover the floor of that well, 200µl of its SN were removed for analysis by ELISA (6.4.12). Refeeding of wells with colonies nearing this state was postponed to prevent dilution of the SN. SN's were either tested immediately (6.4.12), or stored at -20°C for later testing, after accumulation of a suitable number of samples. At any testing, 200µl of supernatant were also removed from a control splenocyte-only well.

After removal of a SN sample, cells from that well were resuspended in remaining SN and transferred to a well of a 24-well plate with 1ml of $RPMI_{15}HAT$ (6.1.3).

6.4.12. Analysis of hybridoma supernatants:-

Neat SN's of initial (6.4.11) and cloned (6.4.13) hybridomas were analysed by ELISA. After a preliminary ELISA to optimise coating conditions, all subsequent ELISA's were performed against rovTNFα immobilised as follows:- rovTNFα (6.3.7) was diluted to 300ng/ml in carbonate coating-buffer (6.1.3) and plated out, at 100µl/well in an ELISA plate (Dynatech Immulon no.4) then incubated at 4°C for at least 16h.

All subsequent incubations were performed at 37°C, in a sealed container, with the exception of the final colour development step which occurred at room T° in open plates. After emptying the coated plates, unused binding sites were blocked by adding 200µl of 2% w/v BSA in PBS (6.1.3)/well and incubating for 1h. Plates were then washed 3 times with PBS before adding 100µl of sample to a well. After a further 1h incubation plates were washed 4 times in PBS. 100µl of an horseradish peroxidase-conjugated, rabbit anti-[mouse immunoglobulin G, whole molecule] preparation, which had been diluted 1:1000 in PBS with 0.25% w/v BSA, were then added to each well before a further 30min incubation. Plates were next washed 6 times in PBS. 100µl of peroxide substrate solution (6.1.3) were then added and the plates left for up to 30min whilst watching for appropriate colour development. Further reaction was stopped by adding 100µl of 12.5% v/v sulphuric acid and the OD_{492} of each well was measured on an ELISA plate reader (Titertek).

SN's of initial colonies, as well as those derived after 2 or 3 cloning procedures were assayed in duplicate. After a first cloning, however, whilst 100µl of SN was assessed exactly as above, the second 100µl of sample was assessed in an ELISA on wells coated, by overnight incubation at 4°C, with 100µl of yeast extract/factor Xa control preparation (6.3.8), diluted in PBS, by the same degree required to dilute rovTNFα stock (6.3.7) to 1µg/ml. All subsequent steps in this ELISA were as above.

Wells whose SN ELISA OD readings were >3x the OD obtained using medium alone were scored as positive. Where these readings were also > the OD obtained using splenocyte SN, wells were considered strongly positive.

6.4.13. Cloning and storage of hybridomas:-

The fate of hybridoma cells initially transferred to 24-well tissue culture plates was dependent on the ELISA scores obtained from their SN's. Cells from all positive wells (6.4.12) were used to produce frozen stocks whilst cells from strongly-positive wells were also used in a first cloning procedure (see below).

Cells were transferred to wells, containing 1ml of fresh medium, of 24-well tissue culture plates after initial growth (6.4.11) and after cloning procedures (see below). Hybridoma cells of interest thus transferred were refed twice weekly with 0.5mls of fresh medium (after 1 week this required removal of an equivalent volume of SN) and examined daily. When transferred from initial cultures the medium used was $RPMI_{15}HAT$ (6.1.3). Cells transferred after first and subsequent cloning procedures were grown in $RPMI_{15}HT$ and $RPMI_{15}$ (6.1.3), respectively.

Cells selected for cloning were submitted to the cloning procedure described below within 3 days of transfer to a 24-well plate. A mouse was killed by cervical dislocation and its spleen removed aseptically, then homogenised into 10ml of RPMI. Freed splenocytes were pelleted by centrifugation at 1000 rpm for 10min, then resuspended in 7ml of medium (first cloning-$RPMI_{15}HAT$, second cloning-$RPMI_{15}HT$, third cloning-$RPMI_{15}$). After waiting 2min for large aggregates to settle out, the cell suspension was transferred to a fresh tube, counted (6.4.1), then irradiated (2.5krad, ^{137}Cs). The cell suspension was diluted, in the same medium, to 5 x 10^6 cells/ml, plated out at 100µl/well in the central 60 wells of 96-well, flat-bottomed tissue culture plates and incubated to provide 'feeder' cells. 24h later, cells selected for cloning were suspended, harvested by centrifugation at 1000 rpm for 5min, resuspended in 6ml of the same medium used for the splenocytes and counted (6.4.1). (Remaining cells in the emptied well of the 24 well plate were refed with 1ml of the same medium and cultured, to later form a frozen stock [see below].) One of two protocols was then adopted. When sufficient cells were obtained, they were successively diluted to each of three concentrations:- 50,10 and 5 cells /ml. 100µl of each dilution was added to 20 wells with feeder cells. When insufficient cells were obtained, they were simply diluted to an estimated 8 live cells /ml and plated at 100µl /well in each of

60 wells with feeder cells. All plates were then incubated for a further 2-4 weeks. After 7d, wells were examined by light microscopy for the presence of individual hybridoma colonies. Only those wells with a single colony were retained. These were now refed twice weekly with 50µl of fresh medium as above (with removal of an equivalent volume of SN when required) and 'clustered' colonies were dispersed by agitation. Once hybridoma cells in any well reached confluency, 200µl of SN was removed and analysed (6.4.12) whilst the cells were suspended in remaining SN and transferred to a well of a 24-well plate.

After a first cloning, any clones whose SN's gave a positive result in an ELISA against the immobilised negative control preparation (6.4.12) were discarded. Other clones, selected on the basis of rate of growth and magnitude of ELISA results, were submitted to second and third clonings. Some third clones were then similarly chosen to provide cells for larger numbers of frozen stocks. After 48h in one well of a 24-well plate, these were divided 1:3 and 24h later further divided 1:2 (1ml of fresh medium/well at each division), before incubation was continued as below.

Cells destined to form frozen stocks were incubated until they neared 50% confluency, when they were suspended, centrifuged at 1000 rpm for 5min, resuspended in 1ml of freezing mix (6.1.3) and slowly frozen to -70°C before being transferred to liquid nitrogen for storage. Cells remaining in the well were refed with 1ml of fresh medium and regrown as above to allow storage of a second vial.

6.4.14. Production of saturated supernatant:-

1 frozen vial of a selected hybridoma third clone (6.4.13) was quickly thawed and transferred to 20ml of $RPMI_{15}$ (6.1.3) in a $75cm^2$ flask. After 7d growth, this was divided between 2 similar flasks which were then 7/8 filled with fresh medium and incubated for a further 2 weeks. The SN's were clarified by centrifugation at 2,500 rpm for 10min, aliquoted and stored at -20°C until needed.

6.4.15. Isotyping of monoclonal antibodies:-

The isotype of monoclonal antibodies was determined using a commercial kit (Isotype plus, Sigma) according to the manufacturer's instructions.

6.5. Maedi-Visna virus: stocks, analysis and experimental protocols:-

6.5.1. Maedi-Visna virus stocks:-

Aliquots of MVV, strain EV-1 (Sargan et al., 1991a), were obtained from departmental stocks. These comprised the clarified (by centrifugation) SN from a culture of normal, ovine fibroblasts, which had been infected with MVV and incubated, in DME with 5% v/v FCS, penicillin and streptomycin, to 100% cytolysis, then stored at -70°C. This stock contained 10^5 $TCID_{50}$/ ml as had been previously judged by limiting dilution analysis using the same cell line (C.Cousens, personal communication). A conditioned medium was prepared for use as a negative control by similarly clarifying the SN from an uninfected culture of the same fibroblast line grown in the same medium for the same period, then aliquoting it and similarly storing at -70°C.

The level of contamination of these stocks by LPS was estimated using a commercial kit (E-toxate, Sigma) to the manufacturer's instructions.

6.5.2. MVV reverse transcriptase assays:-

RT assays were performed according to Sargan et al. (1991b). 15µl samples for assay were placed, in duplicate, in wells of 96-well, round-bottomed plates. 10µl of 2.5x RT reaction mix (7.1.3) were added and the plate incubated at 37°C for 90min. Reactions were stopped by adding 100µl of 10% w/v TCA, 50µg/ml yeast RNA to each well. Plates were left on ice for 15min before reaction mixes were harvested onto glass fibre mats using a titertek cell harvester. Filters were washed for 40s with 5% w/v TCA, 3% w/v NaPPi and for a further 40s with 70% v/v ethanol, then dried. Radioactivity incorporated into acid-precipitable DNA was then measured by immersing the glass fibre discs in scintillant (Optisafe, LKB) and counting emissions in a rack β-scintillation counter.

6.5.3. Infecting ovine fibroblasts with virus. Addition of recombinant proteins:-

2 experiments were performed to assess the impact of rovTNFα on MVV-infected WSCP cells using slightly different protocols. In the first experiment, WSCP cells (6.4.4) were plated into the wells of 2 duplicate, 96-well, flat-bottomed, tissue culture plates at 10,000 cells /well in 100µl of DME medium with 5% v/v FCS, penicillin and streptomycin. After 2d incubation, 50µl of virus stock or conditioned medium (6.5.1), each diluted 1:3 with the above medium, were added to each well, as were 50µl of sample, 2d later. All samples were assayed in sextuplicate and comprised recombinant proteins (6.3.7,8), at 4x final concentration, in PBS with 0.1% w/v BSA. Some wells contained the above additions but no cells. Plates were then returned to the incubator and assessed daily by light microscopy. 9d PI, 1 plate was frozen at -20°C, thawed, refrozen, and thawed again before well contents were mixed and analysed (6.5.2). SN's were removed from the second plate. The cells on this plate were rinsed with PBS (6.1.3), stained with 0.25% w/v crystal violet in 20% v/v methanol (40µl/well) for 2min, rinsed gently, but thoroughly, in water, and the OD_{540} of each well was measured on an ELISA plate reader.

In a second experiment, WSCP cells were plated at 8,000/well in 100µl of the above medium, again in wells of 2 duplicate 96-well plates. 1 week later, SN's were removed and cells refed with DME + 2% FCS, penicillin and streptomycin (100µl/well). After 6d, 50µl samples (as above but with recombinant proteins at 3x final concentration), all in sextuplicate, were added and plates returned for incubation. 24h later, 50µl of virus stock or uninfected conditioned medium (each diluted as above, but including samples with recombinant proteins at final concentration) were added. Plates were then incubated, frozen (at indicated time-points PI) and thawed twice, and analysed as above.

6.5.4. Infecting ovine lung cells with virus. Addition of recombinant proteins:-

In each experiment involving MVV infection of ovine lung cells, cells from a pair of lungs (6.4.2) were plated into wells of several 24-well tissue culture plates at (initially) 2×10^5 cells/ml, 1ml/well and incubated, with refeeding (6.4.2), for 7-9d before

infection. On infection, all old medium was removed and replaced, in all but one experiment, with 0.75ml of fresh medium and 0.2ml of neat virus stock or conditioned medium (6.5.1).

In the exception (fig.5.8), 2ml of virus stock or conditioned medium (+/- added LPS) were added to a well for 2h, then removed before the wells were washed once with PBS and filled with 0.5ml of fresh medium. These SN's were removed at assorted time points thereafter before the proteins they contained were concentrated (6.3.11) and analysed (6.3.12,14).

In all other cases, 50µl of sample (PBS with 0.1% w/v BSA +/- recombinant proteins [6.3.7,8] at 20x final concentration) were added at the time of infection or 24h later as indicated. Cells were refed 4, 7 and (where applicable) 10 d PI, by adding 250µl of medium containing the same final concentration of sample /well. In these experiments, plates were frozen at -20°C at indicated times after infection, then thawed, refrozen and rethawed before well contents were mixed for analysis (6.5.2). To assess the production of ovine TNFα in one of these experiments (table 5.2, experiment 3), proteins in the remaining contents from cells which had been exposed to samples of PBS with BSA only, were concentrated (6.3.11) and analysed (6.3.12,14).

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APPENDIX 1:- ABBREVIATIONS.

The following list (in alphabetical order) defines all abbreviations used in this thesis, with the exception of standard abbreviations for amino acids, and those abbreviations which have been used to describe some buffers, media and other solutions. The latter are listed in 6.1.3.

A:- adenosine.
aa:- amino acid(s).
abs.:- absorbance.
ACTH:- adrenocorticotrophic hormone.
ADP:- adenosine diphosphate.
AIDS:- acquired immunodeficiency syndrome.
APS:- ammonium persulphate.
ATP:- adenosine triphosphate.
b:- base(s).
BCG:- Bacillus Calmette-Guérrin.
BCIP:- 5-bromo-4-chloro-3-indolyl phosphate.
bp:- base pair(s).
BSA:- bovine serum albumin.
BSOCOES:- bis [2-(succinimido-oxy-carbonyloxy) ethyl] sulphone.
c.:- circa.
C:- cytosine
cAMP:- cyclic adenosine monophosphate.
cDNA:- DNA complementary to mRNA.
CFA:- complete Freund's adjuvant.
cGMP:- cyclic guanidine monophosphate.
CHAPS:- 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulphonate.
CIP:- calf intestinal phosphatase.
CNS:- central nervous system.
conc$^{(n)}$:- concentration.
CPE:- cytopathic effect(s).
cpm (or c.p.m.):- counts per minute.
CSF:- cerebro-spinal fluid.
d:- day(s).
DAG:- diacyl-glycerol.

dGTP:- deoxy-guanidine triphosphate.

dITP:- deoxy-inositol triphosphate.

DME:- Dulbecco's modified Eagles (medium).

DMSO:- dimethyl sulphoxide.

DNA:- deoxy-ribonucleic acid.

dNTP:- deoxy-nucleoside triphosphate.

DOC:- deoxy-cholate (or deoxy-cholic acid).

ds.:- double-stranded.

DTT:- dithiothreitol.

EAE:- experimental allergic encephalomyelitis.

EDTA:- ethylene diamine tetra-acetic acid.

EGF:- epidermal growth factor.

EGTA:- ethylene glycol tetra-acetic acid.

ELAM:- endothelial-leukocyte adhesion molecule.

ELISA:- enzyme-linked immunoabsorbent assay.

EM:- electron microscopy.

eq. dil^n:- equivalent dilution.

EtBr:- ethidium bromide.

Expt:- experiment

FCS:- foetal calf serum.

FSH:- follicle stimulating hormone.

G:- guanidine.

GM-CSF:- granulocyte/macrophage colony stimulating factor.

gp:- glycoprotein.

h:- hour(s).

HBSS:- Hanks' balanced salt solution.

HCG:- human chorionic gonadotrophin.

HIV:- human immunodeficiency virus.

HTLV:- human T-lymphotrophic virus.

I:- inositol.

ICAM:- intercellular adhesion molecule.

IFA:- incomplete Freund's adjuvant.

IFN:- interferon.

Ig:- immunoglobulin.

IL:- interleukin.

inc:- incorporated.

IP:- intraperitoneal(ly).

IPTG:- isopropyl *β*-d-thiogalactopyranoside.

IV:- intravenous(ly).

kb:- kilobase(s).

kDa:- kilodalton(s).

LAK:- lymphokine-activated killer.

LGT:- low gelling-temperature.

LPL:- lipoprotein lipase.

LPS:- lipopolysaccharide.

LT:- lymphotoxin.

LTR:- long terminal repeat.

M-CSF:- macrophage colony stimulating factor.

ME:- mercaptoethanol.

MHC:- major histocompatability complex.

min:- minute(s).

MnSOD:- manganous superoxide dismutase.

moi:- moiety of infection.

mRNA:- messenger RNA.

MS:- multiple sclerosis.

MTM:- mixed thymocyte medium.

MV:- Maedi-Visna.

MVD:- Maedi-Visna disease.

MVV:- Maedi-Visna virus.

NA:- nucleic acid.

NBL:- Northumbria Biologicals Limited.

NBT:- nitro-blue tetrazolium.

neg cntrl:- negative control.

NF:- nuclear factor.

NGF:- nerve growth factor.

NK:- natural killer.

no.s:- numbers.

OAc:- acetate.

OD_x:- optical density at x nm.

OPD:- orthophenylenediamine.

ORF:- open reading frame.

PAF:- platelet activating factor.

PAGE:- polyacrylamide gel electrophoresis.

PBM:- peripheral blood mononuclear (cells).

PBS:- phosphate-buffered saline.

PCR:- polymerase chain reaction.

PDGF:- platelet-derived growth factor.

PEG:- polyethylene glycol.

PG:- prostaglandin.

PHA:- phytohaemagglutinin.

PI:- post-infection.

PL:- phospholipase.

PPi:- pyrophosphate.

r:- recombinant.

-R:- receptor.

rboTNFα:- recombinant bovine TNFα.

RFLP:- restriction fragment length polymorphism.

rhTNFα:- recombinant human TNFα.

RIA:- radioimmunoassay.

RNA:- ribonucleic acid.

rovIL-1β:- recombinant ovine interleukin-1 beta.

rovTNFα:- recombinant ovine TNFα.

RP:- reverse sequencing primer.

rpm:- revolutions per minute.

RT:- reverse transcriptase.

s:- second(s).

SC:- sub-cutaneous(ly).

SCID:- severe combined immunodeficiency.

SD:- standard deviation.

SDS:- sodium dodecyl sulphate.

SIV:- simian immunodeficiency virus.

SN:- supernatant.

ss.:- single-stranded.

SSN:- saturated supernatant.

T:- thymidine.

T°:- temperature.

TB:- tuberculosis.

TCA:- tricarboxylic acid.

$TCID_{50}$:- 50% tissue culture infectious dose (i.e. that dose of infectious agent capable of successfully infecting 50% of susceptible cultures to which it is applied).

TCR:- T-cell receptor.

TEMED:- Tetramethyl ethylene diamine.

TGF:- transforming growth factor.

T_m:- annealing temperature.

TNF:- tumour necrosis factor.

TTP:- thymidine triphosphate.

U:- uracil.

UM:- unconditioned medium.

UTR:- untranslated region.

UV:- ultra violet.

v:- volume.

VCAM:- vascular cell adhesion molecule.

VLP:- virus-like particle.

w:- weight.

WSCP:- Weybridge sheep choroid plexus (cells).

APPENDIX 2:- PAPERS PUBLISHED.

The papers included in the following pages have been published as a result of the work described in this thesis. Publisher's permission has been granted to include a photocopy of the second paper.

Gene, 109 (1991) 203–210

GENE 06184

Sequence of the cDNA encoding ovine tumor necrosis factor-α: problems with cloning by inverse PCR

(Amino acid sequence homology; cachectin; cytokine; gene amplification; polymerase chain reaction; recombinant DNA; RNA induction)

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SUMMARY

We have cloned and sequenced the ovine tumor necrosis factor-α (TNF-α)-encoding cDNA, using gene amplification by polymerase chain reaction (PCR) technology, to aid studies of assorted diseases in this species. We used primers selected from published *TnfA* sequences of other species on a cDNA template prepared from lipopolysaccharide-stimulated ovine alveolar macrophages, to generate a product representing the central region of the molecule. We then used a novel method based on 'inverse PCR' to generate a product containing the 5′ and 3′ ends of the molecule. Here, we present the complete sequence of the ovine TNF-α cDNA and compare it with other published TNF sequences. The cloned cDNA has a leader sequence of 156 bp followed by a protein-coding sequence of 702 bp and a 3′-untranslated region of 800 bp. The protein product of the gene is a protein of M_r = 25 586, 79% homologous to human TNF-α. An mRNA produced by alveolar macrophages, which hybridises to the cloned gene, is induced greatly, with a peak induction time of approx. 135 min, in response to stimulation by lipopolysaccharide and to plating on plastic. We also discuss the resolution of some artefacts of the inverse PCR technique.

INTRODUCTION

Tumor necrosis factor-α (TNF-α, also known as cachectin) is an inflammatory cytokine produced by activated macrophages, Langerhans cells and astrocytes, and in smaller quantities by NK cells and other lymphocytes or lymphocytic cell lines, and by mast cells. TNF-α's cytocidal and lymphocyte activating roles have been extensively reviewed (Maennel, 1986; Beutler and Cerami, 1989), and a very large number of effects on many other cell types have been catalogued. It is present at elevated levels in a variety of disease states including septic shock (reviewed in Cerami and Beutler, 1988), arthritis, and several autoimmune states (see for example Teppo and Murray, 1987), and cachexia associated with various cancers (Balkwill et al., 1987), as well as in a variety of viral, parasitic and other diseases. Sequences of *TnfA* genes from a number of species including human, rabbit, and mouse, as well as partial sequences from pig and cat have been published (Pennica et al., 1984; 1985; Shirai et al., 1985; Ito et al., 1986; McGraw et al., 1990; Pauli et al., 1989; Drews et al., 1990). These sequences code for preproteins of 233–235 aa in length which are cleaved to give 156 or 157 aa mature proteins. These proteins are approx. 80% homologous at the aa level, somewhat better conserved than many other cytokine

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Abbreviations: aa, amino acid(s); bp, base pair(s); BSA, bovine serum albumin; cDNA, DNA complementary to mRNA; ds, double strand(ed); DTT, dithiothreitol; EtdBr, ethidium bromide; kb, kilobase(s) or 1000 bp; LPS, lipopolysaccharide; NK, natural killer; nt, nucleotide(s); PCR, polymerase chain reaction; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase; ss, single strand(ed); SSC, 0.15 M NaCl/0.015 M Na_3 · citrate pH 7.6; TNF, tumor necrosis factor; *TnfA* and *TnfB*, genes encoding TNF-α and TNF-β; u, unit(s).

sequences. TNF-β or lymphotoxin has a similar spectrum of biological activity to TNF-α, but is only 30% homologous at the aa sequence level.

In cloning highly conserved genes from particular species PCR has been used a number of times when a homologous gene from a different species has already been sequenced (e.g., Lee et al., 1988). The approach has been successfully transferred to ovine cytokine genes by this laboratory (Fiskerstrand and Sargan, 1990), and others (Seow et al., 1990; McInnes et al., 1990). A problem with the use of PCR in cloning has been the difficulty of obtaining complete gene sequences including both 5′ and 3′ ends of the transcript. Several approaches to this problem have been described including the use of 'anchored' PCR in which the molecule to be cloned is attached to a vector or terminal oligo of known sequence, and the PCR run off this sequence (Ohara et al., 1989), and 'inverse' PCR in which the molecule to be cloned is circularised and amplified using outward facing primers (Ochman et al., 1988; Triglia et al., 1988).

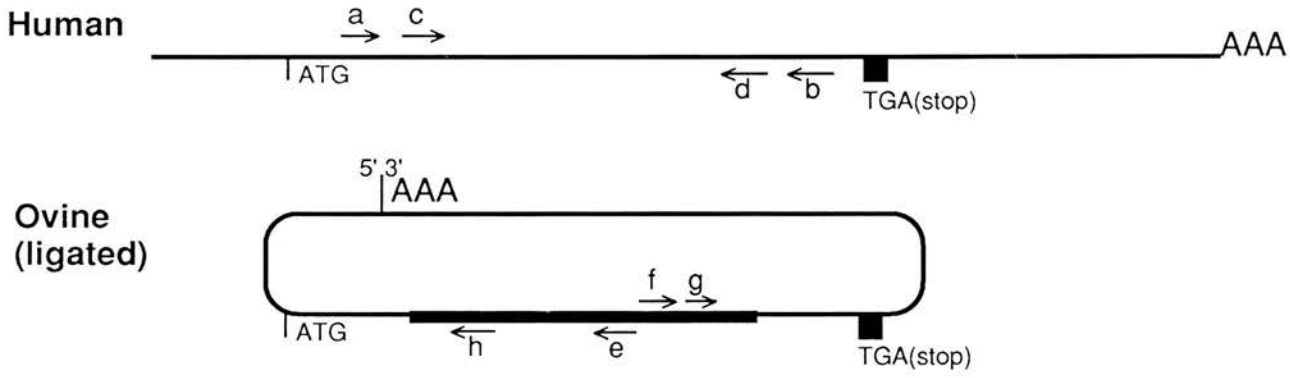
The aim of this study was to clone the complete cDNA encoding ovine TNF-α by using direct and inverse PCR. The derived protein sequence from this molecule is compared with other published TNF-α sequences. Northern-blot analysis is used to study its expression in isolated alveolar macrophages. Problems encountered in cloning using inverse PCR are discussed.

RESULTS AND DISCUSSION

(a) Generation of a *TnfA* gene fragment and analysis of *TnfA* gene expression in macrophages

Published sequences of *TnfA* genes of rabbit, mouse and human were compared to identify areas of the molecules which were highly conserved between these species, and PCR primers were constructed from these areas (primers a, b, c, and d, Fig. 1). Ovine alveolar macrophages were stimulated in vitro with 100 ng LPS/ml for 2 h and used as a source of RNA containing *TnfA* transcripts (Kawakami and Cerami, 1981). An ss cDNA was prepared from this RNA and used as a template for gene amplification using these primers. After two successive rounds of 35 cycles of PCR using nested primers, a band of 493 bp was identified by gel electrophoresis (Fig. 2a), cut out from the gel, and sequenced directly. Some of the DNA was cloned into a phagemid vector. A clone was picked and sequenced to ensure that it was representative of the whole PCR-generated band. The DNA was approx. 80% homologous to the equivalent region of the human *TnfA* gene.

This clone was used as a probe in Northern-blot analysis of RNA collected from ovine alveolar macrophages at various time points after plating out and simultaneously adding LPS (Fig. 2b). A single species of RNA of size approx. 1.85 kb hybridised to the probe. This RNA was inducible,



Primers

```
a) CTC AGC CTC TTC TCI TTC CTG     (sense, 249-269)
b) CCA AAG TAG ACC TGC CCR GAC TC  (antisense, 816-838)
c) CAC CAC GCT CTT CTG CC          (sense, 284-300)
d) GGT CAC CCT TCT CCA ICT GG      (antisense, 752-771)
e) GCA GAG AGG ATG TTG ACC         (antisense, 657-674)
f) ATC AAG AGC CCT TCC CAC AGG     (sense, 676-696)
g) AAG CCC TGG TAC GAA CCC         (sense, 721-738)
h) ACC AGA GGC CTG TTG AAG G       (antisense, 362-380)
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Fig. 1. Cloning strategy and positions of primers used in this paper. The structure of a human *TnfA* cDNA molecule and a circularised ovine *TnfA* cDNA molecule are shown, together with the initial methionine and stop codons of the structural sequences. The positions of primers referred to elsewhere in this paper are shown by numbering relative to the human sequence of Pennica et al. (1984) (primers **a,b,c,d**) or to the cloned ovine sequence given in Fig. 4 (primers **e,f,g,h**). The sequences of primers **a–d** were derived after comparison of the human, murine and rabbit *TnfA* sequences. At positions where these sequences differed in primers **a** and **c** inosine residues (**I**) were incorporated in the primer. In primer **b**, **R** represents a 50 : 50 mixture of A and G residues at this position. The bold line represents the segment of the ovine cDNA cloned using the human derived primers, and sequenced prior to cloning the remainder of the gene using inverse PCR (referred to elsewhere as the ovine *TnfA* central region clone).

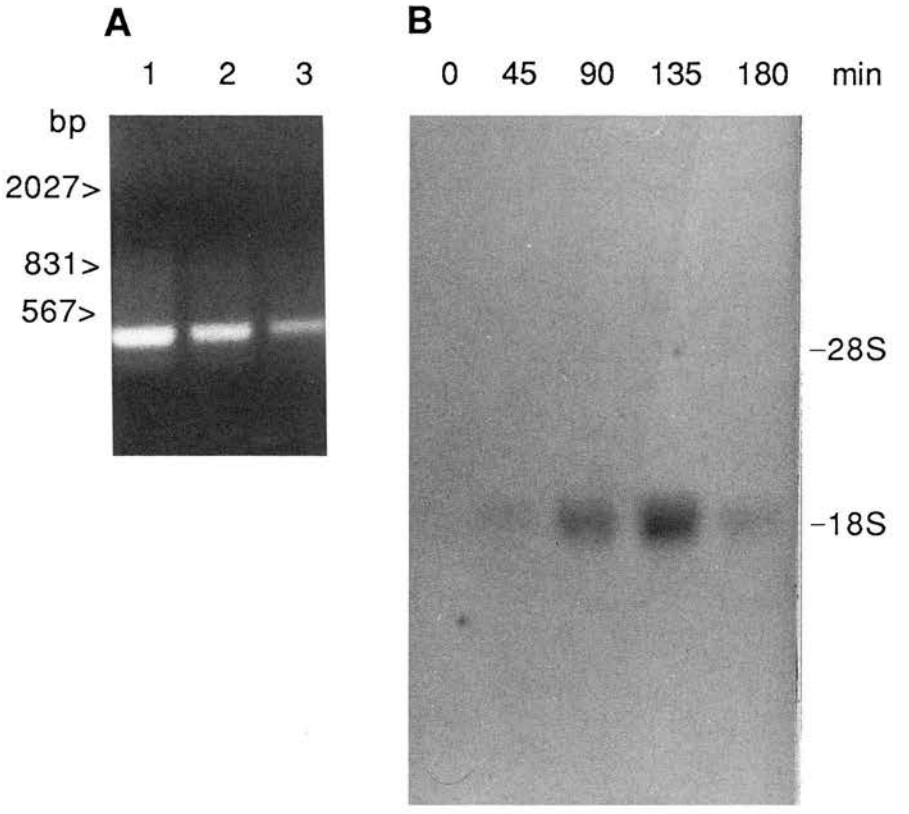

Fig. 2. Production of a clone of the central region of the ovine *TnfA* cDNA and its use to analyse *TnfA* gene expression in alveolar macrophages. (Panel **A**) A *TnfA* cDNA fragment generated by nested PCR on total RNA from stimulated alveolar macrophages. Lanes **1**, **2**, and **3** show products of nested PCR using *TnfA* specific primers on three different cDNA populations. **Methods**. Cell populations rich in alveolar macrophages (typically about 85% pure) were obtained by bronchio-alveolar lavage of Scottish blackface sheep at post mortem. Erythrocytes were removed by hypotonic shock, and the cells washed in Hanks buffered saline solution and transferred into serum-free Iscove's medium, before plating out into 75 cm^2 tissue-culture bottles at a density of 2×10^7 cells/bottle. Macrophages (which are partially stimulated by adherence to plastic; data not shown), were further stimulated by the addition of lipopolysaccharide (from *Salmonella abortus equi*) to 100 ng/ml. RNA was prepared by a slight modification of the method of Chirgwin et al., 1981 (see Sargan et al., 1986). cDNA was synthesised using a dT_{12-18} primer from total RNA. Nested PCR's were performed using primers **a** and **b** followed by **c** and **d** (see Fig. 1). PCR's were carried out in 100 μl samples in 10 mM Tris · HCl pH 8.8/1.5 mM $MgCl_2$/3 mM DTT/50 mM KCl/170 μg/ml BSA/100 μM dNTPs/1 u *Taq* DNA polymerase, using 0.1 μM of each primer and 5–25 ng of cDNA (or 1 μl of a previous PCR). 35 cycles of 0.5 min at 95°C, 0.3 min at 48°C (or 40°C for primers **a** and **b**) and 1.5 min at 70°C were used. The 10-μl samples from these reactions were analysed by electrophoresis on 1% agarose gels containing 40 mM Tris · acetate pH 7.8/1 mM EDTA and staining with 0.5 μg/ml EtdBr. (Panel **B**) Northern-blot analysis of *TnfA* RNA in alveolar macrophages stimulated by LPS and adherence. Alveolar macrophages were prepared and stimulated with LPS as before. At the times after stimulation shown, 10 μg of total RNA were analysed by electrophoresis on 1.2% agarose/7.2% formaldehyde gels containing 20 mM Na · phosphate pH 7.0, blotted onto nitrocellulose, and probed using the ovine *TnfA* central region clone by standard methods (Thomas, 1980). The positions of 28S rRNA (4.7 kb) and 18S rRNA (1.85 kb) are shown.

reaching a maximum concentration at about 135 min after stimulation.

(b) Generation of a clone of the remainder of the *TnfA* gene

RNA from the 135-min time point was used to prepare ds cDNA which was self-ligated. The product was used to drive an inverse PCR (as described by Triglia et al., 1988, and Ochman et al., 1988) with primers e and f. Circularised *TnfA* encoding cDNA molecules can act as templates in PCR to generate the 5′ and 3′ ends of the *TnfA* molecule, whilst retaining the ability to hybridise to probes derived from the cloned central portion of *TnfA*. The main product of this PCR hybridised to the central region probe (Fig. 3, lanes 1, 2, 5 and 6), but was only 1000 bp long instead of the anticipated size of about 1800 bp.

This result might occur in three ways. (*i*) During the self ligation step, bimolecular events causing the joining of cDNA molecules head-to-head or tail-to-tail predominate over intramolecular circularisation or intermolecular head-to-tail joining (these forms would not be of the expected length); (*ii*) the cDNA was incompletely but uniformly extended towards the 5′ end, so that a shortened product was formed; (*iii*) during cDNA synthesis, oligo(dT) mis-priming was occurring off A-rich sequences within *TnfA* cDNA molecules, causing a ds cDNA shortened at the 3′ end to predominate. The first hypothesis is unlikely because

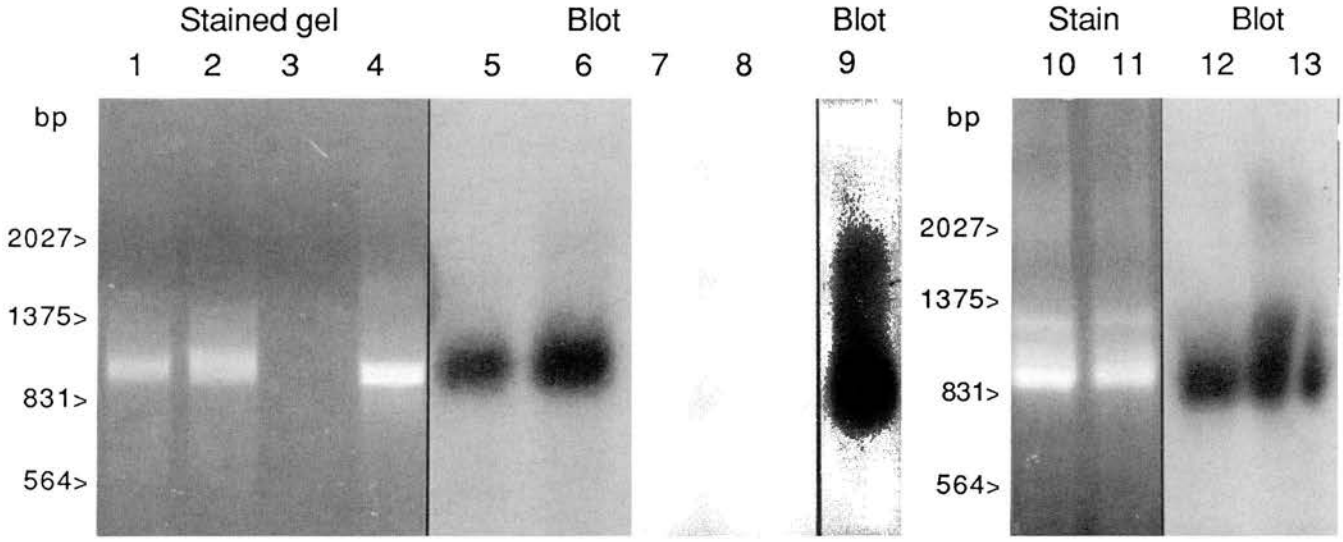

Fig. 3. Products of inverse PCR with different primers. Products of PCR on cDNA from stimulated alveolar macrophages with different sets of primers are shown, both on EtdBr-stained agarose gels (as before), and on Southern blots of these gels. PCR used: lanes **1**, **2**, **5** and **6**, primers **e** and **f** (duplicate reactions); lanes **3** and **7**, primer **e** alone; lanes **4** and **8**, primer **f** alone; lane **9**, primers **e** and **g**; lanes **10–13**, primers **g** and **h** (duplicate reactions). Southern blots were carried out using standard methods (Sambrook et al., 1989). Stringencies for hybridisations were 4 × SSC at 65°C for 16 h, and filters were washed to a final stringency of 0.3 × SSC at 65°C. Lanes **5–9** were probed with the ovine *TnfA* central region clone. A clone containing the 1-kb product from PCR with primers **e** and **g** was used to probe lanes **5** and **6**.

the frequency of bimolecular collisions involving two *TnfA* cDNA molecules rather than a *TnfA* cDNA molecule and a cDNA molecule from a different gene should be very low (from the intensity of Northern-blot signals we estimate that *TnfA* RNA represents about 0.1% of the mRNA from which the cDNA was made). Only the former would allow the possibility of head-to-head or tail-to-tail amplification by primers e or f. Nonetheless the hypothesis was tested by performing a second PCR on 1 μl of the ligated product from the first PCR using either one or other of the two primers e and f, by itself. Neither primer alone was capable of generating a band which could hybridise to our TNF central region clone (though primer f alone did generate a band of about 1000 bp, incapable of so hybridising, and therefore distinct in sequence from at least part of the 1000 bp band generated by primers e and f used together) (Fig. 3, lanes 4 and 8).

A PCR was therefore performed on 1 μl of the product of the e + f-primed inverted PCR using primers e and g (Fig. 3, lane 9). Again this generated a band of 1000 bp which hybridised to the *TnfA* central region probe. Cloning and sequencing of this product showed that it was produced from cDNA molecules incompletely extended to the 5′ end of the *TnfA* gene: sequence just 5′ to primer e was joined to sequence derived from the poly(A) tail. Repeated preparations of cDNA, circularised and subject to repeat PCRs were never successful in producing a larger main product (data not shown). A smear of larger DNA molecules within this PCR product hybridised to the *TnfA* central region probe, including many far larger than the maximum expected size. These might represent molecules produced by polymerase travelling round the original circular template more than once, in a 'rolling circle' fashion. We know of no reports of whether *Taq* DNA polymerase is capable of strand displacement, but such a reaction would be favoured by the high temperature during polymerisation. The DNA in the smear was purified by agarose gel electrophoresis and used as a template in a further round of PCR. The main product was again a band of 1000 bp, consistent with the larger bands containing a concatemer of the 1000 bp molecule (data not shown).

Another test PCR on the 'smear' product, using primers c and e, where primer c is 5′ to the end of the 1000 bp band, produced a product of 390 bp (data not shown), the size anticipated from our known sequence – revealing that amongst these large molecules, there were also molecules which originated from more completely made cDNA molecules. A final PCR was therefore performed using primers h and g where h was selected from as near to the 5′ end of the known central region as possible. (Any product from these two primers would now have little overlap with the original clone, but share 3′ sequence with the 1000 bp clones.) The main product of the PCR was once again a band of approx. 1000 bp. However, another band was present of about 1300 bp. Both hands hybridised to the 1000-bp clones (Fig. 3, lanes 10–13). A cloned copy of the larger band was sequenced to generate all the sequence data 5′ to the central clone. This sequence was checked by direct sequencing of the PCR product. The full sequence is presented in Fig. 4.

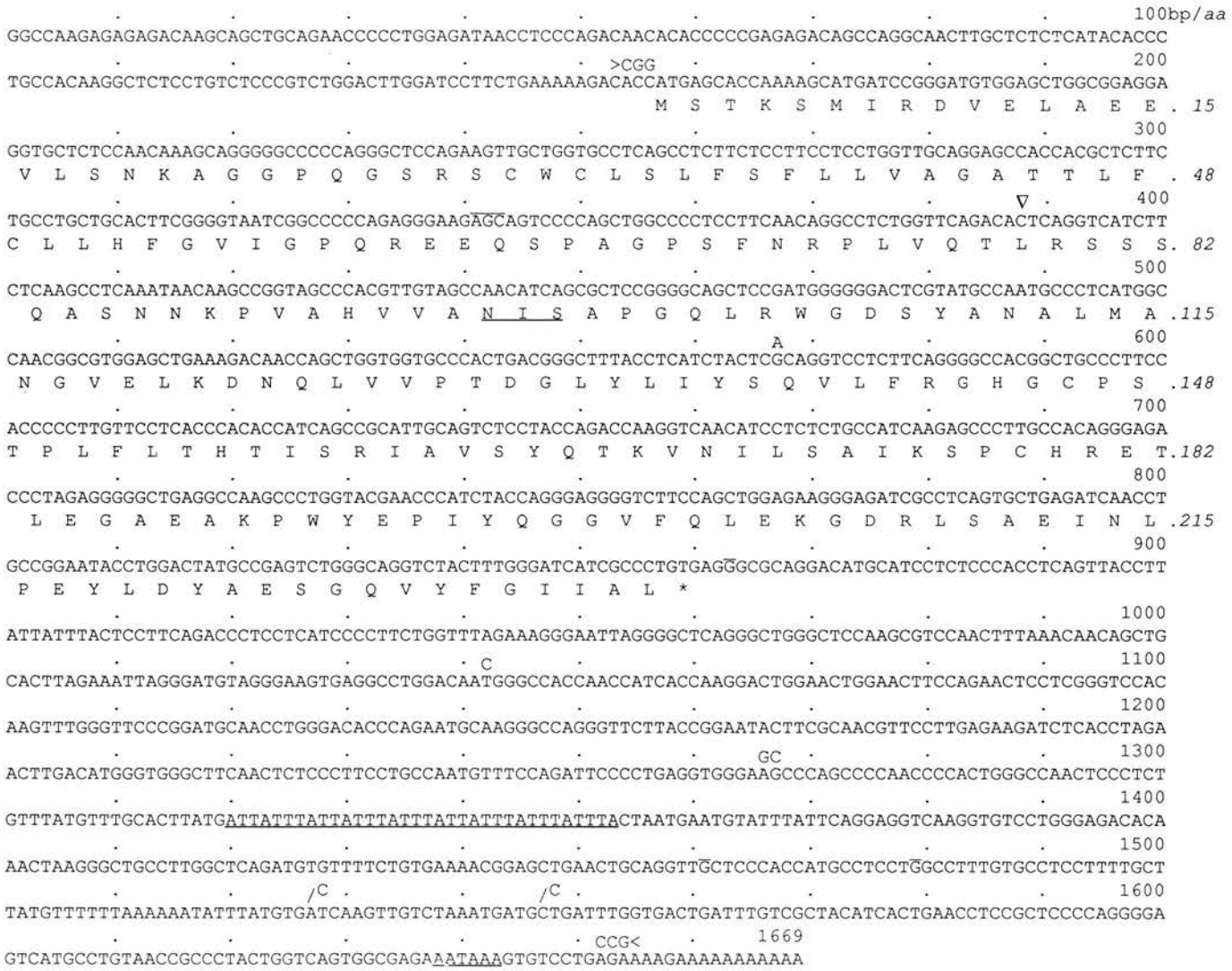

Fig. 4. The complete sequence of ovine *TnfA* cDNA and TNF-α. The nt and derived aa sequences are shown. The positions of a potential glycosylation site (NIS) and of the conserved A + T-rich destabilising signals are underlined, as is the polyadenylation signal (AATAAA). The presumed cleavage site between pre-protein and mature protein (between aa 77 and 78) is shown by an arrowhead at aa 78. Differences from a previously published ovine *TNF* sequence (Young et al., 1990) are shown as superscripts, with overlines representing deletions of the overlined nt [the start and finish positions of the Young et al. (1990) sequence are bracketed by arrowheads at nt positions 153 and 1654]. The asterisk after aa 234 represents a stop codon. **Methods**. For cloning, PCR products were made blunt-ended by filling-in with PolIk and phosphorylated using T4 polynucleotide kinase. After PCR, nucleic acids were recovered from the reaction by extraction with phenol/chloroform and ethanol precipitation, and resuspended in 50 μl of 10 mM Tris · HCl pH 7.5/7 mM $MgCl_2$/0.1 mM EDTA/5 mM DTT/10 mM ATP/30 u of T4 polynucleotide kinase. After 30 min at 37°C, 0.6 mM each of dATP, dCTP, dGTP, TTP and 2.5 u of PolIk were added. Reactions were allowed to proceed for a further 30 min at room temperature. Amplified fragments were purified by preparative electrophoresis on a 1.2% agarose gel and recovered by electroelution (McDonell et al., 1977), phenol/chloroform extraction and ethanol precipitation. They were resuspended in 50 mM Tris · HCl pH 7.4/10 mM $MgCl_2$/2 mM DTT/1 mM spermidine/1 mM ATP and ligated into the *Sma*I site of the phagemid pTZ19R (Pharmacia). Recombinant clones containing an insert of appropriate size were selected by hybridisation to the PCR product. Single-stranded phagemid DNAs were prepared as recommended by Pharmacia and sequenced by the chain-termination method of Sanger et al. (1977), using a commercial kit (U.S. Biochemical Corp., Cleveland, OH). For direct sequencing, ds PCR products were purified by electrophoresis in low-gelling-temperature agarose (1.2%) prior to sequencing by the method of Casanova et al. (1990). Sequences were assembled and analysed using version 6.2 of the University of Wisconsin Genetics Computer Group package (Devereux et al., 1984) and submitted under EMBL database sequence accession No. X55152.

(c) Sequence features of ovine TNF-α

The sequence determined here for the ovine *TnfA* gene product would encode a protein of 234 aa, with an M_r of 25 586. There is one potential Asn-linked glycosylation site. The ovine gene shows 85% homology to human TNF-α in the coding sequence, 80% homology in the RNA leader sequence and 77% in the 3′-untranslated region. The deduced aa sequence is between 72 and 79% identical to

```
                    .         .         .         .         .
OVINE     1 MSTKSMIRDVELAEEVLSNKAGGPQGSRSCWCLSLFSFLLVAGATTLFCL
HUMAN       ...E..........A.PK.T.......R.LF.......I..........
MOUSE       ...E..........E.PQ.M..F.N..R.L....................
RABBIT      ...E.........GP.PK........KR.L....................
                    .    *    .         ∇         .         .
OVINE    51 LHFGVIGPQREEQSPAGPSFNRPLVQ--TLRSSSQASNNKPVAHVVANIS
HUMAN       ............-F.RDL.LIS..A.--AV....RTPSD.........PQ
MOUSE       .N........D.KF.N.LPLISSMA.TL.......N.SD.........HQ
RABBIT      ...R.....E.....NNLHLVN.VA.MV....A.R.LSD..L......PQ
                    .         .         .         .         .
OVINE    99 APGQLRWGDSYANALMANGVELKDNQLVVPTDGLYLIYSQVLFRGHGCPS
HUMAN       .E...Q.LNRR....L......R.......SE...........K.Q....
MOUSE       VEE..E.LSQR....L...MD.........A.....V......K.Q...D
RABBIT      VE...Q.LSQR....L...MK.T.......A............S.Q..R.
                    .         .         .         .         .
OVINE   149 TPLFLTHTISRIAVSYQTKVNILSAIKSPCHRETLEGAEAKPWYEPIYQG
HUMAN       .HVL..................L.........Q...P............L.
MOUSE       Y-VL....V..F.I...E...L...V....PKD.P....L........L.
RABBIT      Y-VL....V..F....PN...L............P.E..PMA......L.
                    .         .         .         .
OVINE   199 GVFQLEKGDRLSAEINLPEYLDYAESGQVYFGIIAL 234
HUMAN       ................R.D...F.............
MOUSE       .........Q....V...K...F.........V...
RABBIT      ............T.V.Q.....L.............
```

Fig. 5. Comparison of ovine TNF-α with other published *TNF* sequences. Dots in the human, murine and rabbit sequences represent residues conserved relative to the ovine sequence. Dashes represent gaps introduced to maximise the alignment. The first aa of the mature protein is shown by an open arrowhead. A single aa insertion (Gln) compared with the sequence of Young et al. (1990), is shown by an asterisk above the residue. The potential glycosylation site (NIS) is shown in bold type. The other sequences shown are taken from Shirai et al. (1985; human); Pennica et al. (1985; mouse) and Ito et al. (1986; rabbit).

previously published mammalian TNF-α sequences (Fig. 5). In contrast to previous studies from other species, the ovine propeptide sequence has diverged more rapidly from the equivalent human, murine or rabbit sequences than has the mature protein, largely because of wide divergence in the 20 or so aa residues upstream from the cleavage site for protein maturation. Two Cys residues believed to form a disulfide bridge in the mature protein are conserved, and there is conservation of the final 13 aa residues, deletion of which is known to abrogate protein function. In a 5′–TTATTTATTTA motif in the 3′-untranslated region 34 nt are completely conserved when compared with the human sequence. This motif probably acts to destabilise the RNA (Shaw and Kamen, 1986; Caput et al., 1986; Beutler et al., 1988).

During the preparation of this manuscript, another laboratory has published an ovine *TnfA* cDNA sequence (Young et al., 1990). That sequence is 99% similar to the one published here, but appears to be incomplete. It lacks 153 nt of 5′-untranslated sequence when compared with the sequence presented here, the other 3 nt of the leader being different from our sequence. Our sequence also displays the following polymorphisms relative to that of Young et al. (1990): an additional 3 nt in the signal peptide region leading to an extra aa; one conservative third nt substitution at position 567 in the coding sequence and six single nt substitutions, as well as three single nt insertions and two deletions in the untranslated tail. The length of the sequence presented here is in reasonable agreement with that measured by Northern blotting, allowing for polyadenylation. From alignment of the sequence with those of human and murine *TnfA* genes, for which the 5′ end of the transcript is well established (Pennica et al., 1984; 1985) it would appear that the sequence is nearly complete, though it may be missing 10–20 nt at the 5′ end (data not shown).

(d) Conclusions

(*1*) We have cloned the ovine *TnfA* cDNA encoding TNF-α using an oligo(dT) primer to generate an ss cDNA followed by the PCR or by inverse PCR. This method allowed the production of a molecule with an intact 3′ end, despite the presence of A + T-rich RNA-destabilising elements.

(*2*) We have demonstrated some potential problems of inverse PCR. A non-TNF-specific major product was generated by one outward-facing primer alone, illustrating that

significant product can come from any abundant cDNA which is cross-reactive with either primer alone, which can form a significant number of head-to-head or tail-to-tail homodimeric molecules after ligation, or which can prime internally from two sites.

(*3*) Examination of our sequence for secondary structure shows the presence of an extremely stable hairpin structure in the RNA in nt positions 436–648 (Fig. 6). This hairpin was probably responsible for the difficulties encountered in extending to the 5′ end of the gene.

(*4*) In the inverse PCR problems will arise if a high proportion of the original cDNA is incomplete because the PCR works more efficiently on shorter amplification products. After PCR it is not sufficient to separate larger molecules for cloning, as many of these may have been generated by concatenation from smaller templates.

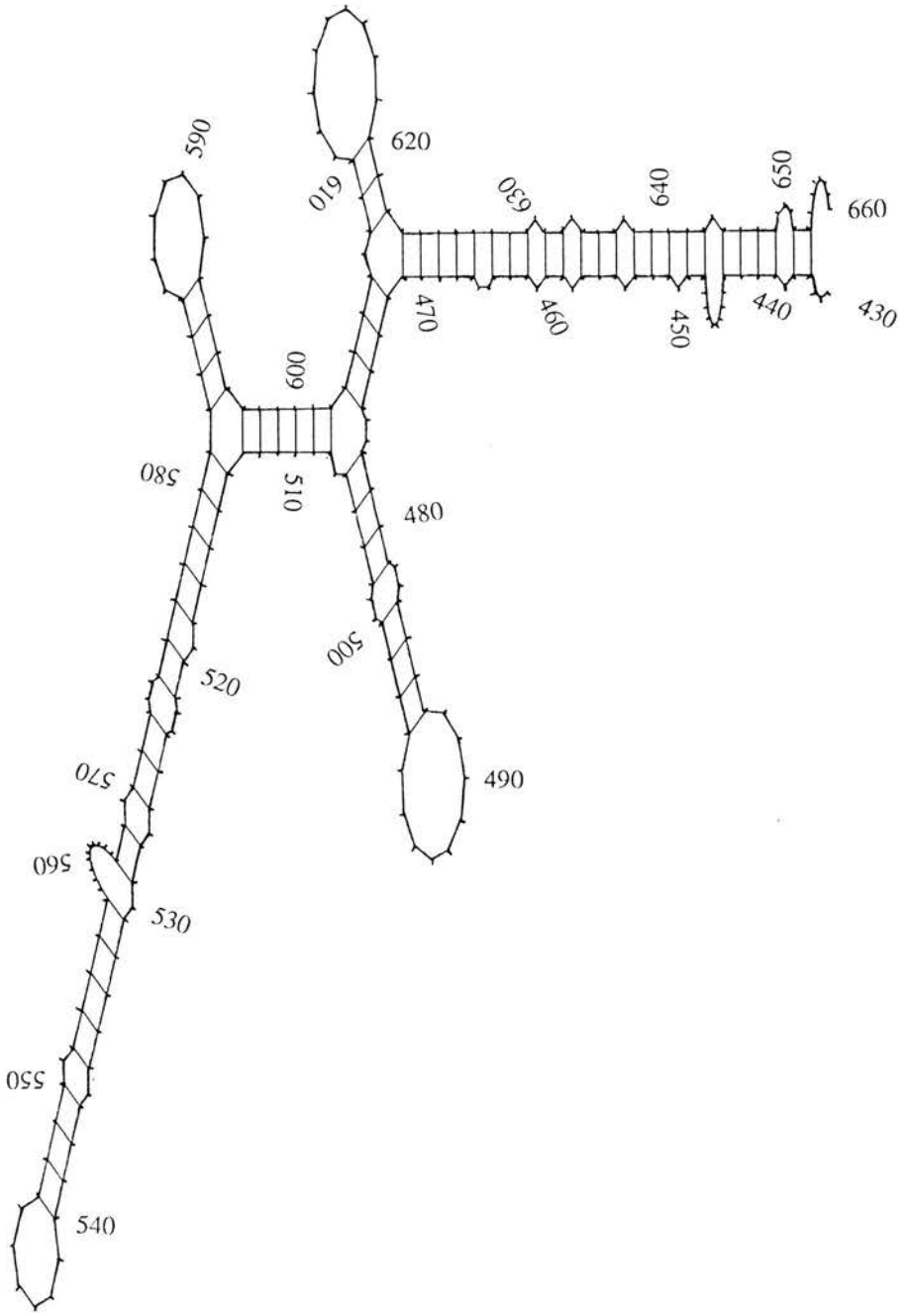

Fig. 6. Potential secondary structure in the RNA transcript of ovine *TnfA*. Secondary structure predictions were derived using the values of Freier et al. (1986), for the ovine *TnfA* transcript. A stable hairpin between nt positions 430 and 660 of the RNA sequence is shown (numbering as in Fig. 4). The stability of the structure is −64.4 kcal/mol.

ACKNOWLEDGEMENTS

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EXPRESSION AND CHARACTERIZATION OF BIOACTIVE RECOMBINANT OVINE TNF-α: SOME SPECIES SPECIFICITY IN CYTOTOXIC RESPONSE TO TNF

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We have expressed and partially purified recombinant ovine tumour necrosis factor α (rovTNF-α) using a yeast Ty, virus like particle, expression system. RovTNF-α is at least as active as recombinant human TNF-α (rhTNF-α) in two different bio-assays performed on ovine material, whilst approximately 1000-fold more rovrTNF-α than rhTNF-α is required to induce the same level of cytotoxicity in TNF-sensitive murine cell lines L929 and WEHI 164 clone 13. When cytotoxic assays are performed on the porcine TNF sensitive cell line PK(15)–1512 rovTNF-α shows about 2 logs greater activity than on murine cells, whilst rhTNF-α is about 1 log more active. A monoclonal antibody, raised against rovTNF-α, has been used to demonstrate the presence of nanogram amounts of an appropriately sized glycoprotein to be native ovine TNF-α in supernatants of LPS stimulated ovine alveolar macrophages. These samples show no detectable cytotoxicity to L929 cells, although they show activity attributable to TNF-α (through neutralization by a polyclonal antiserum raised to rovTNF-α) in an assay on ovine material. The relative lack of activity on murine cells helps to explain previous reports of inability to assay native ovine TNF-α using these cells, in spite of their routine use to assay TNF-α from several other species. The sequence features in ovine TNF-α which might reduce its affinity for the murine TNF type 1 receptor are discussed.

Tumour necrosis factor α (TNF-α), a pleiotropic cytokine produced by lipolysaccharide (LPS) stimulated macrophages and other cell types,[1] is thought to play a central role in the pathogenesis of diseases involving endotoxinaemia[2] and cachexia associated with chronic diseases of assorted aetiologies.[3–5] Its multiple antitumour and proinflammatory properties have been extensively reviewed.[1,6,7] Human TNF-α is secreted as a 17 kDa protein cleaved from a larger precursor[8] and is capable of self associating[9] into its biologically active trimeric[10] or possibly dimeric[11] form.

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TNF-α exerts its effects by interacting with cellular receptors[12] of which two human types (1 and 2) have been identified and cloned[13–15] as have their murine homologs.[16] Whilst type 2 TNF receptor mediated activities, such as thymocyte proliferation in the presence of a comitogen,[17] are highly species specific[18] (possibly reflecting the lower degree of cross species homology of the extracellular domain of this receptor), only minor degrees of species specificity have been noted for activities such as cytotoxicity[19] which are believed to be mediated via the type 1 receptor.[17] Hence TNFs-α from species as diverse as mouse, rabbit, human, ox and horse are all active in cytotoxicity assays using highly sensitive murine cell lines such as L929 or WEHI 164 clone 13, allowing these lines to form the basis of bioassays for TNF in each of these species.[20–25]

Several recent reports have suggested that cytotoxicity to murine TNF sensitive cells is not present even in supernatants from ovine cells which might be expected to contain TNF. We ourselves have consistently obtained disappointing results when attempting to use murine TNF sensitive cells to detect TNF-α in the supernatants of LPS

stimulated, ovine, alveolar macrophages (unpublished observations). To aid in studies of ovine TNF-α, we have therefore expressed recombinant ovine TNF-α (rovTNF-α) using a yeast Ty-VLP expression system.[26] In this system a recombinant protein is expressed as a fusion product with the yeast Ty element protein, P1, whose ability to self assemble into virus like particles (VLPs) is exploited in a simple purification procedure involving successive centrifugations. An ovine TNF-α cDNA expression cassette was produced by a polymerase chain reaction (PCR) and included a specific proteolytic cleavage site between the two fusion partners to allow rovTNF-α to be freed from P1. After cleavage, the latter remains particulate and can therefore be removed by further centrifugation. Recombinant human TNF-α (rhTNF-α) has previously been expressed in yeast and shown to have biological activity[27] whilst the Ty-VLP system has previously been used to express a biologically active cytokine (human α2-IFN).[28] This laboratory has used this system to express other ovine cytokines such as interleukin (IL)-1α and β which are biologically active.[29]

We have assessed the biological activity of the rovTNF-α in a variety of systems, and have used it to raise both polyclonal and monoclonal antibodies capable of identifying production of native ovine TNF-α by stimulated ovine alveolar macrophages. The rovTNF-α protein displays activities characteristic of other TNF proteins and has comparable activity to rhTNF-α in two assays on ovine tissues, but has relatively reduced activity in cytotoxic assays on TNF sensitive murine cell lines. However it is cytotoxic to a porcine kidney cell line.

RESULTS

Expression of Recombinant Ovine TNF-α

Primers were chosen to anneal to the first 21 and final 28 bases of the coding sequence for mature ovine TNF-α.[30] Flanking nucleotides were added to these primers so that in the PCR product coding sequence was preceded by 4 codons encoding a factor Xa recognition sequence[31] and flanked by *Bam*H I restriction endonuclease sites (see Materials and Methods). (No internal factor Xa or *Bam*H I sites exist in the mature ovine TNF-α sequence.) After amplification by PCR of cDNA from ovine alveolar macrophages stimulated with LPS, a product of anticipated size was produced and cloned into pTZ18R. Inserts from 5 clones were completely sequenced. A *Bam*H I excised insert with internal sequence perfectly matching the ovine TNF-α sequence[30] was selected and ligated into the unique *Bam*H I site of the expression plasmid pOGS40,[32] which places fusion protein production under the control of a galactose inducible promoter. Recombinant clones in *E. coli* were sequenced through the insertion site to select a clone with the correct orientation and maintenance of reading frame of insert. Suitable plasmids were chosen for transformation of yeasts.

Crude extracts were prepared from four yeast transformants, after culture and galactose induction, and analysed on Coomassie Blue stained 10% SDS PAGE gels. In a typical extract an extra, heavily stained, protein band was observed at the predicted size of a P_1-TNF fusion protein (Fig. 1A).

One of these transformants was selected for further work. After purification of VLPs from galactose induced cultures of this yeast, a preparation of ~90% pure fusion protein was obtained (Fig. 1B) in which electron microscopy demonstrated typical VLPs (not shown). Cleavage of the fusion protein with factor Xa produced 2 proteins corresponding in weight to P_1 (50 kDa) and TNF (17 kDa) (Fig. 1C). Centrifugation of cleavage solutions and dialysis of the supernatants into PBS left a solution containing the 17 kDa protein at estimated purity 80% (Fig. 1C) as judged by Coomassie Blue staining. However, this protein appears to stain relatively poorly with silver stain (data not shown). (These gels were run under nonreducing conditions to avoid any confusion with the 17 kDa subunit of factor Xa.[33]) Final yields were of the order of 2 mg rovTNF-α per litre of yeast culture. A negative control preparation of yeast transformed with plasmid without insert contained similar levels of other proteins (data not shown). Cross-linking studies suggest that a large proportion of the rovTNF-α is present as a trimer (Fig. 1D).

Cytotoxicity Assays on Murine and Porcine Cell Lines

When used in cytotoxicity assays on L929 cells this, and other, rovTNF-α preparations repeatedly had only limited cytotoxic activity when compared with rhTNF-α (see Fig. 2A) requiring approximately 1000 fold more rovTNF-α to achieve the same cytotoxic effect (CD50 = 8 pg/well for rhTNF-α; 8 ng/well for rovTNF-α). A similar discrepancy in activity was observed for killing of WEHI 164: clone 13 cells (data not shown) as well as for MTT dye reduction assays on the latter cell type (Dr P. Preston, personal communication). No cytotoxicity was observed from the negative control preparation assayed on either cell line at any dilution. Neither was any cytotoxicity observed by the use of rovTNF-α on L929L/R cells (a TNF-resistant L929 derivative[34]) even at concentrations able to induce 100% cytotoxicity on L929 cells (data not shown). To test whether contaminants such as factor Xa might be exerting a protective effect on the cells, the negative

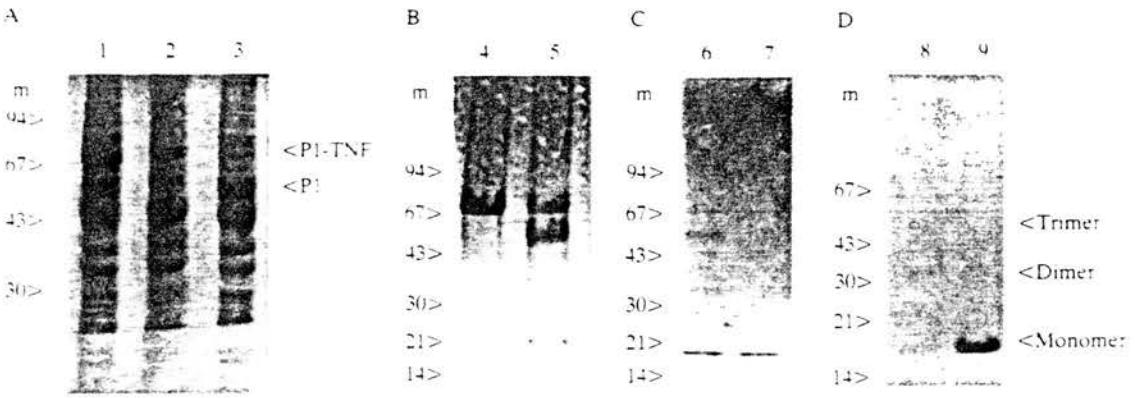

Figure 1. Purification of rovTNF-α.

A–C are photographs of Coomassie Blue stained gels, and D a Western blot, with detection of rovTNF-α by the monoclonal antibody cyt1 as described in the text. Fig. 1A shows a 10% polyacrylamide gel. All other gels were 5–20% gradient gels. Gels in Figs 1A and B were run under reducing conditions and those in 1C and D non-reducing conditions. Lanes are as follows: 1, 2 and 3 – crude extracts of typical pOT40.1, un-, and pMA 5260 transformed yeasts respectively (pOT40.1 encodes an ovine TNF-α-P1 fusion protein, pMA 5260 encodes P1 with no fusion partner); 4 – a pOT40.1 derived VLP preparation; 5 and 6 – partially and fully cleaved (with factor Xa) pOT40.1 derived VLP preparations; 7 – final rovTNF-α preparation after removing P1 by centrifugation; 8 and 9 – rovTNF-α preparation after and before cross-linking; positions of molecular size markers for gels 1a, b, c and d are shown, as are anticipated mobilities of P1 and P1-TNF fusion protein and of putative TNF mono-, di- and tri-mers.

control preparation was mixed with rhTNF-α and compared to the action of pure rhTNF-α. No difference was observed (data not shown).

Very recently a porcine TNF sensitive cell line has been reported.[35] When rovTNF-α was assayed in this cell line a more than 100 fold increase in cytotoxicity was observed (CD50 = 60 pg/well). The cytotoxicity of rhTNF-α is also increased in this cell type in comparison to that observed on L929 cells but the increase is about 10 fold (CD50 = 0.9 pg/well) (Fig. 2B).

Assays for TNF Activity of the Recombinant Protein on Ovine Material

We assessed the activity of our material as a comitogen for ovine thymocytes. In each of 3 assays with thymocytes from different animals it showed

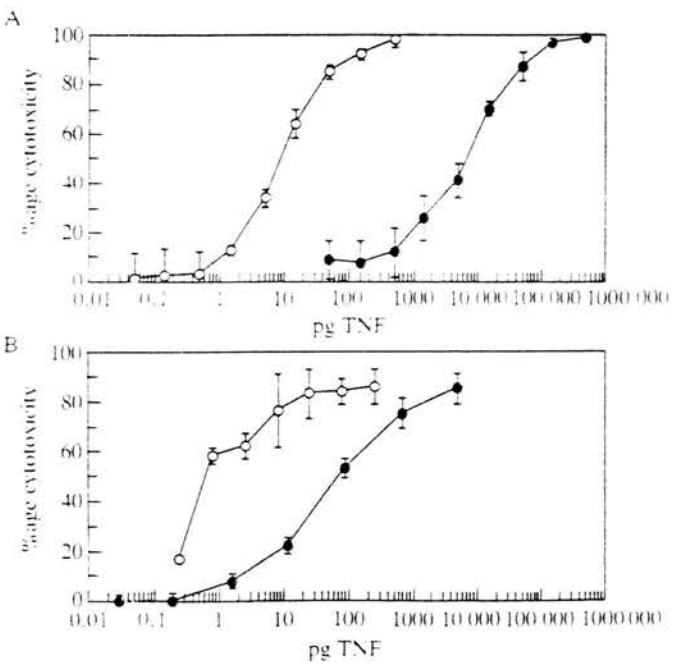

Figure 2. Cytotoxicity of ovine TNF-α to murine and porcine cells.

(A) TNF cytotoxicity towards L929 cells. Error bars show standard deviations in cytotoxicity measurements (in this experiment, $n = 4$). (B) TNF cytotoxicity towards PK(15)- 1512 cells. Error bars show standard deviations in cytotoxicity measurements (in this experiment $n = 2$). Cytotoxicity of the preparations was measured as described. ● rovTNF-α, ○ rhTNF-α. The yeast control preparation had no measurable cytotoxicity in these assays.

activity surpassing that of rhTNF-α. A representative experiment is shown in Fig. 3. In no experiment did a negative control preparation show any activity over the use of media alone. Neither did mixing the negative control preparation with rhTNF-α change the latter's activity.

Another of TNF-α's multiple activities is its ability to promote resorption of cartilage.[36] To further confirm activity on ovine cells we performed 3 cartilage degradation assays on ovine Xiphoid cartilage. A representative experiment is shown in Fig. 4. In all three experiments the rovTNF-α preparation performed similarly to rhTNF-α. Again the negative control preparation showed no activity on its own, nor did it alter the activity of rhTNF-α when they were mixed.

To date, we have not identified any ovine cell lines susceptible to the cytotoxic action of TNF-α.

Antibodies Against the Recombinant Protein Detect Native Ovine TNF-α

A monoclonal antibody, cyt 1, raised against the purified rovTNF-α, recognizes a protein of M_r = 17 000 in the recombinant material but not in negative control preparations. This monoclonal recognizes a M_r = 25 000 protein in 18 h condition medium from LPS-stimulated alveolar macrophages, but not from non-conditioned medium (Fig. 5A). The size of this protein is reduced to more closely approximate that of the recombinant protein by digestion with peptide-*N*-glycosidase F. The recombinant material is unaffected by this treatment (Fig. 5B). The predicted amino acid sequence of mature ovine TNF-α contains one potential Asn-linked glycosylation site.[30]

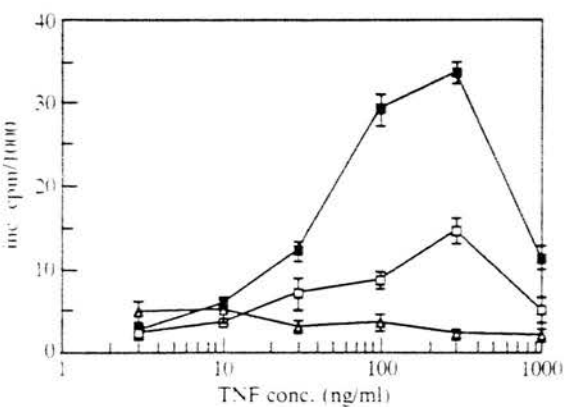

Figure 3. Ovine TNF-α induces proliferation of ovine thymocytes.

The figure shows the result of a representative co-mitogen stimulation assay performed on ovine thymocytes as described. Error bars show standard deviations of incorporated counts (n = 5). The concentrations of TNF shown are those of the added samples (i.e. twice the final concentrations in the assay). ■ rovTNF-α, □ rhTNF-α, △ yeast control preparation.

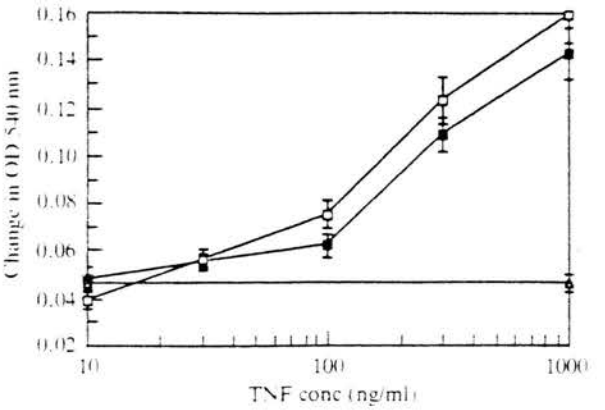

Figure 4. Cartilage degrading activity of ovine TNF-α.

The figure shows the results of a typical cartilage degradation assay performed on ovine xiphoid cartilage disc as described. Error bars show standard deviations of the OD changes measured (n = 5). The concentrations of TNF quoted are those of the added samples (i.e. 4 times the final concentration in the assay). ■ rovTNF-α, □ rhTNF-α, △ yeast control preparation.

Whilst this supernatant, estimated by Western blot to contain more than 1 ng/ml TNF, shows no detectable cytotoxicity to L929 cells in an assay capable of detecting 30 pg/ml human TNF diluted in the same medium (not shown), it did indeed contain bioactive TNF-α, as judged by its ability to induce proliferation in an ovine thymocyte comitogen assay over and above that caused by medium alone. This proliferation could be completely neutralized by a polyclonal antiserum raised against rovTNF-α (Fig. 6). Complete neutralization is somewhat unexpected as the supernatant may contain IL-1 as well as TNF. However, ovine IL-1 is very susceptible to freeze–thaw cycles (unpublished observations). The polyconal antiserum shows no activity against recombinant ovine IL-1 β (not shown).

A time course of TNF-α accumulation in media from LPS stimulated ovine alveolar macrophages is shown in Fig. 7. None of the supernatants were active in L929 cytotoxicity assays. In these extracts, in addition to the predominant 25 kDa protein, the monoclonal cyt1 also detects proteins of M_r = 70 000 (undissociated TNF trimers or TNF-receptor complexes?) and of very high molecular weight (multimeric aggregates?).

DISCUSSION

We have expressed and purified ovine TNF-α protein from a TNF-P1 encoding plasmid in yeast. A significant proportion of the M_r = 17 000 recombinant material appears to be present as dimers and trimers and is therefore potentially active.[10,11]

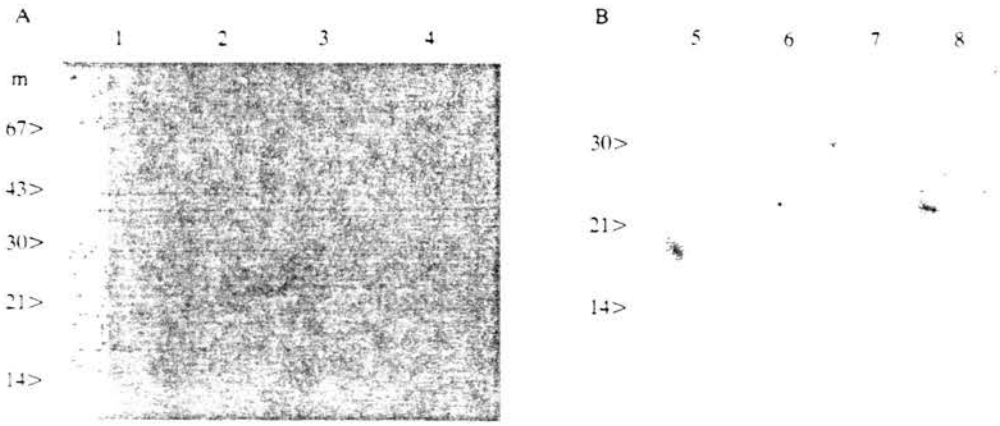

Figure 5. A monoclonal antibody against ovine TNF-α.

Western blots of rovTNF-α and native ovine TNF-α were performed using 5–20% gradient SDS-PAGE gels run under non-reducing conditions. Ovine TNF-α was detected by cyt1. Final visualization was achieved by standard methods in A and by enhanced chemiluminescene (Amersham) in B. Lanes are as follows: 1 and 2 – acetone precipitates of 1 ml of Iscove's/LPS medium and 18 h supernatant from LPS conditioned ovine alveolar lavage cells; 3 – 1ng rovTNF-α; 4 – 1ng equivalent of negative control preparation; 5 and 6 – rovTNF-α without and with peptide-*N*-glycosidase F treatment; 7 and 8 – native ovine TNF-α preparation with and without peptide-*N*-glycosidase F treatment. Positions of molecular size markers for each gel are shown.

Both monoclonal antibodies and a polyclonal antiserum (data not shown) raised to this protein recognize a protein secreted by a population of alveolar lavage cells rich in macrophages when induced by LPS, with a time course in keeping with our own observation of induction of ovine TNF-α mRNA.[30]

Comparison of the mobilities on SDS gels of the recombinant and native proteins, before and after treatment with peptide-*N*-glycosidase F, suggests that the recombinant protein is not glycosylated, but that native TNF-α secreted by ovine macrophages is glycosylated. A potential *N*-linked glycosylation site exists at position 19 of the mature protein.[30] Whilst the function of glycosylation in TNFs-α is unknown, unglycosylated recombinant murine TNF is fully active.[37] (Native murine TNF-α is glycosylated.[38] whilst native human TNF-α is not.[39])

Although rovTNF-α performs as well as, or better than, rhTNF-α in two assays of activity on ovine material, it has very poor cytotoxic activity on two TNF sensitive murine cell lines. On the other hand it is cytotoxic to a porcine TNF sensitive cell line. The concentrations of rovTNF-α required to produce ovine thymocyte proliferation and cartilage degradation are of similar magnitude to those required in other species using homologous rTNF's[18,36,40] suggesting that the ovine rTNF-α is fully functional, but half maximal thymocyte proliferation is seen at a concentration less than a quarter of that required to give half maximal cytotoxicity on murine cells in what is normally a much more sensitive TNF assay.

There are several possible explanations for the relatively poor activity of rovTNF-α in cytotoxicity assays on murine cell lines. It is unlikely that inhibitor(s) exist in the rovTNF-α preparations, or

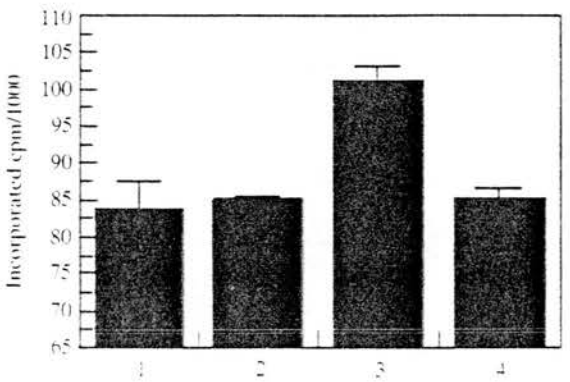

Figure 6. A supernatant from LPS stimulated macrophages enhances the uptake of ^{3}H-thymidine by ovine thymocytes and is neutralized by a polyclonal antiserum raised to rovTNF-α.

Thymocyte comitogen proliferation assays, and TNF neutralization were performed as stated in Materials and methods. Error bars show standard deviations of the incorporated counts (n = 5). Column 1: thymocytes stimulated with fresh Iscove's medium containing 1 μg/ml LPS treated with preimmune rabbit serum; column 2: thymocytes stimulated with fresh Iscove's medium containing 1 μg/ml LPS treated with polyclonal rabbit anti-rovTNF-α serum; column 3: thymocytes treated with 18 h supernatant from alveolar macrophages plated into Iscove's medium containing 1 μg/ml LPS and treated with preimmune rabbit serum; column 4: thymocytes treated with 18 h alveolar macrophage supernatant as before, but treated with polyclonal rabbit anti-rovTNF-α serum.

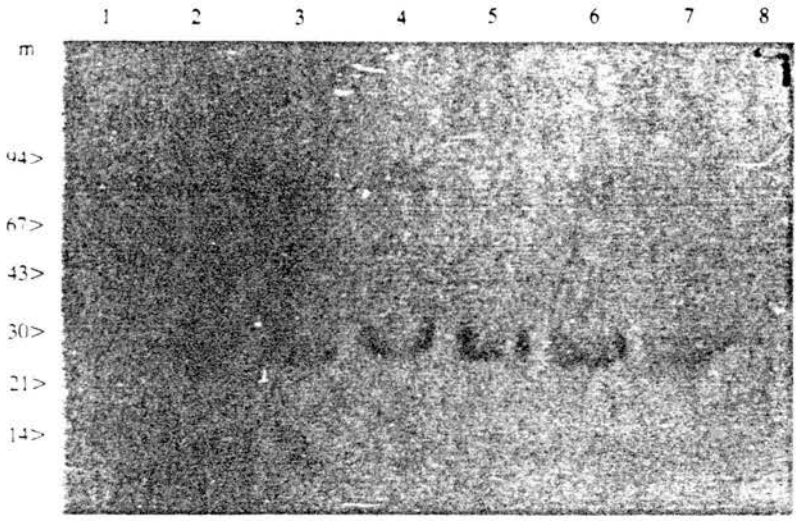

Figure 7. Time course of TNF-α production by ovine lung cells.

Western blot of a 5–20% gradient SDS-PAGE gel run under non-reducing conditions, showing TNF-α production in vitro by ovine lung cells after lavage and stimulation with 1 μg/ml LPS. Ovine TNF-α was detected by the monoclonal antibody cyt1. Lanes 1–7: acetone precipitates of 1 ml supernatants from 0, 2, 4, 6, 18, 25 and 48 h (after stimulation) time points respectively; lane 8: 1 ng rovTNF-α. The position of molecular weight markers is shown.

media used, capable of inhibiting one activity of ovine TNF-α without inhibiting other activities, or the activites of human TNF-α. It is possible that during production of ovine TNF-α by the VLP system the protein becomes folded in such a way as to affect one function (cytotoxicity on murine cells) without affecting others. But this seems unlikely in view of the cytotoxicity of the preparation to porcine cells. It is known that cytotoxic and thymocyte proliferating functions of TNF-α are mediated via different receptors in the mouse[17] and this is likely to be true for other species. Species specificity of the type 2 receptor which mediates thymocyte proliferation has been previously demonstrated (and rovTNF does not act as a comitogen for murine thymocytes, data not shown), but species specificity of the type 1 receptor has not been noted previously.[16] Nonetheless, the most likely explanation for the results presented here is a much reduced affinity of ovine TNF-α for the murine type 1 TNF receptor.

This latter possibility is in keeping with consistently negative attempts to assay ovine TNF-α in the supernatants of stimulated alveolar macrophages in spite of the production of abundant TNF-α mRNA (unpublished observations[30]) and the presence of ovine TNF-α protein detectable by Western blot in concentrations exceeding 1 ng/ml (Figs 5 and 7) in an assay where 300 pg/ml human TNF-α could induce 100% cytotoxicity and 30 pg/ml was readily detectable (not shown). Others have also failed to detect ovine TNF-α using these cells.[41,42] One study[43] does describe some cytotoxicity in ovine macrophage supernatants, but the effect is described as displaying 'considerable variability.' A recent report of expression of a cDNA for ovine TNF-α in *cos* cells suggests that material cytotoxic to WEHI 164 clone 13 cells is found in concentrates derived from the supernatants of these cells. But no indication of the purity of TNF-α in this material or its likely concentration is given, and the effect cannot be observed prior to 15 × concentration of the supernatants.[44]

Human TNF-α has been the subject of several studies by antibody and mutational or deletion analysis to attempt to define functional sites of the molecule (e.g. refs 45–49). These have implicated several amino acids which form a cluster on either side of a groove formed between two subunits of a trimer[45] as well as amino acids at the carboxyl,[47] and near to the amino[48] terminals of the molecule. Three amino acid residues (nos. 9, 67, 106) are conserved in the TNF-α sequences of 5 other species (including pig) whose TNFs-α can be successfully detected by bioassays for cytotoxicity using murine cells, but differ in sheep TNF-α (Fig. 8). Of these residues, that at position 9 has previously been implicated as being important for cytotoxic function on L929 cells in one study[49] in which deletions of the first 8 amino acids of human TNF-α did not affect its cytotoxic activity but deletion of the first 10 amino acids led to a significant decline in this activity. To our knowledge the two other positions have not so far been implicated as being particularly important to function and in the predicted three dimensional structure of human TNF-α[50,51] appear to be somewhat remote from the proposed receptor binding sites though they may be important for the overall conformation of the molecule. Whilst most of the amino acids in the cluster identified by Van Ostade et al.[45] are conserved in the ovine sequence, one at position 32 differs. The amino acid at this position also differs in the ox and pig. It is interesting to note that in one study[23] TNF-α could only be detected in the supernatants of LPS-stimulated bovine alveolar macrophages using WEHI 164 clone

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                      .         .         .≡        .         .
OVINE   -77 MSTKSMIRDVELAEEVLSNKAGGPQGSRSCWCLSLFSFLLVAGATTLFCL
BOVINE      ..................E...........L...................
HUMAN       ...E...........A.PK.T.......R.LF.......I..........
MOUSE       ...E...........E.PQ.M..F.N..R.L...................
RABBIT      ...E..........GP.PK........KR.L...................
PIG         ...E...........A.AK.........R.L...................
                     .  *     .        1 .       ≡  .
OVINE   -27 LHFGVIGPQREEQSPAGPSFNRPLVQ--TLRSSSQASNNKPVAHVVANIS
BOVINE      .............V.S...IS.....--.........S.........D.N
HUMAN       ............-F.RDL.LIS..A.--AV....RTPSD.........PQ
MOUSE       .N........D.KF.N.LPLISSMA.TL.......N.SD.........HQ
RABBIT      ...R.....E.....NNLHLVN.VA.MV....A.R.LSD..L......PQ
PIG         ...E.....K..-F....LSIN..A.--G......T-SD.........VK
            .         .         .         .         .    ≡
OVINE    22 APGQLRWGDSYANALMANGVELKDNQLVVPTDGLYLIYSQVLFRGHGCPS
BOVINE      S......W............K.E.......AE.............Q....
HUMAN       .E...Q.LNRR....L......R.......SE...........K.Q....
MOUSE       VEE..E.LSQR....L...MD.........A.....V......K.Q...D
RABBIT      VE...Q.LSQR....L...MK.T.......A............S.Q..R.
PIG         .E...Q.QSG.....L....K........................Q....
            .         .         .         .   ≡     .
OVINE    72 TPLFLTHTISRIAVSYQTKVNILSAIKSPCHRETLEGAEAKPWYEPIYQG
BOVINE      P.PV..............................P.W.............
HUMAN       .HVL.................L........Q...P.............L.
MOUSE       Y-VL....V..F.I...E...L...V....PKD.P....L........L.
RABBIT      Y-VL....V..F....PN...L............P.E..PMA......L.
PIG         .NV..................L........Q...P.............L.
            .         .         .         .
OVINE   122 GVFQLEKGDRLSAEINLPEYLDYAESGQVYFGIIAL 157
BOVINE      ..................D.................
HUMAN       ................R.D...F.............
MOUSE       .........Q....V...K...F.........V...
RABBIT      ............T.V.Q.....L.............
PIG         .......D..........D...F.............
```

Figure 8. A comparison of predicted amino acid sequences of TNF-α from several species.

(sequences taken from refs 30,36,60,61,62,63). The presumed first amino acid of the mature protein is shown by the number 1. The potential glycosylation site for ovine TNF-α is shown in bold print. Amino acids which are conserved in all other species but differ in the sheep are marked by an overhead ≡.

13 cells not the less sensitive L929 cells. RovTNF-α is more active in cytotoxic assays on porcine than on murine cells, and displays greater sequence similarity to porcine TNF-α than to human or murine TNF-α, but is still less active than rhuTNF-α on either cell type.

From these data, it is clear that TNF-α-sensitive murine cell lines are insufficiently sensitive to ovine TNF-α for them to be used in cytotoxicity assays for routine detection of ovine TNF-α.

Although strict species specificity has been previously demonstrated for type 2 TNF receptors, the present work demonstrates that there may be a higher degree of specificity in the interaction of TNFs-α from some species with the type 1 receptor than has previously been recognized.

MATERIALS AND METHODS

Generation of cDNA Expression Cassette for the Ovine TNF-α Gene

A DNA fragment encoding the structural sequence of mature ovine TNF-α was generated by gene amplification from ovine cDNA, prepared as described previously.[30]

A PCR was performed on this cDNA in the buffer conditions of Ohara et al.[52] using 35 cycles of 95°C, 0.5 min; 48°C, 0.5 min; 71°C, 1.5 min; and a final extension at 71°C for 7.5 min. 1 U Taq polymerase, 5 ng of cDNA and 0.1 μm each of the following primers (see below) were used. PCR products were purified by phenol-chloroform extraction and ethanol precipitation prior to digestion with *Bam*H I. After an intermediate cloning and sequencing step

5′ primer — *Bam*H I: AAG CTT GGA TCC; Factor Xa: ATA GAA GGT AGA; Ovine TNF-α: CTC AGG TCA TCT TCT CAA GCC

AAG CTT GGA TCC ATA GAA GGT AGA CTC AGG TCA TCT TCT CAA GCC

3′ primer — *Bam*H I: AAG CTT GGA TCC; *TCA* (Stop); TNF-α: CAG GGC GAT GAT CCC AAA GTA GAC C

AAG CTT GGA TCC *TCA* CAG GGC GAT GAT CCC AAA GTA GAC C

Stop

via the 'phagemid pTZ18R, (Pharmacia) to eliminate insert fragments containing errors introduced by PCR, the *Bam*H I digested ovine TNF-α expression cassette was ligated into the unique *Bam* H I site in the *TyA* gene of the yeast/*E. coli* shuttle vector pOGS40[32] to form a P1-TNF fusion protein gene. In pOGS40 the *TyA* gene encoding protein P1 has been placed under the control of a strong galactose inducible hybrid promoter PGK-GAL (PAL). The *TyA* gene ends with an unique *Bam*H I site, followed by determination codons in all three reading frames.

After growth in *E. coli* JM83, recombinant clones selected by colony hybridization to an ovine TNF-α probe,[30] were used to prepare plasmid DNA for ds.DNA sequencing through the insertion site using a pOGS40 specific primer. Plasmids with correctly inserted expression cassettes were used in cotransfections of yeast strain BJ2168 (a, leu2$^-$, trp1$^-$, ura3–52, prb1–1122, pep4–3, pcr1–407, gal2)[53] with plasmid pUG41S, which aids galactose inducibility by virtue of its gal4 gene,[54] by standard methods.[55] Double transformants were selected by their ability to grow on minimal media. After initial characterization, one of these (containing the TNF-α expression plasmid pOT40.1) was used for further work.

Production of Recombinant Ovine TNF-α

Selected yeast transformants were incubated at 37°C in minimal medium (6.7 g/l yeast nitrogen base (Difco), 20 mg/l tryptophan) supplemented with glucose (10 g/l) as a sugar source for 72 h before being moved into minimal medium + galactose (10 g/l) for a further 18 h. Yeast cells were harvested by centrifugation and rovTNF-α containing VLPs were prepared by a modification of the method of Adams et al.[26] Crude yeast extracts were made by homogenization with glass beads into TEN buffer at 4°C (TEN is 10 mM Tris pH 7.4, 2 mM EDTA, 140 mM NaCl) (5 ml TEN per 1l culture). These lysates were clarified by centrifugation at 13 000 × ***g*** for 15 min before being centrifuged at 100 000 × ***g*** onto a cushion of 60% sucrose in TEN for 1 h. VLPs at, and below, the cushion interface were collected and dialysed against TEN before being loaded onto 15–45% linear sucrose gradients, with a 60% cushion, in TEN and spun at 53 000 × ***g*** for 3 h.

VLP containing fractions were identified by Coomassie Blue staining of SDS PAGE gels.[56] They were pooled and dialysed into a buffer containing 100 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$. Precipitates formed after dialysis steps were removed by microfuge centrifugation. Total protein concentrations were measured by dye staining[57] and comparing with protein standards. Estimates of protein purity were made by densitometric scanning of stained SDS PAGE gels.

Cleavage of fusion protein was achieved by addition of Bovine Factor Xa (BCL) to the dialysate to which 0.05% CHAPS (Sigma) and 0.05% Na deoxycholate were added, (Factor Xa was used at a ratio of 1 part to 50 parts P1-TNF), and incubated at 25°C for 18 h. After incubation the suspension was clarified by centrifugation at 100 000 × ***g*** for 1 h, then dialysed against several changes of PBS over 18 h. The final dialysate was stored in aliquots at −70°C either with the addition of 0.1% BSA for later use in bioassays, or without BSA, for use in estimating protein concentration, gel analysis and immunizations. rhTNF-α was purchased from British Biotechnology Ltd, Oxford, UK, reconstituted at 1 μg/ml in PBS with 0.1% BSA, aliquoted and frozen at −70°C until use.

Negative control preparations were prepared in an identical manner to the rovTNF-α preparations, from yeast transformed with pMA5260 (a constitutive expressor of P1 protein)[26] with addition of the same concentration of Factor Xa, and were stored and used in assays in the same manner.

Polyclonal Antibodies

Rabbits were immunized with 150 μg of rovTNF-α in CFA by SC injection at several sites, followed by a second immunization of 50 μg of rovTNF-α in IFA SC 3 weeks later with a third injection of 20 μg of rovTNF-α IV after a further 3 weeks. Serum was collected 2 weeks after the third injection.

Monoclonal Antibodies

Balb C mice were immunized SC with 20 μg rovTNF-α in CFA. They were boosted at 3 weeks with 10 μg rovTNF-α in IFA s.c., at 7 weeks with 13 μg i.p., at 10 weeks with 10 μg i.p., and at 13 weeks with 8 μg i.p. and 2 μg i.v. Fusion of splenocytes to NSO myeloma cells was performed 4 days after the last boost using the method of Galfre et al.[58] Positive hybridomas were identified by ELISA against immobilized rovTNF-α preparations. A positive hybridoma designated cyt1 was cloned three times before use of the saturated supernatant as the primary antibody in further assays.

Cytotoxicity Assays

Murine fibrosarcoma lines L929 and L929L/R[34] for cytotoxicity assays were diluted to 5 × 10^4/ml in Iscove's medium containing 5% FBS, L-Glutamine, penicillin and streptomycin, and plated at 100 μl/well into 96 well flat bottomed, tissue culture plates. After 20 h incubation 50 μl of Iscove's medium with 4 μg/ml actinomycin D, and 50 μl of sample in PBS, 0.1% BSA (or 50 μl of conditioned media from macrophages) was added. After a further 18 h incubation, cells were washed once with PBS, fixed and stained for 2 min with 0.25% crystal violet in 20% methanol, rinsed, dried and the optical density of each well was measured at 540 nm.

Figures for cytotoxicity are derived as follows: % cytotoxicity =

$$\frac{\text{Mean absorbance (}-\text{ve control*)} - \text{Mean absorbance (sample)}}{\text{Mean absorbance (}-\text{ve control*)} - \text{Mean absorbance (}+\text{ve control**)}} \times 100$$

*(PBS/BSA only in well)
**(10 ng/ml rhTNF giving 100% cytotoxicity).

Each sample was assayed in quadruplicate.

Cytotoxicity assays using WEHI 164 clone 13 murine fibroblasts[25] were performed as for L929 assays with the following modifications – after plating out and overnight incubation 100 μl sample only were added, plates were incubated a further three days before staining and the medium used was DME 5% FBS.

Cytotoxicity assays on porcine kidney cells PK(15)-1512 were performed as described.[35] Briefly, 2.5×10^4 PK(15) cells were seeded in wells of a 96-well flat-bottomed tissue culture plate in 100 µl EMEM, 7% FBS. After 24 h at 37°C the medium was removed and replaced with 50 µl of basal Iscove's medium supplemented with 7.5% FBS, 0.5% bactopeptone (Difco, Detroit, USA) and containing 3 µg/ml Actinomycin D. After 2 h at 37°C, 50 µl of sample was added to each well, and after an additional 18 h at 37°C the plates were pulsed with 50 µl per well of a 3 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) in dH_2O for 3 h at 37°C. The test was terminated by aspirating off the medium, and solubilizing the contents of the well in dissolving solution (0.5% SDS, 36 mM HCl in isopropanol). Optical density at 590 nm was determined spectrophotometrically. In this case 3 wells per plate were washed with dH_2O to lyse the cells to act as +ve controls for cytotoxicity, since it was not known how much rhTNF would be needed to engender 100% cytotoxicity. Other assays were performed in duplicate. Negative controls and calculation of % age cytotoxicity were as before.

Thymocyte Proliferation Assays

Thymii were obtained from freshly slaughtered sheep. Thymocytes were teased out from small pieces of tissue into RPMI 10% FBS, penicillin and streptomycin, washed, counted and plated out at 2×10^5 viable cells/well of 96 well tissue culture plates in 50 µl medium. To each well was added 50 µl RPMI 10% FBS + phytohaemagglutinin at 36 µg/ml and 100 µl sample. Samples were diluted in PBS + 0.1% BSA and assayed in quintuplicate. After 2 days incubation at 37°C wells were pulsed with 0.25 µCi ^{3}H-thymidine, incubated for a further 18 h, harvested and incorporated radioactivity counted. To measure neutralization of TNF-α by antibodies, TNF-α containing samples, or media, were preincubated with a 1/200 dilution of heat inactivated pre-immune or immune serum for 90 min at room temperature prior to assay.

Cartilage Degradation Assays

Release of glycosaminoglycans from ovine xiphoid cartilage discs was measured in the presence of recombinant TNF-α according to Harkiss et al.[59] Relative concentrations of chondroitin sulphate were assessed by colorimetric change of a 0.0018% solution of dimethylmethylene blue (Pierce) in a formate buffer (2.1 ml formic acid, 2 g Na formate, 5 ml ethanol /l) at 540 nm measured on an ELISA plate reader. Results are expressed as mean absorbance of quintuplicate wells over and above that of medium alone without cartilage.

Preparation of Stimulated Macrophage Supernatants

Alveolar marcrophages were harvested into Hank's balanced saline solution (HBSS) by bronchoalveolar lavage from ovine lungs, washed with HBSS and resuspended in Iscove's medium containing 1 µg/ml LPS 2×10^7 cells in 5 ml media (*c.* 90% macrophages by morphology) were seeded into 25 cm^2 tissue culture flasks and clarified supernatants harvested at the times shown. Supernatants were aliquoted and stored at −70°C till used. For Western blots, 1 ml of supernatant for each time point was precipitated with 4 ml cold acetone, resuspended in 20 µl loading buffer and loaded onto an SDS PAGE gel. Western blotting using monoclonal antibody cyt1 was by standard methods.

Deglycosylation of Ovine TNF-α

Acetone precipitates from 1 ml of supernatant from 18 h LPS stimulated alveolar macrophage cultures, or 20 ng of rovTNFα were bolied for 2 min in 20 µl 1% w/v SDS, then diluted to 200 µl with 100 mM sodium phosphate pH 7.2, 25 mM EDTA, 1% octyl glucoside (Sigma), 0 or 2 units of peptide-*N*-glycosidase F (BCL) were added to duplicate vials, and incubation continued for 18 h. A 20 µl aliquot of each was removed for analysis as before.

Crosslinking of Recombinant Ovine TNF-α

RovTNF-α was cross-linked using bis [2-(succinimido-oxy-carbonyloxy) ethyl] sulfone (BSOCOES, Pierce) under conditions previously used to cross-link human TNF-α.[45]

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