WEIGHT LOSS AND METABOLIC ALTERATIONS INDUCED BY RECOMBINANT TUMOUR NECROSIS FACTOR-ALPHA (TNF)

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SUMMARY

The weight loss and metabolic alterations induced by human recombinant tumour necrosis factor-alpha (TNF) in female NMRI mice were compared with those produced by a cachexia-inducing tumour (the MAC 16 adenocarcinoma), by a restricted food and water intake (pair-feeding), and by mitozolamide, a drug which in toxic doses produces anorexia and weight loss. Methods of reversing the TNF-induced weight loss, and the mechanisms involved in the weight reduction and the TNF-induced hypoglycaemia were investigated.

The weight loss produced by a single injection of TNF was accompanied by anorexia and a decrease in the body water and carcass fat contents of mice. The TNF-induced weight loss was similar to that observed in mitozolamide-treated and pair-fed animals, and thus it appeared to be the result of anorexia. However, tolerance to the weight loss and anorectic effects of TNF occurred after the first injection. The MAC 16 tumour-induced weight loss occurred without anorexia and, in addition to a decline in body water and fat content, muscle catabolism was observed. No catabolic effects of TNF were demonstrated, but rather distinct anabolic actions. TNF was not shown to be produced by the MAC 16 tumour.

TNF induced hypoglycaemia in mice, which was much more pronounced than that seen in MAC 16 tumour-bearing animals. This hypoglycaemia served to maintain an elevated lipogenesis, primarily by the liver.

The weight loss induced by TNF was reversed by rehydrating mice and by prior administration of indomethacin. Although TNF was shown to induce elevated levels of prostaglandin E_2 (PgE_2), and 16,16-dimethyl PgE_2 caused weight loss in mice, PgE_2 did not appear to be involved in the TNF-induced weight loss.

In conclusion, the weight loss produced by TNF was the result of a direct anorectic and dehydrating effect of this agent, in contrast to the complex metabolic alterations observed in cancer cachexia.

KEY WORDS; ANOREXIA, CACHECTIN, CACHEXIA, HYPOGLYCAEMIA, LIPOPROTEIN LIPASE, TUMOUR NECROSIS FACTOR.

TO GRAN, WITH LOVE.

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"GREAT DISCOVERIES AND IMPROVEMENTS INVARIABLY INVOLVE THE CO-OPERATION OF MANY MINDS."

Alexander Graham Bell

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ABBREVIATIONS

Ab	Antibody
ACTH	Adrenocorticotropic hormone
Ag	Antigen
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BCG	*Bacillus Calmette Guerin*
BSA	Bovine serum albumin
CHO	Chinese hampster ovary
Cm	Tissue glucose concentration
cDNA	Complementary deoxyribonucleic acid
Cp	Blood glucose concentration.
2DG	2-Deoxyglucose
2DGP	2-Deoxyglucose-6-phosphate
DMSO	Dimethyl sulphoxide
E. coli	*Escherichia coli*
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetra-acetic acid
EU	European unit
FFA	Free fatty acid
GM-CSF	Granulocyte macrophage (monocyte) colony-stimulating factor
G6P-DH	Glucose-6-phosphate dehydrogenase
hTNF	Human tumour necrosis factor-alpha
IFN	Interferon
IL	Interleukin
i.m.	Intramuscular
Indo	Indomethacin
i.p.	Intraperitoneal
i.v.	Intravenous
Kd	Kilodalton
KRB	Krebs-Ringer bicarbonate (buffer)
LC	Lumped constant
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LT	Lymphotoxin
MCT	Medium chain triglyceride
mRNA	Messenger ribonucleic acid
mTNF	Murine tumour necrosis factor-alpha

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NEFA	Non-esterified fatty acid
PAGE	Polyacrylamide gel electrophoresis
Pg	Prostaglandin
pI	Isoelectric point
PLA_2	Phospholipase A_2
PTH	Parathyroid hormone
r	Correlation coefficient
Rg	Glucose metabolic rate (nmol/g/min)
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute (medium)
SDS	Sodium dodecyl sulphate
S.E.M.	Standard error of the mean
sp. act.	Specific activity
sp. gr.	Specific gravity
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle
Tg	Triglyceride
TNF	Tumour necrosis factor-alpha
U	Unit
VLDL	Very low density lipoprotein
VO_2	Resting oxygen consumption

1. INTRODUCTION

1.1 The discovery of Tumour Necrosis Factor

Late in the nineteenth century, Dr. William Coley, a New York City surgeon, stimulated much of the research which led to the discovery of tumour necrosis factor when he found that he could successfully treat cancer patients by infecting them with live bacteria (Coley, 1891). However, the difficulty in controlling these infections made this therapy highly dangerous. Thus Coley and others subsequently developed vaccines of bacteria-free filtrates from cultures of *Streptococcus* and other organisms, particularly *Serratia marcescens*, which became known as 'Coley's toxins'. These reproduced many of the symptoms of bacterial infection, such as fever and chills, but they did not actually cause an infection in the patients. Although Coley's toxins were employed in the treatment of human malignancy for over 40 years, with favourable results in a number of cases (reviewed by Nauts et al, 1953), results were inconsistent and their use diminished with the advances in radiotherapy and chemotherapy.

Although toxin therapy was no longer favoured in the clinic, investigations into the potential value of microbes as treatments for cancer continued in several laboratories. In 1931 Gratia and Linz demonstrated haemorrhagic necrosis of a transplanted liposarcoma in guinea pigs using *Escherichia coli* culture filtrates. Other investigators also found that haemorrhage could be produced in tumours by the administration of bacterial filtrates, and that the tumours subsequently receded in a significant proportion of cases (reviewed by Shear, 1935). However, these filtrates were toxic to the recipients, often causing disseminated systemic effects leading to circulatory collapse and even death. Thus, Shear and Andervont (1936) attempted to separate the haemorrhage-producing substance from the toxic component. Shear et al. (1943) eventually isolated and purified the active component from cultures of *Serratia* organisms and showed that it was a complex compound composing of both fat and sugar and, as such, they termed this material 'bacterial polysaccharide'. Subsequent work showed that the compound, now known as lipopolysaccharide or endotoxin, is a major constituent of the outer wall of gram-negative bacteria and that this agent, which is responsible for the induction of tumour necrosis, is also the most toxic component of Coley's

toxins, causing shock, coagulopathy and widespread end-organ damage.

The severe toxicity of lipopolysaccharide precluded its use as an antineoplastic agent and stimulated the search for modified molecules lacking this toxicity. In 1962 O'Malley et al. discovered that an endogenous factor was present in the serum of lipopolysaccharide-treated mice that was capable of inducing haemorrhagic necrosis of a tumour grown in another animal. In 1975, Carswell et al. reported a similar observation; a serum factor derived from animals primed with *Bacillus Calmette-Guerin* (BCG) and subsequently injected with endotoxin was found to elicit haemorrhagic necrosis of transplantable tumours *in vivo*, along with cytotoxicity against a variety of tumour cells *in vitro*. In view of the striking damage caused to mouse tumours, they called this substance 'tumour necrosis factor (TNF)' and advanced the hypothesis that endotoxin-induced tumour necrosis is mediated by the release of TNF from activated macrophages.

1.2 The discovery of Cachectin

Cachectin was discovered as a result of investigations by Cerami and co-workers in the late 1970s into the mechanisms of cachexia in chronic disease. They used trypanosomiasis as a model system since it was known that *trypanosome*-infected cattle, rabbits and certain other species developed marked cachexia, despite the presence of a low parasite burden. During the terminal phase of infection these animals develop marked anorexia and lose over half of their initial body weight before dying of infection. They also exhibit a marked hypertriglyceridaemia, mainly in the form of very low density lipoprotein (VLDL), which was found to be caused by a clearing defect due a systemic reduction in the activity of the enzyme lipoprotein lipase (Guy, 1975, and Rouzer and Cerami, 1980).

Kawakami and Cerami (1981) demonstrated that when endotoxin-sensitive (C3H/HeN) mice were injected with bacterial lipopolysaccharide, systemic suppression of lipoprotein lipase (LPL) and hypertriglyceridaemia occurred. These effects were not seen in endotoxin-resistant mice (C3H/HeJ). However, LPL suppression and hypertriglyceridaemia could be induced in endotoxin-

resistant mice by injecting them with the serum of endotoxin-treated C3H/HeN mice. Macrophages from sensitive, but not resistant, mice were shown to produce a factor which was capable of suppressing LPL *in vitro* (Kawakami et al, 1982). Thus, these effects were conferred by a transferable serum factor produced by macrophages. This factor was termed 'cachectin' because of its possible involvement in the pathogenesis of cachexia in chronic disease.

Beutler et al. (1985a) purified murine cachectin to homogeneity from a macrophage cell line and obtained a partial amino acid sequence. Inspection of the amino acid sequences and molecular clones of TNF and cachectin rapidly established that they are identical proteins.

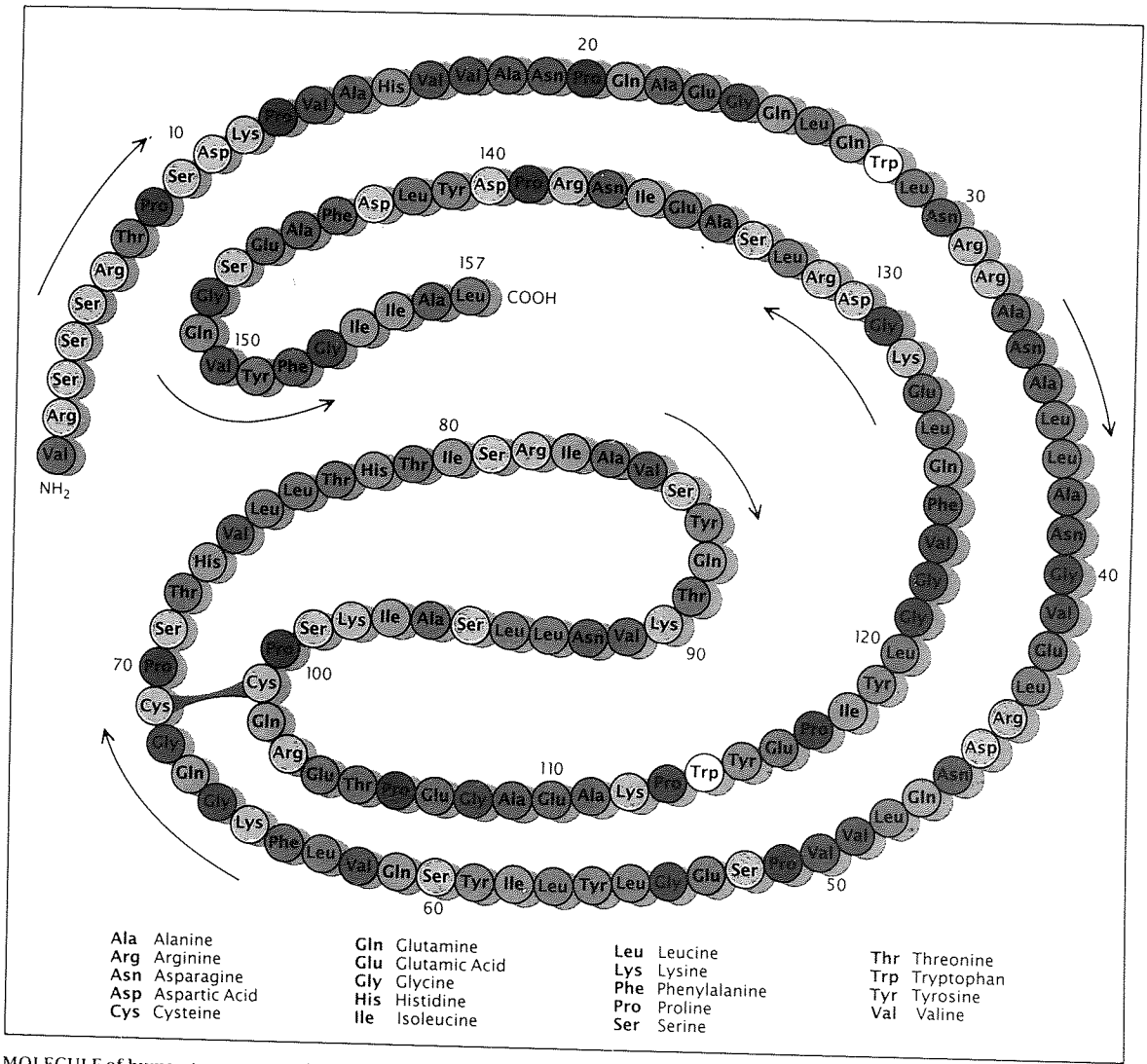
1.3 The structure of TNF

The availability of large quantities of purified TNF was essential in order to determine its structure and to further investigate its biological actions. A human promyelocytic leukaemia cell line, HL-60, which when induced with phorbol myristate acetate produces substantial amounts of TNF, was utilized for the purification of TNF (Aggarwal et al, 1985c, and Wang et al, 1985), and for the molecular cloning of the TNF cDNA (Pennica et al, 1984, and Wang et al, 1985). Human TNF was also cloned and expressed by using cloned rabbit (Shirai et al, 1985) or murine (Marmenout et al, 1985) TNF cDNA as probes.

Based upon the sequence of TNF's cDNA, human TNF (hTNF) is produced as a pro-peptide consisting of 233 amino acids which is cleaved to yield the mature polypeptide of 157 amino acid residues (figure 1.1). The presequence of 76 residues is apparently removed before the release of TNF from the cell (Pennica et al, 1984, and Wang et al, 1985). Human TNF is a non-glycosolated protein with a molecular weight of 17,000, as determined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), and an isoelectric point (pI) of 5.3. Under non-denaturing conditions hTNF has a molecular weight of 45,000, suggesting that the native protein associates in an oligomeric form. In fact, dimers, trimers and pentamers of TNF have been reported in

Figure 1.1

The amino acid sequence of recombinant human tumour necrosis factor



MOLECULE of human tumor necrosis factor is a protein consisting of 157 amino acids. The amino acid sequence was determined in 1984, the year the gene encoding TNF was first cloned by several groups associated with biotechnology companies.

(Old, 1988)

biological fluids or culture medium. Smith and Baglioni (1987) have recently suggested that the active form of the protein is trimeric.

In comparison to murine TNF (mTNF) the human peptide has one additional amino acid, histidine, at position 73 (Pennica et al, 1985). Human TNF also possesses two cysteine residues (positions 69 and 101), which are probably involved in a single intramolecular disulphide bond, even though such a bond does not appear necessary for either the tertiary structure or the biological activity of the protein. The major structural difference between hTNF and mTNF is, however, the presence of a potential N-glycosolation site on mTNF.

Murine TNF is secreted as a 156 amino acid protein, but is initially synthesized as a polypeptide hormone containing 79 extra amino acids appended at the amino terminal end of the molecule (Pennica et al, 1985). While the mature polypeptides are highly conserved among species (79% conservation between human and murine forms) the propeptide segments display even greater conservation (86%). This observation has been interpreted as an indication that a hormonal function is associated with the propeptide segment. However, the propeptide is extensively cleaved in processing to yield the mature 17,000 Dalton protein (Beutler and Cerami, 1986, and Muller et al, 1986). Therefore, it would seem likely that any hormonal function that exists must be associated with small peptide fragments.

1.4 Cellular origin of TNF and inducing stimuli for TNF production

Activated blood monocytes and tissue macrophages are the best sources of TNF so far investigated (Matthews and Watkins, 1978, Mannel et al, 1980, and Ruff and Gifford, 1981). All types of macrophages tested to date have been shown to produce TNF in response to LPS. These include macrophages of hepatic, pulmonary, peritoneal and bone marrow origin (Decker et al, 1987). It would appear, however, that cell maturation and differentiation along the mononuclear phagocytic pathway are essential for TNF biosynthesis, since peripheral blood monocytes and bone marrow-derived macrophages produce

relatively small amounts of TNF in response to LPS unless they are first primed with agents such as interferon.

Other cell types have also been shown to produce TNF, although in much smaller quantities than produced by macrophages and monocytes. These include T lymphocytes (Cuturi et al, 1987), natural killer cells (Degliantoni et al, 1985), bone marrow-derived mast cells (Kasper and Tharp, 1987, and Tharp and Kasper, 1987), and human myeloid leukaemia cell lines (Gifford et al, 1984, and Aggarwal et al, 1985b).

LPS is the most potent stimulus for the production of TNF. *In vivo* TNF production is usually achieved by priming macrophages with BCG and then injecting LPS (Carswell et al, 1975, and Matthews and Watkins, 1978). Cultured monocytes or macrophages synthesize TNF *in vitro* after activation with LPS, phorbol esters, and certain viruses and viral particles, including *Sendai* virus and influenza virus (Ruff and Gifford, 1981, Kull and Cuatrecasas, 1984, Aderka et al, 1986, and Beutler et al, 1986a).*Trypanosome* or *plasmodium* cell lysates and certain gram-positive organisms, including *Staphlococci*, also induce small amounts of TNF (Hotez et al, 1984). Other cytokines, including interferon-gamma (Beutler et al, 1986b) and interleukin-2 (IL-2) (Nedwin et al, 1985) have been shown to augment LPS-induced TNF secretion, and granulocyte macrophage colony-stimulating factor (GM-CSF) has been demonstrated to stimulate TNF mRNA production (Cannistra et al, 1987).

1.5 **Comparison with lymphotoxin**

Lymphotoxin (LT), also known as TNF-β, is a lymphocyte-derived cytolytic factor which binds to the same receptor as TNF and evokes a spectrum of bioactivities very similar to those attributed to TNF (Evans, 1982, Granger et al, 1985, and Tsujimoto et al, 1985). In humans, the genes encoding these two proteins are closely linked (approximately 1100 base pairs apart) and a high degree of homology exists between the two compounds; 46% on the nucleotide level and 28% on the amino acid level (Pennica et al, 1984). Structural dissimilarities include the possession of an internal disulphide

bond by TNF but not LT, and the presence of extensive glycosolation in LT but not in TNF. LT also possesses 18 more N-terminal amino acids than TNF and, since both compounds specifically lyse certain tumour cells, it is suggested that this region is not required for cytotoxic activity.

Whilst TNF is almost exclusively produced by macrophages in response to LPS and other stimuli (section 1.4), lymphotoxin is a product of both T cells and B lymphoblastoid cell lines (Ruddle et al, 1983, Conta et al, 1983, and Aggarwal et al, 1985b), although several T cell lines and clones have been reported to make both LT and TNF (Ruddle et al, 1989). LT is produced in response to specific antigenic challenge, certain viruses (Wong and Goeddel, 1986), or exposure to a variety of lymphocyte mitogens (Granger and Williams, 1968, and Ruddle et al, 1983). Macrophages do not produce LT in response to stimuli capable of eliciting its production by lymphocytes, nor do lymphocytes produce LT when exposed to endotoxin. TNF is produced more quickly than LT (Ruddle and Waksman, 1968, and Beutler et al, 1985b) and in greater quantities. Possibly while secretion of either of these cytokines may elicit many of the same biological effects, one cytokine may be produced to the exclusion of the other in response to different pathological stimuli.

1.6 **Antineoplastic properties of TNF**

1.6.1 Laboratory studies

In vitro studies have demonstrated that TNF has direct cytostatic and cytotoxic activities against a variety of human and murine tumour cell lines (Carswell et al, 1975, Haranaka and Satomi, 1981, Sugarman et al, 1985, and Fransen et al, 1986b) at concentrations as low as 500pg/ml. In contrast, non-transformed cell lines are usually resistant to this antiproliferative action (Helson et al, 1975, Haranaka and Satomi, 1981, Williamson et al, 1983, Haranaka et al, 1984, and Fransen et al, 1986b) and, in fact, TNF has been shown to be a potent mitogen for normal fibroblasts in culture (Sugarman et al, 1985, and Vilcek et al, 1986). However, several investigators have reported that TNF shows cytotoxic activities against certain subpopulations of normal

mouse and human lymphocytes (Playfair et al, 1982, and Umeda et al, 1983) and that TNF reveals suppressive effects on human haematopoietic stem cells (Degliantoni et al, 1985, and Murase et al, 1987). Preclinical studies with TNF have demonstrated *in vivo* tumour regression in established syngeneic murine tumours and in several human and murine tumour xenografts transplanted in athymic mice (Haranaka et al, 1984).

Inhibitors of RNA synthesis, such as actinomycin D and mitomycin C, and protein synthesis, such as cycloheximide, have been shown to greatly increase the susceptibility of tumour cells to TNF cytotoxicity, and even to cause lysis of cells which are normally resistant to TNF (Matthews and Watkins, 1978, Ruff and Gifford, 1981, and Hahn et al, 1985). A similar increase in TNF cytotoxicity is seen in tumour cells incubated at 39-40°C instead of 37°C (Matthews, 1983, and Tsujimoto et al, 1985). In addition, marked synergistic antiproliferative activity has been demonstrated whilst using TNF in combination with interferon-gamma on both murine and human cells *in vitro* (Williamson et al, 1983, and Sugarman et al, 1985). Increased TNF receptor expression by interferon-gamma may contribute to this synergy (Aggarwal et al, 1985a, Ruggiero et al, 1986, and Tsujimoto et al, 1986a).

The resistance of most normal cells and many tumour cell lines to the lytic effects of TNF is neither due to a lack of TNF receptors nor to a low binding affinity for the ligand (Sugarman et al, 1985, and Tsujimoto et al, 1986a). The augmenting effect of RNA and protein synthesis inhibitors indicates that escape from the cytolytic effect of TNF requires the synthesis of certain critical proteins, and that these proteins are not made in sufficient quantities by cells which are susceptible to the effects of TNF. In fact, a variety of proteins are synthesized by normal cells in response to TNF and these may be potential candidates for the fulfillment of this protective role (Kirstein and Baglioni, 1986, and Kirstein et al, 1986).

1.6.2. Clinical trials

A plethora of phase I clinical trials of recombinant human TNF in patients with advanced cancer, using a variety of dose schedules, have been reported

during the last two years (Spriggs et al, 1987, Blick et al, 1987, Chapman et al, 1987, Kimura et al, 1987, Selby et al, 1987, Feinberg et al, 1988, Sherman et al, 1988, and Spriggs et al, 1988), and phase II trials are, at present, being performed at several institutions.

The most common toxicities observed in the phase I studies reported to date are fever, chills, fatigue and hypotension. Others include haematological toxicity, hepatotoxicity and changes in serum zinc levels and plasma cortisol levels. Younger asymptomatic patients appear to tolerate TNF with fewer episodes of severe toxicity.

Dose-limiting toxicities in schedules using bolus administration of TNF include severe hypotension, transient mild thrombocytopenia and hepatotoxicity, manifested as elevated serum transaminases (Kimura et al, 1987, and Selby et al, 1987). A trial of alternating intramuscular (i.m.) and intravenous (i.v.) bolus injections of TNF was associated with decreases in haemoglobin, lymphocytes and platelets (Blick et al, 1987). Administration of TNF as a continuous i.v. infusion over a 24h period resulted in hypotension as the dose-limiting toxicity (Spriggs et al, 1988), whereas, administration as a 5 day continuous infusion resulted in rapid but transient thrombocytopenia as the toxicity which led to cessation of treatment (Sherman et al, 1988).

Few beneficial clinical responses have been observed during the clinical trials undertaken so far. Although determination of efficacy is not an objective in undertaking phase I clinical trials, the small response rate observed in these trials is disappointing. However, animal studies have shown that TNF is a more effective antineoplastic agent when administered in conjunction with interferon-gamma (Williamson et al, 1983, Broukaert et al, 1986, Fransen et al, 1986b, and Balkwill et al, 1987b), and clinical trials are presently being undertaken using this combination approach.

1.7 Biological activities of TNF

TNF not only exerts numerous effects on malignant cells, but it is also an important modulator of the metabolic activities of most normal cells. Virtually all somatic tissues, with the exception of erythrocytes, possess a receptor for TNF, either in the resting cell or after inducement with a priming stimulus (Scheurich et al, 1987) and, although receptor number does not usually bear any relationship to susceptibility to the cytotoxic effect of TNF (Sugarman et al, 1985, and Tsujimoto, 1986a), it may have a bearing on the other metabolic effects induced by this cytokine. New biological activities of TNF are continually being discovered and the list is growing longer by the day. Below is a brief description of some of those activities already attributed to TNF, although this list is by no means exhaustive.

1.7.1 Neutrophil activation

TNF exerts a multitude of effects on polymorphonuclear neutrophils and, as such, is thought to play a major role in inflammatory responses and host defense mechanisms. TNF has been reported to augment neutrophil phagocytic activity (Klebanoff et al, 1986), increase cytotoxicity to certain micro-organisms (Djeu et al, 1986), cause neutrophil degranulation (Klebanoff et al, 1986), and production of the superoxide anion (Tsujimoto et al, 1986b) and hydrogen peroxide (Klebanoff et al, 1986). TNF has also been reported to enhance neutrophil adherence to endothelial monolayers (Gamble et al, 1985, and Klebanoff et al, 1986) by a mechanism involving the regulation of the expression of cell surface molecules both on the neutrophil and on the vascular endothelial cells. This mechanism is very rapid, the full effect being observed in less than 5min, and does not require protein synthesis on the part of the neutrophil. It is assumed that, not only the beneficial host defence and anti-neoplastic effects of TNF, but also the morbidity that accompanies TNF infusion, can be attributed in some measure to these neutrophil responses.

1.7.2 Effects on endothelial cells

TNF also exerts its regulatory action on the inflammatory process by a direct

effect on the vascular endothelium, causing the adherence of neutrophils to endothelial monolayers (Gamble et al, 1985, and Pohlman et al, 1986). This process requires protein synthesis on the part of the endothelial cell and may involve the expression of a number of antigens related to intercellular adhesion. TNF has been demonstrated to cause endothelial rearrangement (Stolpen et al, 1986) in tissue explants and to exert a direct cytotoxic effect on the endothelium (Sato et al, 1986). In addition, it causes endothelial cells to produce a pro-coagulant factor and to down-regulate the expression of thrombomodulin (Stern et al, 1985), thus favouring coagulation and thrombus formation. This effect may be enhanced by the TNF-induced IL-1 production by endothelial cells (Stern et al, 1985, and Nawroth et al, 1986) and may be involved in the haemorrhagic necrosis of certain tumours by TNF.

1.7.3 Effects on haematopoietic progenitor cells

TNF appears to play a role in the homeostasis of haematopoiesis by exerting both inhibitory and stimulatory effects on haematopoietic progenitor cells. On the one hand, TNF has been reported to be capable of inhibiting the proliferation and differentiation of a number of these cells *in vitro* (Shah et al, 1978, Degliantoni et al, 1985, Broxmeyer et al, 1986, and Lu et al, 1986) and, as such, has been implicated in the anaemia of chronic disease. However, TNF has also been shown to stimulate the production of granulocyte-monocyte colony-stimulating factor (GM-CSF) by cultured normal lung fibroblasts, vascular endothelial cells, smooth muscle cells and several malignant cell types (Munker et al, 1986). GM-CSF, in turn, stimulates the production of granulocytes and monocytes, augments the function of mature cells and enhances the growth of pluripotent and erythroid stem cells (Metcalf, 1985). Thus, stimulation of GM-CSF production by TNF exerts a positive effect on haematopoiesis. TNF has also been reported to induce myeloid cells to develop into terminally differentiated myelomonocytic cells (Trinchieri et al, 1986). Thus, TNF may play a significant role in the regulation of haematopoiesis, but this role has yet to be fully defined.

1.7.4 Effects on bone and connective tissue

TNF stimulates bone resorption and inhibits the synthesis of collagen. In bone this is achieved by accelerating the actions of osteoclasts while suppressing osteoblast formation (Bertolini et al, 1986, and Tashjian et al, 1987), and can be completely inhibited by salmon calcitonin, a specific inhibitor of osteoclast activity. In cartilage, TNF inhibits the synthesis, and stimulates resorption, of proteoglycan, an essential component of the matrix of cartilage (Saklatvala, 1986). Loss of proteoglycan, which occurs in rheumatoid arthritis, osteoarthritis and other joint diseases, results in severe impairment of cartilage function.

TNF has a catabolic effect on synovial cells and dermal fibroblasts, triggering the production of collagenase and prostaglandin E_2 (PgE_2) (Dayer et al, 1985). Whereas collagenase functions to disrupt the extracellular collagen matrix in inflamed tissues, PgE_2 apparently stimulates the production of intracellular proteases. Thus, TNF may play an important role in the pathogenesis of inflammatory diseases involving bones and joints. In fact, it has been reported that TNF may be detected in the synovial fluid of patients suffering from a variety of inflammatory joint diseases (Duff et al, 1987a) and it is produced *in vitro* in response to stimuli that trigger joint inflammation (Duff et al, 1987b).

1.7.5 Mediation of endotoxic shock

When large doses of TNF are administered to animals many of the biological effects mentioned above, and others unmentioned so far, become 'exaggerated', leading to a state of shock and even death. This shock-like state is very similar to that observed following administration of LPS to animals (Tracey et al, 1986). It would thus appear that TNF is not only an essential element in the response to LPS but that it is also capable of mimicking many of the effects of endotoxin poisoning.

TNF administration to rats (Tracey et al, 1986) resulted in a number of physiological changes including the development of metabolic acidosis,

marked haemoconcentration, hypotension, hyperglycaemia followed by hypoglycaemia, haemorrhagic infarction of the gastrointestinal tract, pancreatic and adrenal haemorrhage, acute renal tubular necrosis, and a severe interstitial pneumonitis, often leading to ventilatory arrest and death. Dogs exhibited similar symptoms, as well as a generalised stress response characterised by elevated levels of catecholamines, glucocorticoids and glucagon (Tracey et al, 1987).

TNF has been shown to be an endogenous pyrogen, being capable of causing fever through a direct effect on hypothalamic neurons, and indirectly by triggering peripheral production of IL-1. Hence, a biphasic fever curve is obtained following i.v. administration of TNF to rabbits (Dinarello et al, 1986). However, a monophasic curve has been seen in humans (Selby et al, 1987).

TNF-induced shock is completely reversible by prior administration of prostaglandin synthesis inhibitors (Kettlehut et al, 1987). Thus, it would appear that arachidonate metabolites are involved in the histopathologic changes observed following TNF infusion.

1.7.6 Antimalarial and antiviral activity

TNF has been associated with the pathogenesis of cerebral complications in murine malaria (Grau et al, 1987), however, it also appears to fulfill a protective function by exerting a toxic effect on the parasites (Clark et al, 1987). TNF has been detected in the serum of severely ill malaria-infected animals and patients, and on endothelial cells of the brains of patients with cerebral malaria (Clark et al, 1989). In addition, TNF, when administered as an i.v. injection, can mimic the pathology seen in this disease (Clark et al, 1989).

TNF has also been demonstrated to inhibit the growth of certain viruses in culture, apparently by a selective lytic effect on virus-infected cells (Mestan et al, 1986, Wong and Goeddel, 1986, and Koff and Fann, 1986), and thus may play a role in the host defense against viruses. This conclusion is further supported by the demonstration that viruses can act as inducers of TNF synthesis (Aderka et al, 1986, and Wong and Goeddel, 1986).

1.8 Comparison with Interleukin-1 (IL-1)

Although TNF and IL-1 are biochemically and immunologically distinct proteins, sharing no amino acid homology, and recognising and binding to different receptors, striking similarities in their biological activities have been observed (summarised in table 1.1). They are both macrophage products, have similar molecular weights (17Kd), can both affect the growth of many malignant cell lines and can regulate the metabolism of non-malignant cells (section 1.7, Mizel et al, 1981, Baracos et al, 1983, Gowen et al, 1983, Bevilacqua et al, 1984, Dinarello, 1984, Lovett et al, 1986, and Tsai and Gaffney, 1986). In fact, IL-1 has been reported to show similar effects on neutrophils, endothelial cells, haematopoietic progenitor cells, bone and connective tissue, body temperature and pathogens as those described above for TNF. Slight differences have been observed however and these are described below.

IL-1 alpha (pI 5.0, 159 amino acids) and beta (pI 7.0, 153 amino acids) have been isolated and cloned to date (Lomedico et al, 1984, and March et al, 1985), although it is thought that other members of this family exist. Both these forms of IL-1 have a molecular weight of 17Kd and they bind to the same receptor. A 26% homology exists between the amino acid sequences of IL-1 alpha and beta and a 45% homology exists between their nucleotide sequences (March et al, 1985, and Gubler et al, 1986).

As described previously (section 1.7.1) TNF is a direct stimulus for the neutrophil respiratory burst and degranulation. Although IL-1 is also implicated in these effects, unlike TNF, it does not exert a direct effect but results in the discharge of the neutrophils' secondary granules (Klempner et al, 1978). Human neutrophils have been reported to produce both an IL-1-like factor and an inhibitor of IL-1. The inhibitor is constitutively present in neutrophils and may be important as a negative regulator of IL-1 function (Tiku et al, 1986). It is not known, however, if a similar inhibitor exists for TNF activity.

Although both TNF and IL-1 are capable of stimulating the production of collagenase and PgE_2 from synovial cells and dermal fibroblasts (section 1.7.4,

Mizel et al, 1981, and Dayer et al, 1984), it would appear that human synovial cells are more responsive to TNF than IL-1 and that dermal fibroblasts are more sensitive to IL-1 than TNF (Dayer et al, 1985).

IL-1 has not been demonstrated to cause the shock-like symptoms observed after high-dose TNF administration (section 1.7.5), although it is an endogenous pyrogen and produces fever by increasing PgE_2 synthesis in or near the anterior hypothalamus (Dinarello, 1985).

The most striking difference, however, in the biological activities of TNF and IL-1 is seen in their effects on T cells. IL-1 has been reported to activate T lymphocytes in response to antigen or mitogen by stimulating IL-2 synthesis (Larsson et al, 1980, Smith et al, 1980, and Gillis and Mizel, 1981), and the expression of IL-2 receptors (Williams et al, 1984, and Schwab et al, 1985), on T cells. Resting lymphocytes apparently lack cellular receptors for TNF (Kull et al, 1985) and, as such, have been thought to be unresponsive to TNF. However, Scheurich et al. (1987) have shown that human peripheral T lymphocytes can be activated to express TNF receptors and that TNF treatment results in increased expression of the Tac antigen (IL-2 receptor).

Table 1.1

Overlapping biological activities of TNF and IL-1

FUNCTION	TNF	IL-1
T cell activation	±	+
Cytotoxic/cytostatic action on tumour cells	+	±
Inhibition of lipoprotein lipase	+	+
Pyrogenic action	+	+
Activation of neutrophils	+	+
Osteoclast activation	+	+
Induction of procoagulant activity on endothelial cells	+	+
Induction of surface antigens on endothelial cells	+	+
Mitogenic action in fibroblasts	+	+
Induction of IFN-β_2	+	+
Induction of *c-myc* and *c-fos*	+	+
Induction of EGF receptor	+	?
Induction of IL-1 synthesis	+	+
Induction of TNF synthesis	+	+
Induction of GM-CSF	+	+
Increased prostaglandin and collagenase synthesis	+	+
Induction of acute phase protein (C3)	+	+
Decreased hepatic albumin synthesis	+	+
Toxicity to β-islet of Langerhans cells	+	+
Mediator of septic shock	+	-

(adapted from Le and Vilcek, 1987)

1.9 The Role of TNF in the Cachexia and Anorexia of Cancer

TNF (cachectin) has been implicated in the pathogenesis of cachexia in animals infected with *Trypanosomes* (Rouzer and Cerami, 1980) and this role has since been extended to incorporate cachexia in general, including cancer cachexia and the cachexia associated with other chronic diseases.

The term cachexia is derived from the Greek kakos, 'bad' and hexis, 'condition'. Cancer cachexia is the state of malnutrition and wasting which is probably the most debilitating feature, as well as the major cause of death, in patients suffering from cancer (Harnet, 1952, Inagaki et al, 1974, and Warnold et al, 1978). Although numerous different definitions of cancer cachexia can be found in the literature, it can probably best be described as a metabolic syndrome characterized by anorexia (decreased calorie intake), asthenia, anaemia, altered host metabolism and an increased energy expenditure, with progressive weight loss resulting from significant losses in body fat, protein and other body compartments (Costa, 1977, and Bozzetti, 1984).

Although cancer cachexia is often accompanied by some degree of anorexia it does not appear to be the result of semi-starvation alone, but represents a more complex metabolic problem, with anorexia appearing as just one of several symptoms presenting themselves in this syndrome. In fact, some cancer patients present with cachexia without any signs of anorexia (Brennan, 1977, and Lawson et al, 1982) and, even with forced-feeding and hyperalimentation of cancer patients, the process of wasting is only temporarily reversed (Terepika and Waterhouse, 1956). In addition, although cachexia and anorexia are similar with respect to weight loss, there are several important differences which exist between the two conditions, particularly in relation to metabolism.

The anorexia of cancer may be further complicated by the presence of a mechanical obstruction preventing the entry of food into the gastrointestinal tract, as often occurs in malignant diseases affecting the head and neck, oesophagus, or upper gastrointestinal tract, and by malabsorption, which appears to represent, not only local, but also distant systemic effects of some

tumours (Blackwell, 1961, Kelley et al, 1961, and Schein et al, 1975). Many cancer patients find that their taste for food is altered and its palatability is changed and this can also affect the appetite of these patients (Carson and Gormican, 1977, Gorshein, 1977, DeWys, 1978, and Williams and Cohen, 1978). Insulin response to glucose is both depressed and delayed in cancer patients (Kisner et al, 1978) and, since increased insulin stimulates appetite, this blunted insulin response in cancer patients may lead to a decreased appetite. Alterations of metabolism in cancer patients may also depress appetite; glucose uptake by the liver is sensed by vagal fibers which send impulses to the central food intake control centre. It is possible that the increased metabolic activity of the liver (glucose uptake for Cori cycle activity, section 1.9.2) and the increased liver weight that occurs in cancer (Theologides and Pegelow, 1970) may result in increased firing of hepatic glucoreceptors which would lead to suppression of appetite. Increased blood lactate levels (Pitts and McClure, 1967, and Weinhouse, 1972) and fatty acid levels (Waterhouse et al, 1964) in cancer patients may also lead to decreased eating. In addition, pain, fever, chemotherapy, radiotherapy and anxiety may all contribute to the decrease in food consumption (Donaldson, 1977, Ohnuma and Holland, 1977, and Donaldson and Lenon, 1979).

In order to determine whether TNF is involved in the cachexia or anorexia of cancer one must understand the basic mechanisms underlying these disorders. Below is a brief description of the metabolic effects induced by anorexia and how these differ from those observed in cancer cachexia.

1.9.1 Metabolic effects of anorexia

Anorexia is a hypometabolic condition in which there is a decrease in the turnover of carbohydrate, fat and protein stores and a decrease in energy requirement, attained by a slowing of spontaneous activity. The fuel sources in anorectic conditions are shown in figure 1.2.

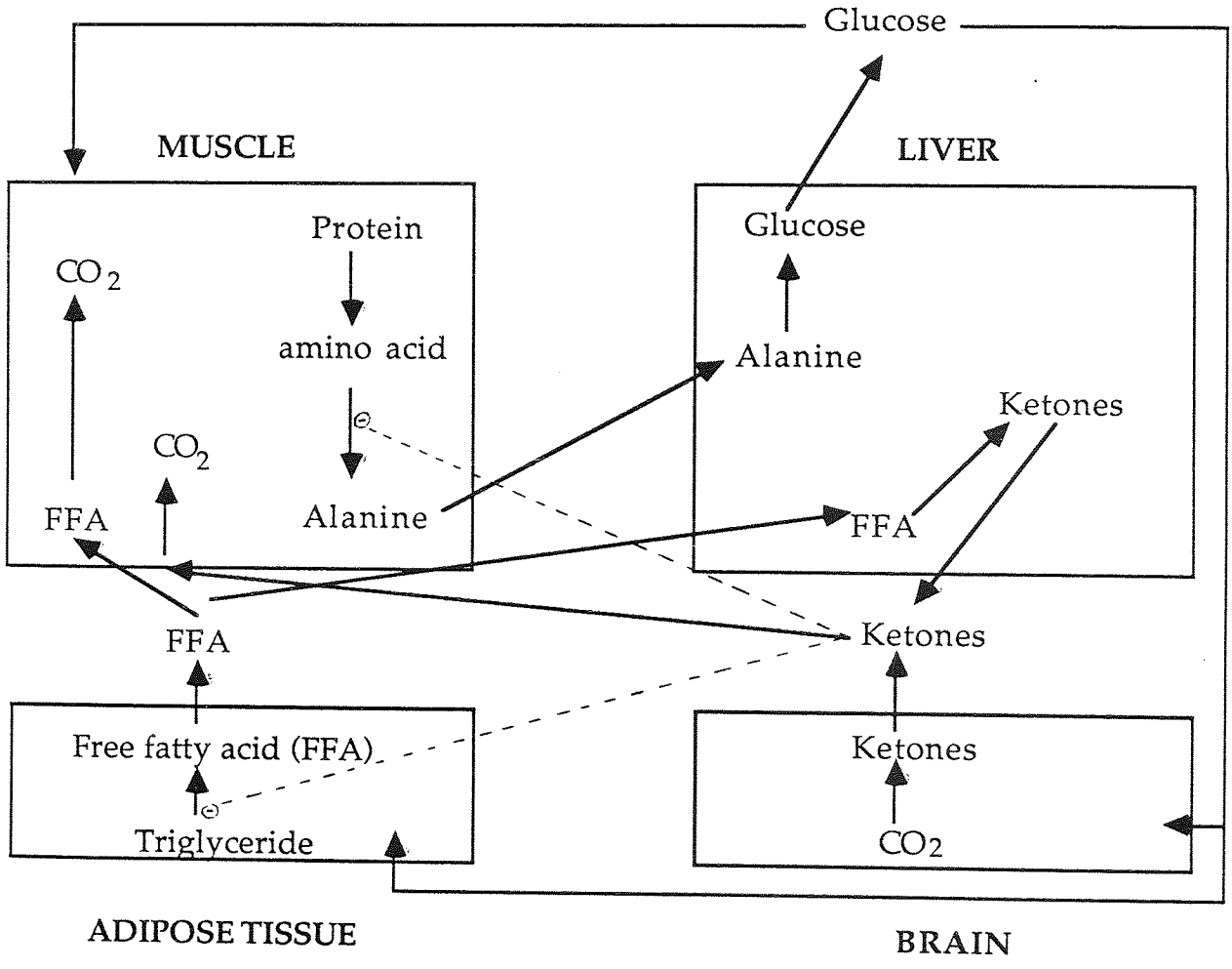
During the early stages of anorexia glucose is synthesized from amino acids (alanine) derived from muscle protein, mainly in the liver, but as the anorexia progresses synthesis also occurs in the cortex of the kidney. The

Figure 1.2

Fuel sources under anorectic conditions

The formation of ketone bodies in prolonged anorexia results in:

1. The sparing of glucose oxidation by brain due to cerebral ketone body metabolism.
2. A decrease in the rate of FFA mobilisation from adipose tissue.
3. The inhibition of protein breakdown in muscle allowing protein conservation and decreasing the rate of gluconeogenesis to maintain glucose homeostasis.



(Tisdale, 1982)

urinary losses of urea, and of calcium, magnesium and potassium from the cells as intracellular protein is utilized cause an accompanying loss of water, and this is mainly responsible for the initial rapid weight loss accompanying anorexia.

At the same time as amino acids from proteins are utilized for gluconeogenesis, free fatty acids (FFA) are mobilised from adipose tissue. Both of these effects are the result of a fall in plasma insulin levels and a rise in glucagon levels which occurs in the early stages of anorexia, triggered by an initial drop in blood glucose. This early hormonal response to anorexia serves to maintain glucose production to meet the needs of the brain.

Excess FFA are converted into ketone bodies (acetoacetate and 3-hydroxybutyrate) in the liver, these pass into the blood and are utilized as energy sources by other tissues, including the brain. Under normal conditions the brain is a major consumer of glucose, but during prolonged anorexia the brain adapts to utilize ketone bodies as the predominant metabolic fuel (Owen et al, 1967). High ketone body levels also stimulate insulin secretion by the β-islet cells of the pancreas and, since insulin is a potent anti-lipolytic hormone, this decreases the rate of FFA mobilisation from adipose tissue. There is also evidence that high ketone body levels can directly reduce lipolysis (Hawkins et al, 1971), thus regulating to some extent their own production.

Ketone bodies also play an important role in the inhibition of protein breakdown during prolonged anorexia, decreasing plasma alanine concentrations and urinary nitrogen excretion (Sherwin et al, 1975). The precise mechanism of this effect is not clear but may be due to the inhibition of the oxidation of branched chain amino acids by ketone bodies (Buse et al, 1972). Since these amino acids are the precursors of both the carbon and nitrogen portions of alanine, and are themselves known to inhibit protein catabolism in muscle, their accumulation could mediate the observed inhibition of protein breakdown.

1.9.2 Metabolic effects of cachexia

As described above, both humans and animals adapt to the insult of anorexia by decreasing their basal metabolic rates, hence reducing energy requirements, and by the production of ketone bodies. These protective mechanisms are not, however, observed in patients and animals with cancer cachexia. Many patients with cancer cachexia appear to have an inappropriately high energy expenditure despite a decrease in calorie intake (Young, 1977) and this may contribute to the progressive wasting that occurs in this condition. The absence of ketosis (Conyers et al, 1979) may also explain the loss of muscle protein and low protein synthesis, prolonged breakdown of adipose tissue, and decreased insulin secreting capacity observed in cancer cachexia. Figure 1.3 shows the fuel sources in cachectic cancer patients.

An explanation for the increased energy expenditure, and possibly the lack of ketosis, in cancer cachexia is an elevated Cori cycle activity (conversion of lactate to glucose in the liver) which has been found to be high in patients with metastatic carcinoma and progressive weight loss (Holroyde et al, 1975). Since this is a particularly inefficient process, consuming 6ATP's per Cori lactate-glucose cycle, the large energy consumption involved may contribute to the wasting observed in cancer cachexia (Gold, 1974). Gluconeogenesis from alanine (Waterhouse et al, 1979) and from glycerol (Lundholm et al, 1982) is also higher in patients with progressive malignant disease and this is probably due, at least in part, to the excess energy demands imposed on the host by the tumour. Glucose intolerance in cancer cachexia appears to be related both to abnormal insulin production and to insulin resistance without a reduction in the number of insulin receptors on peripheral tissues (Kisner et al, 1978).

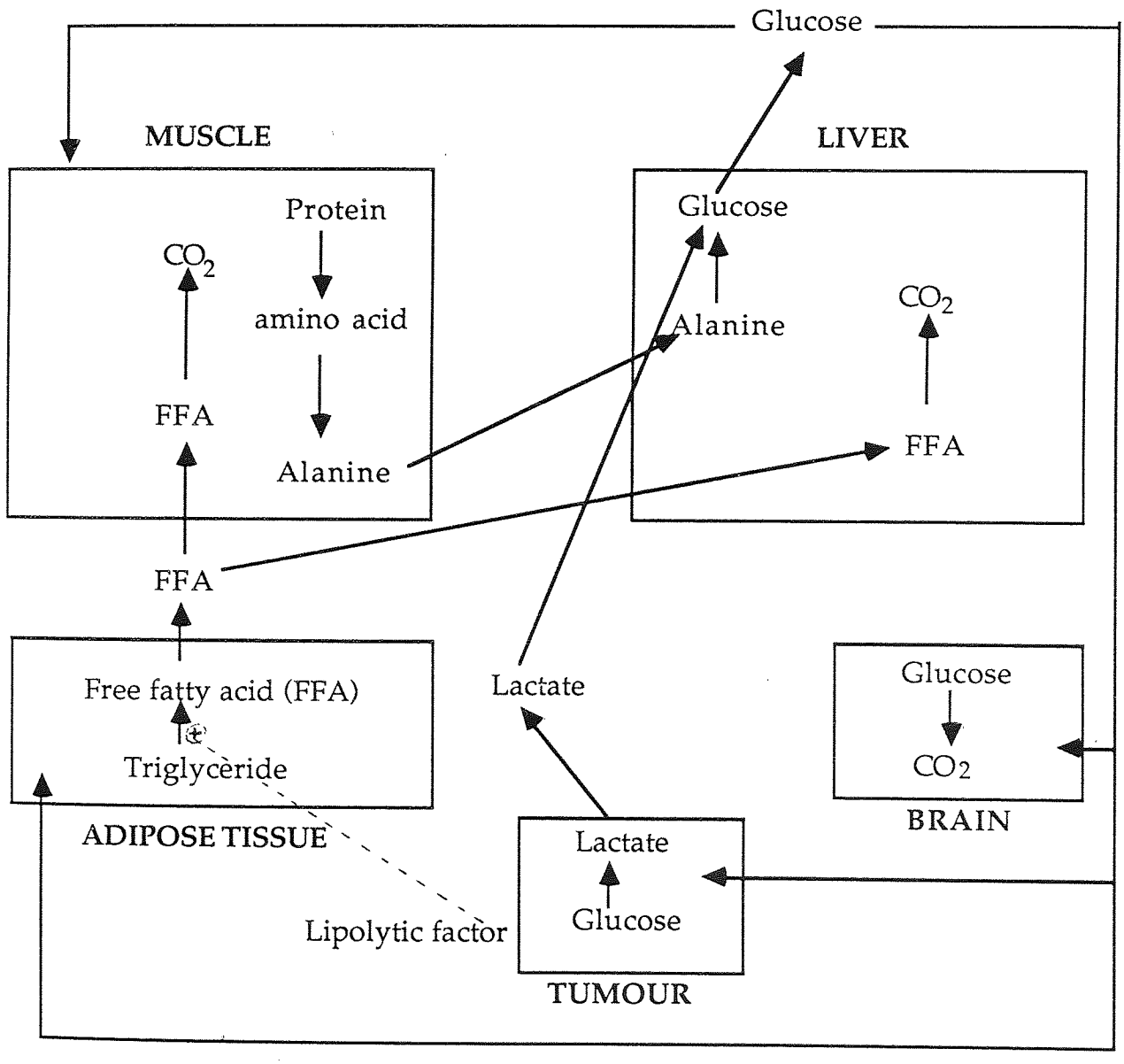
Skeletal muscle depletion and a negative nitrogen balance are other hallmarks of cancer-associated cachexia (Clark and Goodlad, 1971, Goodlad and Clark, 1972, and Theologides, 1972) and are contributed to by the lack of ketosis in this condition. In addition, cancer patients show anomalous plasma amino acid patterns (Feldman and Plonk, 1976), possibly arising from the tumour removing one or more of the essential amino acids it requires from

Figure 1.3

Fuel sources in cachectic cancer patients

The absence of ketosis may explain:

1. The loss of muscle protein and low protein synthesis.
2. The prolonged breakdown of adipose tissue.
3. The decreased insulin secreting capacity.



(Tisdale, 1982)

the amino acid pool of the host, and this may lead to a breakdown of muscle proteins. Thus, unlike anorexia, where the total plasma amino acid concentration may be low, it is often not reduced in cachexia, although the pattern of the individual amino acids is different from control. However, as tumour cells can concentrate amino acids more efficiently than do normal cells (Wiseman and Ghadially, 1955, and Copeland et al, 1979), this will inevitably lead to competition between host and tumour for nitrogen compounds and result in a negative nitrogen balance in the host.

The progressive decrease in carcass lipids observed in cancer cachexia, even when there is a very small tumour burden, is due to the mobilisation of FFA from the host's adipose tissue and is often associated with an increase in plasma lipids, probably due to a decreased fractional clearance of triacylglycerols (Redgrave et al, 1984). Insulin resistance in adipose tissue may favour decreased lipid synthesis and increased lipolysis, but it is also thought that the mobilisation of the host's FFA is the result of secretion of a lipolytic factor by the tumour.

1.9.3 Factors implicated in the pathogenesis of cancer cachexia

A variety of host-derived and tumour cell products have been identified in the serum of cancer patients and many of these have been suggested to be capable of producing the catabolic alterations observed in cancer cachexia. Peptide hormones, including ACTH, PTH, cortisol, glucagon and prostaglandins (Ellison and Neville, 1973, Benson et al, 1974, Mallinson et al, 1974, and Odell and Wolfsen, 1980) are secreted by some tumour cells and can all induce physiological changes that lead to weight loss. In 1980 Ketada et al. identified a 'lipid metabolizing factor' in the serum of AKR mice. Then, in 1981, Masuno et al. isolated Toxohormone-L from tumour extracts and body fluids of patients and animals with tumours. Injection of this factor into the lateral ventrical of rats caused lipid depletion and anorexia in these animals (Masuno et al, 1984). TNF is, however, the factor which is receiving the greatest interest at the present time and is under the most scrutiny to determine whether it is capable of mediating the effects of cancer cachexia.

1.9.4 TNF and cancer cachexia

As described previously (section 1.2), TNF was initially suggested to be a mediator of the cachexia associated with *Trypanosome* infection (Rouzer and Cerami, 1980). However, on the basis of two lines of evidence, TNF has since been implicated in the pathogenesis of cancer-associated cachexia and cachexia associated with other chronic illnesses (Beutler and Cerami, 1986).

The first line of evidence leading to the classification of TNF as a cachectic agent (and the name 'cachectin') was the observation that TNF is a potent inhibitor of lipoprotein lipase (LPL) activity both in cell culture and animals (Beutler et al, 1985a, and Semb et al, 1987). LPL activity is also depressed in cancer patients (Vlassara et al, 1986). This enzyme, which is also known as 'clearing factor', catalyses the hydrolysis of triglycerides in chylomicrons and very low density lipoproteins (VLDL) to form fatty acids and glycerol (figure 1.4). The triacylglycerol is hydrolysed progressively through a diacylglycerol to a monoacylglycerol which is finally hydrolysed by a separate monoacylglycerol hydrolase. Some of the released FFA's return to the circulation, but the bulk are transported into the tissue. This process is essential for the normal utilization of fat as an energy source since triglycerides in chylomicrons or in VLDL cannot be taken up intact by tissues. After entry into the tissues, FFA's are re-esterified to triacylglycerols or oxidised as fuel. The enzyme has almost no effect on free triglycerides.

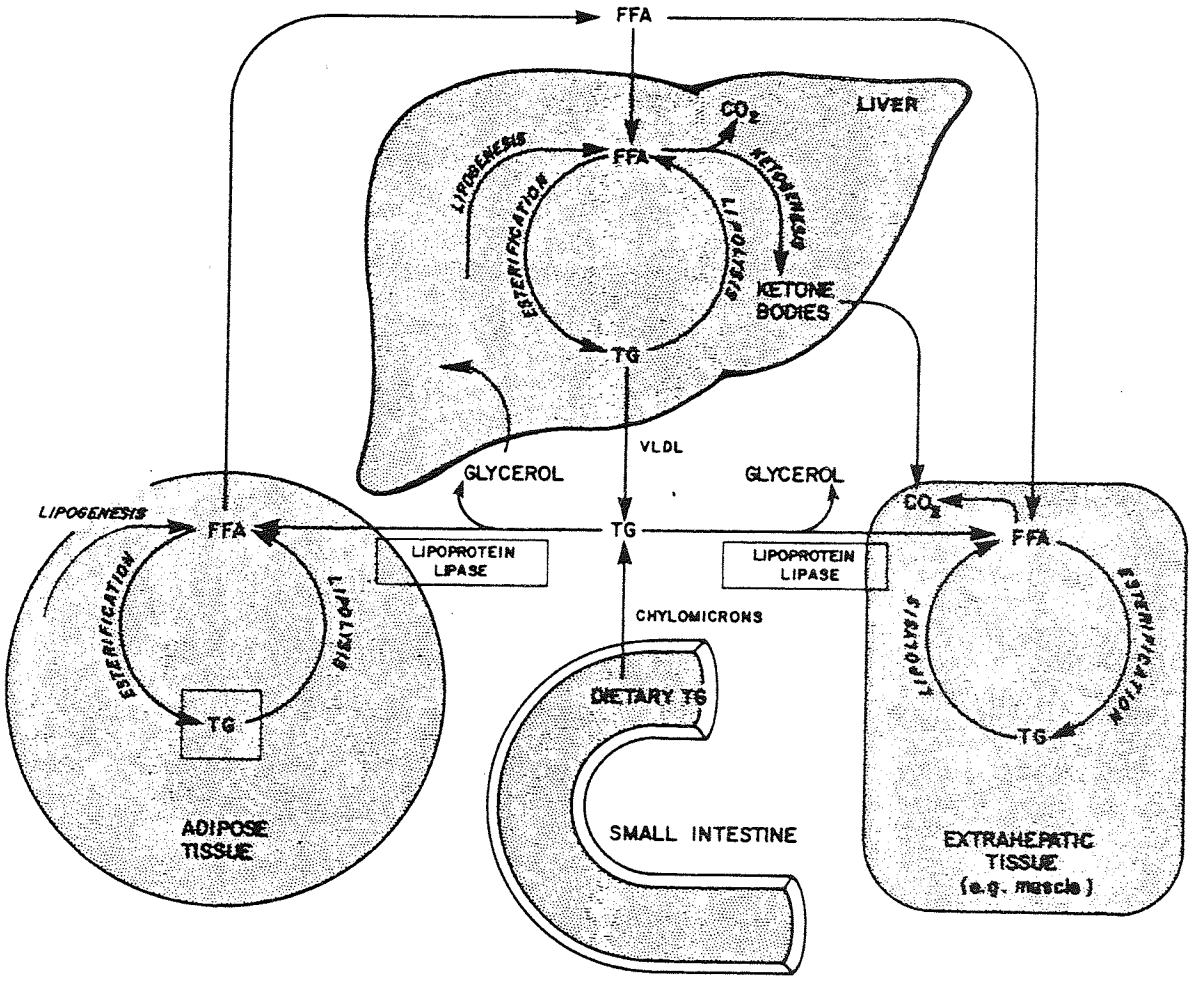
LPL is synthesized principally in adipose tissue and muscle cells but is found in varying quantities in most extrahepatic tissues. It is then secreted in the active form on the capillary endothelia of these tissues. Hydrolysis of triacylglycerols takes place while the lipoproteins are attached to the enzyme on the endothelium.

Normal blood does not contain appreciable quantities of this enzyme, but following injection of heparin, LPL is released from the tissues into the circulation (Spooner et al, 1979, Ashby and Robinson, 1980, and Eckel et al, 1984). Thus, it is thought that heparin is linked to LPL, or that it may enhance

Figure 1.4

The role of lipoprotein lipase in lipid metabolism

Lipoprotein triacylglycerol (TG) in chylomicrons or in very low density lipoproteins (VLDL) cannot be taken up intact by tissues but must first undergo hydrolysis by lipoprotein lipase, an enzyme situated in the capillary endothelium of extrahepatic tissues, prior to uptake of the released free fatty acids (FFA).



(Harper et al, 1979)

the binding of the enzyme to chylomicrons and VLDL's.

Decreases in the levels of adipocyte LPL have been demonstrated in such disorders as diabetes (Kessler, 1963, and Schnatz and Williams, 1963) and starvation (Nilsson-Ehle et al, 1980). Inhibition of this enzyme would lead to accumulation of triglycerides in the plasma and hypertriglyceridaemia similar to that which was observed by Rouzer and Cerami (1980) in *Trypanosome*-infected rabbits. It was hypothesized that the TNF-induced inhibition of adipocyte LPL was responsible for this hypertriglyceridaemia and, as triglycerides cannot be taken up intact by fat cells, this resulted in a reduction in the amount of fat which could be utilized for energy by these cells, thus leading to the lipid resorption, wasting and weight loss associated with cachexia.

The second line of evidence leading to the suggestion that TNF is capable of inducing cachexia was the observation that animals given daily injections of dialysed conditioned medium from lipopolysaccharide-induced peritoneal macrophages developed weight loss and anorexia, and they died if sufficient quantities of medium were administered (Cerami et al, 1985). It was suggested that the macrophage secretory products contained impure cachectin/TNF, and that this played a central role in the weight loss and anorexia observed in treated animals.

Since the publication of these findings by Cerami et al. (1985), and in parallel with this study, several more investigations have been undertaken to determine the role of TNF in cancer-associated cachexia and these will be reviewed in section 5 of this thesis.

1.10 Aims and scope of the present investigation

On initiation of this investigation the role of TNF in the pathogenesis of cancer cachexia was not clear and very few studies had been published regarding this possible toxic effect of TNF. However, in view of the potential antineoplastic effects of TNF and its administration to humans in clinical trials, it became evident that a better understanding of the metabolic effects and possible cachectic effects of TNF were required. In addition, it was realised that if TNF was identified as the factor responsible for the pathogenesis of cancer cachexia, reversal of the effects of this syndrome could possibly be achieved by administration of inhibitors of TNF action.

This study aimed to compare the effects of TNF administration with those observed in cancer cachexia. In order to achieve this objective an animal model of cachexia, the MAC 16 adenocarcinoma passaged in NMRI mice, was utilized. The MAC 16 adenocarcinoma is one of a series of colonic tumours originally induced in NMRI mice by 1,2-dimethylhydrazine (Double et al, 1975), and is considered to be a good model of human cachexia since host weight loss occurs with small tumour masses. This is similar to the human situation where the tumour burden rarely exceeds 5% of the total body weight (Costa, 1977). There are very few experimental models of cachexia where weight loss occurs with such small tumour burdens; most models utilize rapidly growing transplantable rodent tumours which only show signs of cachexia when the tumour represents 30-40% of the total body weight (Strain et al, 1980). The MAC 16 model does not cause anorexia (Beck and Tisdale, 1987) and is therefore an ideal model for looking at the biochemical effects of cachexia without the complications of anorexia. Thus, the effects of TNF administration in NMRI mice were compared with the effects of this cachexia-inducing tumour in the same mouse strain.

The initial study comparing TNF with the MAC 16 adenocarcinoma was expanded to include investigations into other metabolic effects of this cytokine, including its effects on glucose metabolism and lipogenesis, and methods of reversing the TNF-induced weight loss observed during this study.

2. MATERIALS

2.1 Animals

Pure strain inbred NMRI mice (age 6-8 weeks) were purchased from Bantin and Kingman, Hull, U.K. or were bred in the animal house of Aston University, Birmingham. Animals were fed *ad libitum* a rat and mouse breeding diet (Pilsbury's, Birmingham, West Midlands) and were given free access to water. Unless otherwise stated, mice were kept in a 12h light (8am-8pm) / dark (8pm-8am) cycle and, in all the experiments where weight changes and food and water consumption were measured, mice were housed in individual cages.

BALB/C mice (24-28g) were purchased from Bantin and Kingman, Hull, U.K.

2.1.1 Tumour system

The MAC tumours are a series of transplantable adenocarcinomas of the large bowel of mice from primary tumours induced by prolonged administration of 1,2-dimethylhydrazine (Double and Ball, 1975). Tumours were passaged in pure strain NMRI mice (age 6-8 weeks). MAC 16 or MAC 13 tumours (doubling times; 10 and 7 days respectively) were excised from donor NMRI mice, placed in sterile 0.9% NaCl and cut into fragments 1 X 2mm in size. Fragments were then implanted subcutaneously into the flank of the right limbs of mice using a trocar (Mike Wynter, Aston University). All experiments involving the MAC 16 adenocarcinoma were performed in collaboration with S.A. Beck, Aston University.

2.2 Cell lines

The L929, sensitive to TNF, and the L929R, with induced resistance to TNF, cell lines were kindly donated by Dr. N. Matthews, Dept. of Microbiology, UWIST, Cardiff, Wales.

2.3 Gases and agents for anaesthesia

The following medical gases were purchased from BOC Ltd, London:
nitrogen
nitrous oxide
oxygen
air : carbon dioxide (95 : 5)
nitrogen : carbon dioxide (95 : 5)
oxygen : carbon dioxide (95 : 5)

Fluothane (halothane) inhalation anaesthetic was obtained from ICI, Macclesfield, Cheshire.

2.4 Purchased materials, chemicals and reagents

Alpha Laboratories Ltd, Hampshire
Wako NEFA C kit, for the determination of free fatty acids in serum or plasma.

Amersham International, Buckinghamshire
2-deoxy-D-[2,6-^{3}H]-glucose (sp. act. 42Ci/mmol)
2-[1-^{14}C]-deoxy-D-glucose (sp. act. 56mCi/mmol)
D-[U-^{14}C]-glucose (sp. act. 270mCi/mmol)
[U-^{14}C]-palmitic acid (sp. act. 850mCi/mmol)

BDH Chemicals Ltd, Poole, Dorset
ammonia solution (sp. gr. 0.88, analar)
calcium carbonate
ethanolamine (Scintran(R))
2-ethoxyethanol (Scintran(R))
ethylenediaminetetra-acetic acid (EDTA), disodium salt
formic acid 90% (analar)
glycine (analar)
hyamine 10-X hydroxide solution (about 10% w/v in methanol)
2-methoxyethanol

perchloric acid
potassium chloride
potassium hydrogen carbonate ($KHCO_3$)
potassium dihydrogen orthophosphate (KH_2PO_4)
potassium hydroxide pellets
sodium acetate, trihydrate
sodium hydrogen carbonate (analar)
trichloroacetic acid (TCA)
zinc sulphate

BOC Ltd, London
Liquid nitrogen

Burroughs Ltd, London
absolute ethanol

Evans Medical Ltd, Beaconsfield, Buckinghamshire
heparin sodium injection BP, 25000U/ml

Fisons Scientific Equipment, Loughborough, Leicestershire
acetic acid (glacial)
barium hydroxide (analar)
calcium chloride (analar)
citric acid (analar)
dipotassium hydrogen orthophosphate, trihydrate ($K_2HPO_4.3H_2O$)
ethylacetate
hydrochloric acid (HCl) (sp. gr. 1.16, about 32% w/v)
magnesium sulphate ($MgSO_4.7H_20$)
Optiphase scintillation fluid
petroleum ether, light (b.p. 40-60°C)
n-propanol
sodium chloride
sodium hydroxide pellets (analar)
sulphuric acid

Gibco, Paisley, Scotland

foetal calf serum

RPMI 1640 medium (with 25mM HEPES and L-glutamine)

Macarthy's Ltd, Romford, Essex

arachis oil B.P.

May and Baker, Loughborough, Leicestershire

chloroform

diethyl ether

New England Nuclear, Du Pont (UK) Ltd, Southampton

prostaglandin E_2 [^{125}I] Radioimmunoassay kit

Scientific Hospital Supplies Ltd, Liverpool

Liquigen, a medium chain triglyceride (MCT) emulsion containing 52% MCT and 48% water, calorific value 1.7MJ% (400KCal).

Sigma Chemical Company Ltd, Poole, Dorset

actinomycin D-mannitol

adenosine 5'-triphosphate (ATP), trisodium salt

alanine dehydrogenase, suspension in 2.4M $(NH_4)2SO_4$, pH7.0

amyloglucosidase, from *Aspergillus niger*. Suspension in 3.2M $(NH_4)2SO_4$, pH6.0

α-1 antitrypsin

bovine serum albumin

creatinine diagnostic kit, for the determination of creatinine in plasma, serum or urine

crystal violet

16,16-dimethylprostaglandin E_2

dimethyl sulphoxide (DMSO)

Dowex-1-X8 (200-400mesh), formate form

Dowex-50-X8 (200-400 mesh), H+ form

α-D-(+)-glucose

glucose-6-phosphate dehydrogenase (G6P-DH), from bakers yeast, type VII
glycogen
hexokinase, from bakers yeast, type C-130
hydrazine hydrate
β-hydroxybutyrate dehydrogenase, from *Rhodopseudomonas spheroides*, type II
indomethacin, crystalline
L-lactic dehydrogenase, from rabbit muscle, type II
lipopolysaccharide from *E. coli* 055:B5 (TCA extract)
nicotinamide adenine dinucleotide (NAD), grade IV
nicotinamide adenine dinucleotide, reduced form (NADH), grade III
nicotinamide adenine dinucleotide phosphate (NADP), from yeast β-NAD
ninhydrin (2,2-dihydroxy-1,3-indanedione)
phosphate-buffered saline (PBS)
prostaglandins E_1, E_2, F_1-α and F_2-α
o-toluidine reagent kit, for the determination of glucose in plasma, serum and whole blood
triethanolamine hydrochloride
triglyceride diagnostic kit, for the determination of triglycerides in plasma and serum
triolein
Trizma base (Tris-(hydroxymethyl)-aminomethane)
trypsin
urea nitrogen reagent kit, for the determination of urea in plasma, serum or urine

<u>Tennants Ltd, West Bromich, West Midlands</u>
acetone
methanol

2.5 Donated chemicals

Mitozolamide was synthesized and donated by May and Baker, Dagenham and was stored at 25°C. Fresh solutions of mitozolamide in arachis oil containing 10% DMSO were made up daily and a concentration of 20mg/Kg (in 200µl) was injected i.p. into mice. Control mice were injected with 200µl arachis oil containing 10% DMSO.

Recombinant human Tumour Necrosis Factor-alpha (TNF), 6 X 10^7 U/mg, was donated by Boehringer Ingelheim Ltd, Bracknell, Berkshire and was stored at 4°C. The endotoxin content was less than 0.125 EU/ml. Fresh solutions of TNF were made up daily in 0.9% NaCl and 200µl of the stated concentration of TNF was injected i.v. into the tail veins of NMRI mice. Injections were performed between 8am and 11am. Control mice were injected i.v. with 200µl of 0.9% NaCl.

Recombinant murine Tumour Necrosis Factor-alpha (mTNF), 2 X 10^8 U/mg, was donated by Professor W. Fiers, Biogent, Switzerland and was stored at -70°C. The endotoxin content was 36ng/ml. Fresh solutions of mTNF were made up daily in 0.9% NaCl and 200µl of the stated concentration of mTNF was injected i.v. into the tail veins of NMRI mice. Control mice were injected i.v. with 200µl of 0.9% NaCl.

2.6. Buffers

<u>0.2M Acetate buffer, pH 4.8.</u>

acetic acid, 96%	4.8ml
sodium acetate	9.75g
distilled water	to 1000ml

<u>0.2M Citrate buffer, pH5.0</u>

citric acid	21.0g
1N NaOH	200ml

The pH was adjusted to 5 with 1N NaOH and the final volume was made up

to 500ml with distilled water.

Hydazine-glycine buffer, pH 9 (0.4M hydrazine; 0.5M glycine)

glycine	11.4g
hydrazine hydrate	25ml
distilled water	200ml

The pH was adjusted to 9 with concentrated hydrochloric acid and the final volume was made up to 300ml with distilled water.

Hydrazine-tris buffer, pH 8.5

hydrazine hydrate	1ml
EDTA-$Na_2H_2.2H_20$	20mg
1N HCl	5ml
0.1M tris buffer, pH 8.5	to 20ml

Prepared freshly every day.

Hydrazine-tris buffer, pH 10 (40mM tris; 1M hydrazine; 1.4mM EDTA)

hydrazine hydrate	5ml
0.2M tris solution	20ml
EDTA-$Na_2H_2.2H_2O$	50mg
distilled water	40ml

The pH was adjusted to 10 with 1N HCl and the final volume was made up to 100ml with distilled water.

Krebs-Ringer bicarbonate buffer (KRB), pH 7.6

Sodium chloride (NaCl), 0.9%	769.23ml
Potassium chloride (KCl), 1.15%	30.77ml
Potassium dihydrogen orthophosphate, (KH_2PO_4), 2.11%	23.08ml
Magnesium sulphate ($MgSO_4.7H_2O$), 3.82%	7.70ml
Sodium hydrogen carbonate ($NaHCO_3$), 1.3%	7.70ml
Calcium chloride ($CaCl_2$), 1.22%	161.54ml

0.1M Phosphate buffer, pH 6.8

SOLUTION A:

potassium dihydrogen orthophosphate (KH_2PO_4)	13.6g
distilled water	to 1000ml

SOLUTION B:

di-potassium hydrogen orthophosphate (K_2HPO_4)	17.4g
distilled water	to 1000ml

Equal volumes of solutions A and B were mixed to give a solution of pH 6.8.

0.3M Triethanolamine buffer, pH 7.5 (containing 4.05mM $MgSO_4$)

triethanolamine hydrochloride	5.6g
magnesium sulphate ($MgSO_4.7H_2O$)	100mg
distilled water	50ml

The pH was adjusted to 7.5 with approximately 12ml of 1N KOH and the final volume made up to 100ml with distilled water.

0.5M Triethanolamine buffer, pH 7.6 (containing 5mM EDTA)

triethanolamine hydrochloride	23.3g
EDTA-$Na_2H_2.2H_20$	0.47g
distilled water	200ml

The pH was adjusted to 7.6 with approximately 20ml of 2N NaOH and the final volume made up to 250ml with distilled water.

0.1M Tris buffer, pH 8.5

Trizma base	2.42g
distilled water	100ml

The pH was adjusted to 8.5 with approximately 6ml of 1N HCl and the final volume was made up to 200ml with distilled water.

0.2M Tris solution

Trizma base	4.84g
distilled water	200ml

2.7 Reagents

Ninhydrin reagent

SOLUTION A:	
ninhydrin	0.08g
0.2M citrate buffer, pH 5.0	50ml
SOLUTION B:	
ninhydrin	2.0g
2-methoxyethanol	50ml

Solutions A and B were mixed together and the resulting reagent was kept in the dark. Ninhydrin reagent was made up freshly each day.

3. METHODS

3.1 Comparison of TNF with a cachexia-inducing tumour

3.1.1 Treatment of animals

Female NMRI mice (20±1g) were injected with TNF (1.5, 3.0, 4.5, 6.0 or 7.5 X 10^7 U/Kg) (200µl, i.v.). Controls were injected with 0.9% NaCl (200µl, i.v.). Body weights and food and water intake were monitored over a 24h period. A second injection of the same concentration of TNF or 200µl of 0.9% NaCl was given 24h after the first injection. Male NMRI mice (26-28g) were transplanted with the MAC 16 adenocarcinoma as described previously (2.1.1) and body weights and food and water intake were monitored daily. Experiments were terminated if mice lost >30% of their original body mass. Non-tumour-bearing male NMRI mice were used as controls.

3.1.2 Food and water consumption

Food intake was measured by weighing the pellets remaining. Food wastage was minimal using pelleted food. Water consumption was determined by volume. Water bottles contained a ball valve to prevent dripping.

3.1.3 Metabolite determinations

The levels of various metabolites in the blood of mice 90min after the second TNF injection were measured and compared with those in controls 90min after the second injection of 0.9% NaCl. Blood metabolite levels in mice bearing the MAC 16 adenocarcinoma were determined when tumour weights were 0.372±0.035g (days 28-35 after transplantation).

3.1.3.1 Collection of blood samples

Mice were anaesthetised with a mixture of halothane (2.5%), oxygen (0.5cc/min) and nitrous oxide (0.7cc/min). Approximately 600µl of blood was collected by cardiac puncture into syringes containing 0.05ml heparin solution (15mg/ml). Blood samples were then transferred to microfuge tubes on ice and the levels of the following metabolites were measured using a Beckman

DU7 spectrophotometer:

Glucose

Free fatty acid

Triglyceride

3.1.3.2 Determination of Glucose

Glucose levels were determined in samples of whole blood with the use of a Sigma o-toluidine reagent kit. In the presence of heat and acid o-toluidine reacts readily with glucose to form a blue-green complex which may be measured spectrophotometrically at 635nm. The intensity of this colour is proportional to the concentration of glucose in the sample (Hyvarinen and Nikkila, 1962 and Feteris, 1965). 100µl of whole blood was used in each assay.

3.1.3.3 Determination of Free Fatty Acid

Free fatty acid (FFA) levels were determined in plasma obtained by centrifuging whole blood in a Beckman microfuge for 30sec. A Wako NEFA C kit, which is an enzymatic colorimetric method for the quantitative determination of FFA in serum and plasma, was used. This method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl CoA synthetase. The acyl CoA thus produced is oxidized by added acyl CoA oxidase, with the generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase, permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine to form a purple dye which can be measured colorimetrically at 550nm. The intensity of the dye is proportional to the concentration of FFA in the sample. 20µl of plasma was used in each assay.

3.1.3.4 Determination of Triglyceride

Triglyceride levels were determined in plasma obtained by centrifuging whole blood in a Beckman microfuge for 30sec. Determinations were carried out with the use of a Sigma triglyceride diagnostic kit. In this method triglycerides are extracted from plasma with isopropanol. Interfering

substances, including glucose, phosphatases, bilirubin and glycerol, are removed by a solid absorbent. The triglyceride-containing extract is saponified with potassium hydroxide to glycerol and FFA. The glycerol is then oxidized in the presence of periodate to form formaldehyde. Formaldehyde, in the presence of ammonium acetate and acetylacetone, forms diacetyldihydrolutidine, a yellow dye, which can be measured colorimetrically at 410nm. The intensity of the dye is proportional to the concentration of triglyceride in the sample. 50µl of plasma was used in each assay.

3.1.4 Body Composition analysis

The gastrocnemius and thigh muscles from the left hind limb of mouse carcasses were carefully dissected out and weighed together with the whole carcass. The carcass plus muscles were heated at 80°C until a constant weight was achieved. The carcass and muscles were then reweighed separately to obtain the dry weight. The water content for the muscles and carcass was determined from the difference between the wet and dry weights. Total carcass fat was determined by the method of Lundholm et al, (1980). Each carcass was broken up into small pieces and the fat was extracted successively with 25ml of each of the following; acetone : ethanol (1 : 1v/v), chloroform : methanol (1 : 1v/v) and diethyl ether. The extracts were combined, transferred to a pre-weighed round-bottom flask, and the solvents were evaporated off using a Buchi rotary evaporator to leave a fatty residue. The flask was reweighed and the amount of fat extracted from the carcass was calculated.

3.1.5 Measurement of serum and tumour TNF Levels

3.1.5.1 Primed TNF production

Non-tumour-bearing and MAC 16 and MAC 13 tumour-bearing male NMRI mice (24±2g) were administered 1.25mg/Kg *E. coli* lipopolysaccharide (in 200µl of 0.9% NaCl) into the tail veins. Mice were anaesthetised 90min later with a mixture of halothane (2.5%), oxygen (0.5cc/min) and nitrous oxide

(0.7cc/min) and approximately 800µl of blood was collected in unheparinized syringes by cardiac puncture, transferred to microfuge tubes and allowed to clot at room temperature. Blood samples were then centrifuged for 30sec in a Beckman microfuge, and the resulting serum was used for TNF determinations. Tumours were carefully dissected out of tumour-bearing mice and homogenised in 0.5ml/100mg tissue weight of distilled water using a CAMLAB 563C homogeniser (speed 8) fitted with a teflon pestle. The homogenates were then transferred to microfuge tubes, centrifuged for 30sec in a Beckman microfuge and the supernatants were used directly for the determination of TNF.

3.1.5.2 TNF assay

TNF was determined by an *in vitro* cell cytotoxicity assay similar to that described by Ruff and Gifford (1981). L929 cells were seeded at a concentration of 3×10^4 per well into 96-well flat-bottom microtitre trays in 100µl RPMI 1640 medium containing 10% foetal calf serum, and incubated at 37°C overnight under an atmosphere of 5% CO_2 in air. The medium was then removed and was replaced with varying dilutions (from 1 to 11) of mouse serum or tumour extract in RPMI 1640 medium and actinomycin D (1µg/ml), to a final volume of 100µl. Controls contained only RPMI 1640 medium and actinomycin D. Internal standards contained RPMI 1640 medium with 1U of recombinant human TNF and actinomycin D. The plates were re-incubated for 16 to 18h and were then rinsed with phosphate-buffered saline. The cells were fixed with 200µl of methanol for 10min and were then stained for 10min with 1% w/v crystal violet. Plates were rinsed with distilled water and left to dry in a stream of warm air. 50µl of 33% acetic acid was added to each well to elute the dye and plates were enumerated spectrophotometrically at 570nm on a Titertek Multiskan. The percentage cell cytotoxicity was calculated using the following formula, as described by Flick and Gifford (1984, 1986):

$$\% \text{ cell cytotoxicity}_{dil} = (A_{con} - A_{dil}) / A_{con} \times 100$$

Where % cell cytotoxicity$_{dil}$ is the amount of cell destruction at any particular

dilution of the TNF-containing serum or tumour extract, A_{con} is the absorbance of control wells, and A_{dil} is the absorbance of wells at a particular dilution of TNF. Dose-response curves were generated from assays performed in duplicate. The dilution of TNF-containing serum or tumour extract giving the same cell destruction as 1U of recombinant human TNF was recorded.

All serum samples and tumour extracts were similarly assayed on the TNF-resistant cell line, L929R, in order to ensure that any cell cytotoxicity was solely due to TNF.

3.1.6 Determination of *in vitro* lipolytic activity

Male BALB/C mice were killed by cervical dislocation and the epididymal adipose tissue was carefully dissected out, weighed and minced in Krebs-Ringer bicarbonate buffer, pH 7.6. Approximately 50-100mg of the adipose tissue was transferred to vials containing either 100µl of the MAC 16 tumour supernatant (equivalent to 0.32mg of protein) or TNF (4×10^3, 4×10^4, or 4×10^5 U/ml of assay mixture) in a total volume of 0.25ml of the Krebs-Ringer buffer. The vials were gassed for 20sec with 5% CO_2 in air and incubated for 2h in a shaking water bath at 37°C. At the end of the incubation vials were placed on ice and 50µl of the cell-free supernatant was removed and immediately used for the determination of FFA concentration with the use of a Wako NEFA C kit, as described previously (3.1.3.3). Controls containing adipose tissue and buffer alone were included in each experiment and the spontaneous release of FFA was subtracted from the values obtained with tumour or TNF present.

3.1.7 Determination of *in vitro* proteolytic activity

Male BALB/C mice were killed by cervical dislocation and the diaphragms were carefully dissected out, blotted on filter paper, cut in half and weighed. Each half was placed in a vial containing 0.75ml Krebs-Ringer bicarbonate buffer, pH 7.6, and gassed for 20sec with 5% CO_2 in air. Pre-incubations were carried out for 30min in a shaking water bath at 37°C, and the diaphragms were then blotted and transferred to clean vials containing the following in

Krebs-Ringer bicarbonate buffer, to a total volume of 0.75ml:

A 10^3U TNF
B 10^4U TNF
C 10^5U TNF
D 10^5U TNF + 1mM indomethacin
E 10^5U TNF + 1mg/ml α-1 antitrypsin
F 100µl MAC 16 tumour extract (2.9mg protein/ml)
G 100µl MAC 16 tumour extract + 1mM indomethacin
H 100µl MAC 16 tumour extract + 1mg/ml α-1 antitrypsin
I 100µl MAC 16 tumour extract + 1mM indomethacin + 1mg/ml α-1 antitrypsin
J 0.1mg/ml trypsin
K 0.1mg/ml trypsin + 1mM indomethacin

The vials were gassed for 20sec with 5% CO_2 in air and incubated for a further 2h at 37°C. Incubations were terminated by the addition of 0.5ml of the assay mixture to 0.125ml of cold 50% TCA, mixing and centrifuging for 10min in a Beckman bench centrifuge at 3000rpm. The supernatants were then neutralised to pH 6-7 with 1N NaOH, centrifuged for 5min at 3000rpm and the final volume was measured. 0.2ml of the neutralised sample was mixed with 1ml of ninhydrin reagent, held in a boiling water bath for 20min and diluted to 5ml with n-propanol : water (1 : 1v/v). The absorbance was determined spectrophotometrically at 570nm and the concentration of amino acids was read from a standard curve utilizing alanine as the reference. The spontaneous release of amino acids (alanine) from the diaphragms in the absence of any additions was subtracted from the final readings.

3.1.8 The effect of prostaglandins on the release of amino acids from mouse diaphragms

The method used was essentially that described above (3.1.7). In this case mouse hemi-diaphragms were incubated with 5, 10 and 20µg/ml of assay mixture of each of the following prostaglandins; PgE_1, PgE_2, PgF_1-α and PgF_2-α, and the release of amino acids was determined.

3.2 Determination of urea nitrogen and creatinine

Female NMRI mice (19±1g) were placed in metabolic cages immediately after a single injection of TNF (7.5 X 10^7U/Kg, i.v.) or 0.9% NaCl (200µl, i.v.). Urine was then collected over a 24h period and used for the determination of urea nitrogen and creatinine levels.

3.2.1 Urea nitrogen

Urea nitrogen levels were determined in mouse urine with the use of a Sigma urea nitrogen diagnostic kit, which is a colorimetric method for the quantitative determination of urea in serum, plasma or urine. This method relies upon the hydrolysis of urea by urease to ammonia and carbon dioxide. Ammonia then reacts with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside, to form indophenol which is blue in colour. The concentration of ammonia is directly proportional to the absorbance of indophenol, which is measured spectrophotometrically at 570nm. 10µl of urine, which had been diluted 100 fold with water, was used in each assay.

3.2.2 Creatinine

Creatinine levels were determined in mouse urine with the use of a Sigma creatinine diagnostic kit, which is a colorimetric method for the quantitative determination of creatinine in serum, plasma or urine. This method relies upon the formation of a yellow/orange colour when creatinine is treated with alkaline picrate. This reaction is not, however, specific and a number of substances including proteins in body fluids will interfere. The reaction is made specific for creatinine by using the fact that under acid conditions the creatinine-picrate colour fades faster than the interfering chromogens. Thus, the colour derived from creatinine is destroyed at acid pH and the difference in colour intensity measured spectrophotometrically at 500nm before and after acidification is proportional to the creatinine concentration in the sample. 300µl of urine, which had been diluted 10 fold with water, was used in each assay.

3.3 Comparison of the effects of human TNF with those of murine TNF

Female NMRI mice (19.2±0.5g) were injected i.v. with 0.25, 0.5, 0.75, 1.0 or 1.25mg/Kg of either human TNF (hTNF) or murine TNF (mTNF). Controls were injected with 200µl of 0.9% NaCl. Body weights and food and water consumption were measured over a 24h period (3.1.2).

3.4 The effect of TNF on MAC 13 tumour-bearing mice

Female NMRI mice (20.6±0.3g) were transplanted with the MAC 13 adenocarcinoma as described previously (2.1.1). On day 16 after transplantation mice were injected i.v. with 200µl of TNF (4.5 or 7.5 X 10^7U/Kg) or 0.9% NaCl (controls), and the body weight change over a 24h period was determined.

3.5 Comparison of TNF with Mitozolomide

Female NMRI mice (19.3±0.15g) were injected i.p. with 20mg/Kg of mitozolamide (2mg/ml) in arachis oil containing 10% DMSO, or with 200µl arachis oil containing 10% DMSO (controls), and body weights were monitored over a 24h period. Food and water consumption, blood glucose, plasma FFA and triglyceride levels, and body compositions were determined as described previously (3.1.2, 3.1.3 and 3.1.4 respectively).

3.6 Chronic TNF administration

Female NMRI mice (19-22g) were injected i.v. with TNF (1.5, 3.0, 4.5, 6.0 or 7.5 X 10^7 U/Kg) at the same time each day (9am) for 5 days. Controls were injected with 200µl of 0.9% NaCl. Body weights and food and water intake were monitored daily as described previously (3.1.2). The levels of blood glucose and plasma FFA and triglycerides of mice 90min after the final injection of

TNF were determined as described before (3.1.3). Body composition analysis was performed on day 5 (3.1.4).

3.7 Comparison of TNF with pair-fed mice

Female NMRI mice (19.2±0.46g) were given the same amount of food and water, both over a 24h period (acute) and over a 5 day period (chronic), as that consumed by mice following injection of 7.5 X 10^7U/Kg of TNF. Food was given as weighed pellets. Water was left in small containers attached to the inside of the animals' cages. The quantities of food and water given were as follows:

TIME		7am	11am	5pm	11pm
DAY 1:	food (g)	0	0	0.4	0.8
	water (ml)	0	0	1.1	1.1
DAY 2:	food (g)	0.3	0.2	0.3	1.0
	water (ml)	0.3	0.5	1.5	1.5
DAY 3:	food (g)	0.5	0.2	0.3	1.5
	water (ml)	0.5	1.0	1.0	2.0
DAY 4:	food (g)	0.3	0.2	0.3	1.5
	water (ml)	0.5	1.0	1.0	2.0
DAY 5:	food (g)	0.3			
	water (ml)	0.5			

Body weights were monitored at 11am each day. Food and water consumption, blood glucose, plasma FFA and triglyceride levels, and body compositions were determined for both acute (11am, day 2) and chronic (11am, day 5) pair-feeding as described previously (3.1.2, 3.1.3 and 3.1.4 respectively).

3.8 The effect of force-feeding on the TNF-induced weight loss

In this experiment the 12h light-dark cycle in which the animals were normally kept was reversed so that the mice were kept in the dark from 8am until 8pm. Experiments were started each morning at 10am. Female NMRI mice were administered i.v. 7.5 X 10^7 U/Kg TNF or 200µl of 0.9% NaCl. Mice were then dosed orally, by means of a stomach tube, at 45min or 2h intervals over an 8h period, with 100µl or 800µl of either water, 40% w/v glucose solution or 40% v/v MCT (medium chain triglyceride, Liquigen$^{(R)}$) solution, this being of equal calorific value to the glucose solution. Body weights and food and water intake were monitored over the 8h period (3.1.2) and body composition analysis was performed (3.1.4).

3.9 Investigation of the role of prostaglandin E_2 and indomethacin in the TNF-induced weight loss

3.9.1 Prostaglandin E_2 (PgE_2) administration

Fresh solutions of 16,16-dimethyl PgE_2 dissolved in triolein were made up in 0.9% NaCl, to give a final concentration of 0.125mg/ml, and were administered i.p. (0.5mg/Kg) at 6h intervals (11am, 5pm and 11pm) to female NMRI mice (19±1g). Controls were injected with 200µl of 0.9% NaCl containing triolein. Body weights and food and water intake were monitored over a 24h period (3.1.2) and the total body water content was determined as described previously (3.1.4).

3.9.1.1 Measurement of urine and faeces production

Mice were administered three i.p. injections of either 0.5mg/Kg of 16,16-dimethyl PgE_2 (200µl) or 200µl of 0.9% NaCl containing triolein as described above (3.9.1). In order to determine the amount of urine and faeces excreted over a 24h period mice were placed in metabolic cages immediately after the first injection and the urine and faeces were collected. After 24h the faeces were weighed to give the wet weight. They were then dried overnight in an

oven at 80°C and weighed again to give the dry weight. The amount of urine produced was calculated by volume and the difference between the wet and dry weights of the faeces was added to this to give the total urine production.

3.9.2 Indomethacin

Fresh solutions of indomethacin (2mg/ml) in arachis oil containing 10% DMSO were made up and 10mg/Kg were injected i.p. into female NMRI mice (19-22g). In order to determine the effect of indomethacin on the TNF-induced weight loss mice were divided into 6 groups and injected as follows:

1. 200µl of 0.9% NaCl, i.v.
2. 10mg/Kg indomethacin, i.p., 2h prior to 200µl of 0.9% NaCl, i.v.
3. 7.5 X 10^7U/Kg TNF, i.v.
4. 10mg/Kg indomethacin, i.p., 2h prior to 7.5 X 10^7U/Kg TNF, i.v.
5. 10mg/Kg indomethacin, i.p., 1.5h prior to 7.5 X 10^7U/Kg TNF, i.v.
6. 10mg/Kg indomethacin, i.p., 0.5h prior to 7.5 X 10^7U/Kg TNF, i.v.

Body weights and food and water intake were monitored over a 24h period (3.1.2) and the total body water content was determined as described previously (3.1.4).

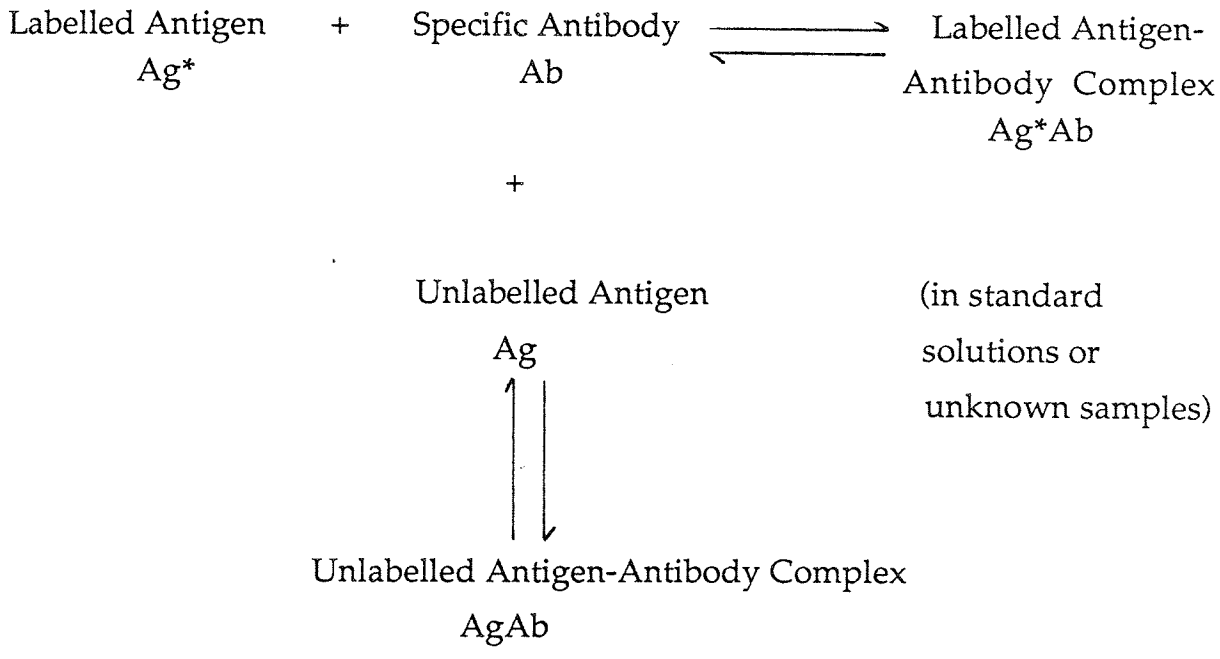
3.9.2.1 Measurement of urine and faeces production

Mice were divided into groups 1 to 4 as described above (3.9.2). Immediately after the first injections mice were placed in metabolic cages and the amount of urine and faeces produced over a 24h period was determined as described previously (3.9.1.1).

3.9.3 PgE_2 assay

PgE_2 levels were determined in mouse spleen using the Du Pont Prostaglandin E_2 [^{125}I] Radioimmunoassay (RIA) Kit. The basic principle of this radioimmunoassay is competitve binding, where a radioactive antigen competes with a non-radioactive antigen for a fixed number of antibody

binding sites. When unlabelled antigen from standards or samples and a fixed amount of tracer (labelled antigen) are allowed to react with a constant and limiting amount of antibody, decreasing amounts of tracer are bound to the antibody as the amount of unlabelled antigen is increased. In this kit, separation of the antibody-antigen complexes is achieved by precipitation of the antibody-bound tracer with polyethylene glycol in the presence of carrier immunoglobulin. After centrifugation, the supernatant containing the unbound antigen is decanted, and the pellet containing the antibody-antigen complex is counted in a gamma counter. Results obtained for the standards are used to construct a standard (dose-resonse) curve from which the unknowns are read by interpolation.



3.9.3.1 Treatment of animals

PgE_2 was measured in the spleens of male NMRI mice (20-26g) 0.5, 1, 4 and 6h after a single injection of either 7.5 X 10^7U/Kg TNF (200µl, i.v.) or 0.9% NaCl (200µl, i.v.). Measurements were also performed 2h after the following treatments:

1. 200µl of 0.9% NaCl, i.v.
2. 10mg/Kg indomethacin, i.p., 2h prior to 200µl of 0.9% NaCl, i.v.
3. 7.5 X 10^7U/Kg TNF, i.v.
4. 10mg/Kg indomethacin, i.p., 0.5, 1.5 and 2h prior to 7.5 X 10^7U/Kg TNF, i.v.
5. 7.5 X 10^7U/Kg TNF, i.v., daily for 2, 3 or 5 days.

3.9.3.2 Preparation of spleens

Mice were killed by cervical dislocation and the spleens were rapidly removed and weighed. Spleens were sliced with a scalpel on filter paper moistened with cold 0.85% NaCl and were then placed in 25ml flasks containing 2ml of Krebs-Ringer bicarbonate buffer, with 1mg/ml each of glucose and bovine serum albumin. The slices were first incubated at 37°C for 20min in a gas phase of N_2 : CO_2 (95 : 5) and then with O_2 : CO_2 (95 : 5) for the final 15min. At the end of the incubation spleens were removed and the medium was frozen in liquid nitrogen (-196°C) and only defrosted immediately prior to extraction.

3.9.3.3 Extraction of PgE_2

1ml of thawed medium was removed from each flask, adjusted to pH 3-3.5 with 2N HCl and extracted twice by vortexing for 5min with 3ml ethylacetate, centrifuging in a Beckman bench centrifuge at 3000rpm for 5min and separating and combining the top layers. Extracts were evaporated to dryness at room temperature under a stream of nitrogen, reconstituted with 2ml assay buffer from the Prostaglandin E_2 [^{125}I] RIA Kit, and then diluted 100 times using the same assay buffer. 100µl of this final diluted sample was used in the assay. Radioactivity was measured using a 1282 Compugamma universal gamma counter.

3.10 The effect of TNF on glucose metabolism

3.10.1 Treatment of animals

Female NMRI mice (19±1g) were injected i.v. with 7.5 X 10^7U/Kg TNF. Controls were injected with 200µl of 0.9% NaCl and were pair-fed throughout the experiment as described previously (3.7).

3.10.2 Blood metabolite determinations

At various time points (0.5, 1, 2, 4, 8, 12 and 24h) after administration of TNF or 0.9% NaCl blood was removed from mice by cardiac puncture as described previously (3.1.3.1) and the levels of the following metabolites were measured using a Beckman DU7 spectrophotometer:

Glucose
FFA
Triglyceride
Acetoacetate
D-(-)-3-Hydroxybutyrate
L-(+)-Lactate
L-Alanine
Pyruvate

The levels of blood glucose and plasma FFA and triglycerides were determined as described previously (3.1.3.2, 3.1.3.3 and 3.1.3.4 respectively).

3.10.2.1 Determination of blood Acetoacetate

Acetoacetate levels were determined in whole blood by the method of Mellanby and Williamson (1974), which relies on the formation of NAD^+ and D-(-)-3-hydroxybutyrate in the presence of 3-hydroxybutyrate dehydrogenase and NADH. The decrease in extinction at 340nm due to the oxidation of NADH is proportional to the amount of acetoacetate present.

3.10.2.1.1 Deproteinisation of blood

Immediately after collection 100µl of whole blood was transferred to a microfuge tube on ice and deproteinised by the addition of 100µl of ice-cold 10% w/v perchloric acid. After thorough mixing and centrifuging for 30sec in a Beckman microfuge the supernatant was removed and the volume was measured. The pH was adjusted to 7 with 20% w/v potassium hydroxide solution and the neutralised supernatant was allowed to stand in an ice-bath for approximately 30min before being sedimented in a Beckman microfuge for 30sec. The supernatant was carefully decanted, the volume was measured again, and the supernatant was used for the determination of acetoacetate.

3.10.2.1.2 Acetoacetate assay

Assay cuvettes were made up containing 33mM phosphate buffer, pH 6.8, 0.2mM NADH and 50µl neutralised and deproteinised sample, in a total volume of 3.1ml. A blank was prepared containing 50µl distilled water in place of the sample. The reaction was initiated by the addition of 10µl of 3-hydroxybutyrate dehyrogenase (150mU) and the decrease in extinction at 340nm and a temperature of 25°C was measured for 20min.

3.10.2.2 Determination of blood D-(-)-3-Hydroxybutyrate

D-(-)-3-Hydroxybuyrate levels were determined in whole blood by the method of Williamson and Mellanby (1974), which relies on the oxidation of D-(-)-3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase in the presence of NAD^+ to form NADH and acetoacetate. The increase in extinction at 340nm due to the formation of NADH is a measure of the amount of D-(-)-3-hydroxybutyrate present.

3.10.2.2.1 Deproteinisation of blood

Blood was treated in the same manner as for the determination of acetoacetate (3.10.2.1.1).

3.10.2.2.2 D-(-)-3-Hydroxybutyrate assay

Assay cuvettes were made up containing 0.32mM hydrazine hydrate - 16mM Tris buffer, pH 8.5, 0.4mM NAD and 100µl deproteinised and neutralised sample, in a total volume of 3.1ml. A blank was prepared containing 100µl distilled water in place of the sample. The reaction was initiated by the addition of 10µl of 3-hydroxybutyrate dehydrogenase (150mU) and the increase in extinction at 340nm and a temperature of 25°C was measured for 20min.

3.10.2.3 Determination of blood L-(+)-Lactate

L-(+)-Lactate levels were determined in whole blood by the method of Gutman and Wahlefeld (1974), which relies on the oxidation of L-(+)-lactate by NAD to pyruvate and NADH in the enzymatic reaction catalysed by lactate dehydrogenase. The increase in absorbance at 340nm due to the formation of NADH is a measure of the amount of L-(+)-lactate in the sample.

3.10.2.3.1 Deproteinisation of blood

Immediately after collection 200µl of whole blood was transferred to a microfuge tube on ice and deproteinised by the addition of 400µl ice-cold 1N perchloric acid. After thorough mixing the deproteinised sample was sedimented in a Beckman microfuge for 30sec and the supernatant formed was used for the determination of L-(+)-lactate.

3.10.2.3.2 L-(+)-Lactate assay

Assay cuvettes were made up containing 0.34M hydrazine hydrate - 0.43M glycine buffer, pH9, 2.75mM NAD and 100µl protein-free sample, in a total volume of 2.9ml. A blank was prepared containing 100µl of 1N perchloric acid in place of the sample. The reaction was initiated by the addition of 20µl lactate dehydrogenase (55U) and the increase in extinction at 340nm and a

temperature of 37°C was measured for 30min.

3.10.2.4 Determination of blood L-Alanine

L-Alanine levels were determined in whole blood by the method of Williamson (1974), which relies on the oxidation of L-alanine by NAD to pyruvate and NADH in the enzymatic reaction catalysed by alanine dehydrogenase. The increase in absorbance at 340nm due to the formation of NADH is a measure of the amount of L-alanine in the sample.

3.10.2.4.1 Deproteinisation of blood

Blood was treated in the same manner as for the determination of L-(+)-lactate (3.10.2.3.1) except that 50µl of blood was deproteinised by the addition of 100µl ice-cold 1N perchloric acid.

3.10.2.4.2 L-Alanine assay

Assay cuvettes were made up containing 0.33M hydrazine hydrate - 13.3mM Tris buffer with 0.47mM EDTA, pH10, 0.8mM NAD and 20µl deproteinised sample, in a total volume of 3.1ml. A blank was prepared containing 20µl distilled water in place of the sample. The reaction was initiated by the addition of 10µl alanine dehydrogenase (124mU) and the increase in extinction at 340nm and a temperature of 25°C was measured for 45min.

3.10.2.5 Determination of blood Pyruvate

Pyruvate levels were determined in whole blood by the method of Czok and Lamprecht (1974), which relies on the reduction of pyruvate by NADH to lactate and NAD in the enzymatic reaction catalysed by lactate dehydrogenase. The decrease in absorbance at 365nm due to the oxidation of NADH is proportional to the amount of pyruvate in the sample.

3.10.2.5.1 Deproteinisation of blood

Immediately after collection 200µl of blood was transferred to a microfuge tube on ice and deproteinised by the addition of 600µl ice-cold 0.6N perchloric acid. After thorough mixing the sample was left to stand in an ice-bath for 10min and was then centrifuged in a Beckman microfuge for 30sec. The pH of the supernatant was adjusted to 2.5-5.0 with 2N potassium hydroxide and, after thorough mixing, the sample was again left to stand in an ice-bath for 10min to allow precipitation of potassium perchlorate. The sample was re-centrifuged for 30sec in a Beckman microfuge, the volume of supernatant was measured and, because pyruvate is unstable, the supernatant was used immediately for the assay of pyruvate.

3.10.2.5.2 Pyruvate assay

Assay cuvettes were made up containing 300mM triethanolamine buffer, pH 7.6, 3mM EDTA, 0.1mM NADH and 300µl deproteinised and neutralised sample, in a total volume of 2.0ml. A blank was prepared containing no sample and half the volume of NADH solution. The change in extinction at 365nm and a temperature of 25°C was read until constant (E_1). 20µl lactate dehydrogenase (5.5U) was added, the change in extinction was recorded for a further 25min (E_2) and the decrease in extinction ($E_2 - E_1$) was calculated.

3.10.3 Determination of liver Glycogen

Liver glycogen levels were determined by the method of Keppler and Decker (1974), which relies on the hydrolysis of glycogen to glucose by amyloglucosidase. The glucose thus formed is specifically determined with hexokinase, which, in the presence of ATP and $MgSO_4$, forms ADP and glucose-6-phosphate. This glucose-6-phosphate then reacts with NADP and glucose-6-phosphate dehydrogenase to give 6-phosphogluconolactone and NADPH. The glucose thus liberated after the hydrolysis of glycogen is proportional to the increase of NADPH and is measured by the extinction change at 340nm.

3.10.3.1 Preparation of tissue homogenate

At various times points (0.5, 1, 2, 4, 8 and 12h) after the injection of TNF or 0.9% NaCl (3.10.1) mice were killed by cervical dislocation and the livers were rapidly removed. The livers were weighed and thoroughly homogenised using a CAMLAB 563C homogeniser (speed 8) with 5 parts of ice-cold 0.6N perchloric acid and the resulting homogenates were stored at 0-4°C until they were assayed.

3.10.3.2 Glycogen hydrolysis

0.2ml of the acid tissue homogenate was incubated in a shaking water bath at 40°C for 2h with 0.1ml of 1M potassium hydrogen carbonate solution and 2ml of a solution of amyloglucosidase (10mg/ml) in 0.2M acetate buffer, pH 4.8. The incubation was stopped by addition of 1ml of 0.6N perchloric acid and, after centrifugation for 5min at 3000rpm in a Beckman bench centrifuge, 0.05ml of the acid supernatant fluid was taken for the determination of glucose. An homogenate glucose blank was also prepared by centrifuging the acid tissue homogenate for 15min at 3000rpm in a Beckman bench centrifuge, neutralising the supernatant to pH 6-7 with solid potassium hydrogen carbonate, and taking 0.05ml for the determination of glucose.

3.10.3.3 Liver Glycogen assay

Assay cuvettes were made up containing 0.3M triethanolamine buffer with 4.0mM $MgSO_4$, pH7.5, 1mM ATP, 0.9mM NADP, 5µg glucose-6-phosphate dehydrogenase (G6P-DH) and 0.05ml deproteinised sample, in a total volume of 1.05ml. A blank was prepared containing 0.05ml glucose blank in place of the sample. The increase in extinction at 340nm and a temperature of 25°C (oxidation of glucose-6-phosphate) was read until constant (E_1). 5µl hexokinase (1.5U) was added and the increase in extinction was again read until constant (E_2). The change in extinction ($E_2 - E_1$), after subtraction of the glucose blank (free glucose before hydrolysis), corresponds to the glycogen content of the sample.

3.11 Body Temperature

Female NMRI mice (19±1g) were injected i.v. with either 3.0 X 10^7U/Kg or 7.5 X 10^7U/Kg of TNF. Controls were injected i.v. with 200µl of 0.9% NaCl. Rectal body temperatures (°C) were then measured at various time points (0.5, 1, 2, 3, 4, 5 and 6h) with the use of a thermocouple.

3.12 The effect of TNF on $^{14}CO_2$ production from ^{14}C-labelled glucose and palmitic acid

Immediately after a single i.v. injection of 7.5 X 10^7U/Kg TNF or 200µl of 0.9% NaCl female NMRI mice (19-20g) were injected i.p. with 50µCi/Kg of either D-[U-^{14}C]-glucose or [U-^{14}C]-palmitic acid in 200µl of 0.9% NaCl. Animals were then placed in airtight metabolic cages into which air was pumped through solid calcium carbonate to absorb any CO_2. Metabolically produced CO_2 was trapped in glass test-tubes containing 20ml of a mixture of ethanolamine : ethoxyethanol (1 : 4). At specified time intervals (0.5, 1, 2, 4 and 8h) 500µl aliquots of the ethanolamine - ethoxyethanol mixture were taken, transferred to scintillation vials containing 10ml Optiphase scintillation fluid and the radioactivity was measured in a Packard TRI-CARB 2000CA liquid scintillation analyser.

3.13 Determination of resting oxygen consumption (VO_2)

Female NMRI mice (18-20g) were injected i.v. with either 7.5 X 10^7 U/Kg TNF or 0.9% NaCl. The average oxygen consumption of 2 mice in each chamber was determined by indirect, closed circuit calorimetry (Stock, 1975). The animals were starved throughout the experiment and measurements were taken 1, 2 and 24h after the injections of TNF or saline.

3.14 The effect of TNF on glucose utilization

The extent of glucose utilization by different tissues was investigated using the method of Sokoloff et al (1977), which involves the *in vivo* administration of radioactive 2-deoxy-D-glucose (2DG). The transport, cellular uptake and phosphorylation by hexokinase of this analogue correlate with those of glucose, but, because 2-deoxyglucose-6-phosphate (2DGP) cannot be readily metabolised further, it can be detected in tissues containing little or no glucose-6-phosphatase activity, like brain and muscle (Lackner et al, 1984, and Jenkins et al, 1986). Glucose utilization was calculated according to the following equation:

$$Rg = \frac{Cm^*(T)}{LC \int_0^T \frac{Cp^*}{Cp}\, dt}$$

where Rg is the tissue glucose metabolic rate (nmol/g/min); Cm*(T) is the concentration of phosphorylated metabolites of 2-deoxyglucose in the tissue (dpm/g) at t=60min; Cp is the blood glucose concentration (nmol/ml); Cp* is the concentration of radioactive 2-deoxyglucose in the blood (dpm/ml) and LC is the lumped constant which was determined as described below (3.14.4).

3.14.1 Treatment of animals

Female NMRI mice (18-20g) were starved overnight and throughout the experiment, but given water *ad libitum*. The following morning the mice were injected i.v. with 7.5 X 10^7U/Kg TNF or 200µl of 0.9% NaCl, and 1h later they were injected i.v. with 50µCi/Kg 2-deoxy-D-[2,6-^{3}H]-glucose in 200µl of 0.9% NaCl. In order to determine the retention of 2-deoxyglucose-6-phosphate by the different tissues a third i.v. injection of 5µCi/Kg 2-[1-^{14}C]-deoxy-D-glucose in 200µl of 0.9% NaCl was administered 35min after the injection of the tritiated glucose.

3.14.2 Blood glucose concentration and the decay of radioactivity (Cp*) in the blood

Blood was removed by cardiac puncture from animals under anaesthesia at specified time intervals (2, 10, 20, 37, 45 and 60min after injection of the 2-deoxy-D-[2,6-^{3}H]-glucose) as described previously (3.1.3.1). Blood glucose concentration was measured by the o-toluidine method (3.1.3.2). The decay of radioactivity in the blood was measured by a modification of the method of Meszaros et al (1987a); immediately after collection 100µl blood was transferred to a microfuge tube containing 100µl ice-cold 0.5M perchloric acid and, after thorough mixing, the deproteinised sample was centrifuged in a Beckman microfuge for 30sec. The supernatant was carefully transferred to a clean microfuge tube and the pH was adjusted to 7 with 1N potassium hydroxide solution. The volume of supernatant present after neutralisation was measured and 20µl was transferred to a scintillation vial containing 10ml Optiphase scintillation fluid. The radioactivity in each sample was read in duplicate using a dual ^{14}C / ^{3}H channel analyser by means of a Packard TRI-CARB 2000CA liquid scintillation analyser.

3.14.3 Accumulation of radioactive 2-deoxyglucose-6-phosphate by tissues (Cm*)

The accumulation of phosphorylated metabolites of 2-deoxyglucose was measured in selected tissues 60min after the injection of 2-deoxy-D-[2,6-^{3}H]-glucose. Mice were killed by cervical dislocation and the following tissues were carefully dissected out and weighed; liver, brain, spleen, kidneys, pancreas, thigh muscles, gastrocnemius muscles, diaphragm, lungs, stomach and colon. Each tissue was homogenised in 0.4-0.8ml/100mg tissue weight of ice-cold 0.5N perchloric acid using a CAMLAB 563C homogeniser (speed 8) fitted with a teflon pestle. The homogenate was then transferred to a centrifuge tube and centrifuged at 3000rpm in a Beckman bench centrifuge for 15min. The supernatant was carefully transferred to a clean centrifuge tube, neutralised to pH 7 with 10% w/v potassium hydroxide solution, re-centrifuged at 3000rpm for 5min and the final volume of neutral supernatant was measured. 100-500µl of this neutral extract was then transferred to

scintillation vials containing 10ml Optiphase scintillation fluid and the radioactivity in each sample was measured in duplicate using a dual ^{14}C / ^{3}H channel analyser by means of a Packard TRI-CARB 2000CA liquid scintillation analyser. This gave a measure of the total 2-deoxyglucose radioactivity present in the tissue.

Removal of 2-deoxyglucose-6-phosphate from the neutral extract was accomplished by use of the Somogyi (1945) reagent; 200µl of the neutral extract was transferred to a clean microfuge tube and 100µl of 0.175M zinc sulphate solution, followed by 100µl of 0.15M barium hydroxide solution, were added, yielding a neutral mixture. The resulting precipitate, which absorbed any 2-deoxyglucose-6-phosphate, was then sedimented in a Beckman microfuge for 30sec. 100µl of the supernatant was transferred to a scintillation vial containing 10ml Optiphase scintillation fluid and the radioactivity was measured in duplicate using a dual ^{14}C / ^{3}H channel analyser by means of a Packard TRI-CARB 2000CA liquid scintillation analyser. This gave a measure of the free 2-deoxyglucose content of the tissue.

The difference between the total radioactivity of the neutral extract and the radioactivity present after the zinc sulphate / barium hydroxide treatment represented the 2-deoxyglucose-6-phosphate content of the tissue.

3.14.4 Determination of lumped constant (LC) for liver and muscle

The lumped constant is a dimensionless correction factor for discrimination against 2-deoxyglucose in glucose metabolic pathways, which is required in order to be able to calculate the glucose utilization by tissues. The lumped constant was determined by a modification of the *in vitro* method of Ferre et al (1985) which employs the following equation:-

$$LC = \frac{\text{tissue 2-deoxyglucose-6-phosphate / 2-deoxyglucose in medium}}{\text{glucose utilization / glucose in medium}}$$

This formula corresponds to the fractional extraction of 2-deoxyglucose by the tissue divided by the fractional extraction of glucose (Sokoloff et al, 1977). Glucose utilization by the liver and muscle was taken as the sum of the D-[U-^{14}C]-glucose converted into CO_2, lactate, alanine and lipids, and into CO_2, lactate and glycogen respectively, as described below.

3.14.4.1 Preparation of tissues

Female NMRI mice (20-21g) were killed by cervical dislocation and the livers, gastrocnemius muscles and thigh muscles were carefully dissected out and weighed. The livers and muscles were finely sliced with a scalpel and transferred to 25ml flasks containing 2ml Krebs-Ringer bicarbonate buffer, pH 7.4, with 1% BSA, 5mM glucose, 1μCi of D-[U-^{14}C]-glucose and 2μCi of 2-deoxy-D-[2,6-^{3}H]-glucose. The medium was gassed for 2min with a mixture of O_2 : CO_2 (95 : 5) and the flasks were fitted with 1ml centre wells, sealed with rubber seals and incubated at 37°C in a shaking water bath for 1h. After the incubation the liver and muscle slices were removed, dried by blotting with filter paper and re-weighed. Half of the tissue slices were assayed for their radioactive 2-deoxyglucose-6-phosphate content (3.14.3). The other half of the liver slices were assayed for their ^{14}C-labelled lipid content (3.15), whereas the muscles were assayed for their ^{14}C-labelled glycogen content (3.14.4.4). After determining the radioactive CO_2 production by the tissues (3.14.4.2), the incubation medium was frozen at -20°C and only defrosted immediately prior to use for the determination of labelled alanine and/or lactate.

3.14.4.2 $^{14}CO_2$ production

After removing the liver and muscle slices, the rubber seals were rapidly replaced and 0.5ml Hyamine was injected through the seals into the centre wells of the incubation flasks. CO_2 was liberated from the medium by injecting 0.5ml of 40% w/v perchloric acid through the rubber seals into the incubation medium. After allowing the flasks to stand, with gentle mixing for 15min, the Hyamine was removed and transferred to scintillation vials containing 10ml Optiphase scintillation fluid. The ^{14}C-labelled CO_2 thus

produced was measured in a Packard TRI-CARB 2000CA liquid scintillation analyser.

3.14.4.3 Labelled alanine and lactate content

Labelled alanine and lactate were determined in the thawed incubation medium by ion-exchange chromatography using the method of Ferre et al (1978). 1ml incubation medium was transferred to a clean centrifuge tube and neutralised to pH 7 by the addition of 10% w/v potassium hydroxide solution. To this was added 500µl of 0.175M zinc sulphate solution followed by 500µl of 0.15M barium hydroxide solution. After thorough mixing, the precipitate, which contained 2-deoxyglucose-6-phosphate, was sedimented by centrifuging at 3000rpm in a Beckman bench centrifuge for 10min. The volume of the supernatant thus formed was measured and all of the supernatant was passed on to a 10 x 0.7cm layered bed resin column consisting of Dowex-1-X8 (200-400 mesh) in the formate form and Dowex-50-X8 (200-400 mesh) in the H^+ form. The sample was eluted with water to remove labelled glucose. After each elution 500µl of the eluate was transferred to a scintillation vial containing 10ml Optiphase scintillation fluid and assayed for radioactivity using a Packard TRI-CARB 2000CA liquid scintillation analyser. This was repeated several times until no labelled glucose could be measured in the eluate. The column was then washed sequentially with 0.1M formic acid, 0.2M formic acid, water, and 2M ammonium hydroxide solution, each solution being washed through the column several times until no radioactivity could be detected in the eluate before the following solution was added. The 0.2M formic acid eluates have previously been found to contain labelled lactate by this method and the ammonium hydroxide eluates to contain labelled alanine (Kreisberg et al, 1972). Only lactate was determined from the muscle slices and thus, in this case, the experiment was terminated following the addition of 0.2M formic acid to the column.

3.14.4.4 Labelled glycogen content

Labelled glycogen was determined by the method of Stauffacher and Renold (1969) after hydrolysis of the muscle in 0.5ml of 1N sodium hydroxide at 70°C

for 30min. Carrier glycogen (10mg) was added to the hydrolysates and glycogen was precipitated at -20°C for 1h with 66% ethanol. The glycogen precipitate was washed twice with 66% ethanol and dissolved in 0.5ml water. The radioactivity was then counted in 10ml Optiphase scintillation fluid using a Packard TRI-CARB 2000CA liquid scintillation analyser.

3.15 The effect of TNF on lipogenesis from glucose

3.15.1 Treatment of animals

D-[U-^{14}C]-glucose in 200µl of 0.9% NaCl was administered i.p. (250µCi/Kg) to female NMRI mice (19±1g), either immediately or 1h after an i.v. injection of 7.5 X 10^7U/Kg TNF. Controls were pair-fed during the experiment as described previously (3.7) and were injected i.v. with 200µl of 0.9% NaCl at the same time or 1h before the ^{14}C tracer.

3.15.2 Preparation of tissues

After injection of the D-[U-^{14}C]-glucose (2h) blood was removed by cardiac puncture from animals under anaesthesia as described previously (3.1.3.1), transferred to microfuge tubes on ice and the volume noted. The livers, spleens, epididymal fat pads and colons were then carefully dissected out and weighed.

3.15.3 Extraction of lipids

Lipids were extracted from all of the above organs and the blood by the method of Stansbie et al (1976). Briefly, the organs and blood samples were transferred to glass scintillation vials containing 3ml of 30% w/v potassium hydroxide and heated to 70°C for 15min. 3ml of 95% w/v ethanol was then added to each vial and heating at 70°C was continued for a further 2h. Vials were then cooled and the saponified material was acidified with 3ml of 9M sulphuric acid. Acid-soluble lipids were extracted three times by shaking with light petroleum ether (b.p. 40-60°C), centrifuging at 3000rpm in a Beckman

bench centrifuge for 15min and removing the supernatants. The three petroleum fractions (supernatants) were combined and evaporated to dryness at 40°C under a stream of nitrogen. The lipid extracts were then redissolved in 10ml Optiphase scintillation fluid and the radioactivity was measured using a Packard TRI-CARB 2000CA liquid scintillation analyser.

4. RESULTS

4.1 Experiments to compare TNF with the cachexia-inducing MAC 16 adenocarcinoma

Inbred NMRI mice were utilized for studies reported in this thesis as the cachexia-inducing MAC 16 adenocarcinoma was originally passaged in these mice and, as such, it was thought that they would be sensitive to any cachectic effects of TNF.

4.1.1 Body weight and food and water consumption

The characteristics of weight loss produced by the MAC 16 adenocarcinoma passaged in male NMRI mice have previously been reported (Bibby et al, 1987, and Beck and Tisdale, 1987). Briefly, weight loss started to occur when the tumour mass exceeded 0.1g and reached 10g in a 30g mouse when the tumour mass was 0.7g. This represented a 33% weight loss with a tumour accounting for only 2% of the weight of the animal. The average food intake in the MAC 16 tumour-bearing animals (15±0.6Kcal/day) was not significantly different from that in non-tumour-bearing mice (14.9±0.9Kcal/day). Also the water intake in tumour-bearing animals (4.6±0.27ml/day) did not differ from that of controls (4.8±0.6ml/day).

Recombinant human TNF, when administered as a single i.v. injection, caused a dose-related weight loss over a 24h period, which was significantly greater than saline-injected controls at all concentrations of TNF employed (figure 4.1). At the highest concentration of TNF injected (7.5×10^7 U/Kg) the weight loss reached 2g in a 20g female mouse, representing a 10% weight loss. No mortality was observed with any of the concentrations of TNF employed. This weight loss was accompanied by a dose-dependent decrease in both the food and the water consumption of TNF-treated mice over a 24h period (figure 4.2); the average food intake in animals treated with the highest dose of TNF being 50-55% of that of controls and the average water intake being 45-50% of that of saline-injected animals. The decrease in both the food and the water consumption of TNF-treated mice was directly proportional to the decrease in body weight of these animals (figure 4.3).

Figure 4.1

The effect of a single injection of TNF on the weight of female NMRI mice

Recombinant human TNF was administered as a single i.v. injection to female NMRI mice (20±1g) and the animals were weighed 24h later. The values represent the means ± S.E.M. of 4 to 11 animals for each concentration of TNF. *p≤0.01 and **p≤0.001 from saline-injected controls by Student's t-test.

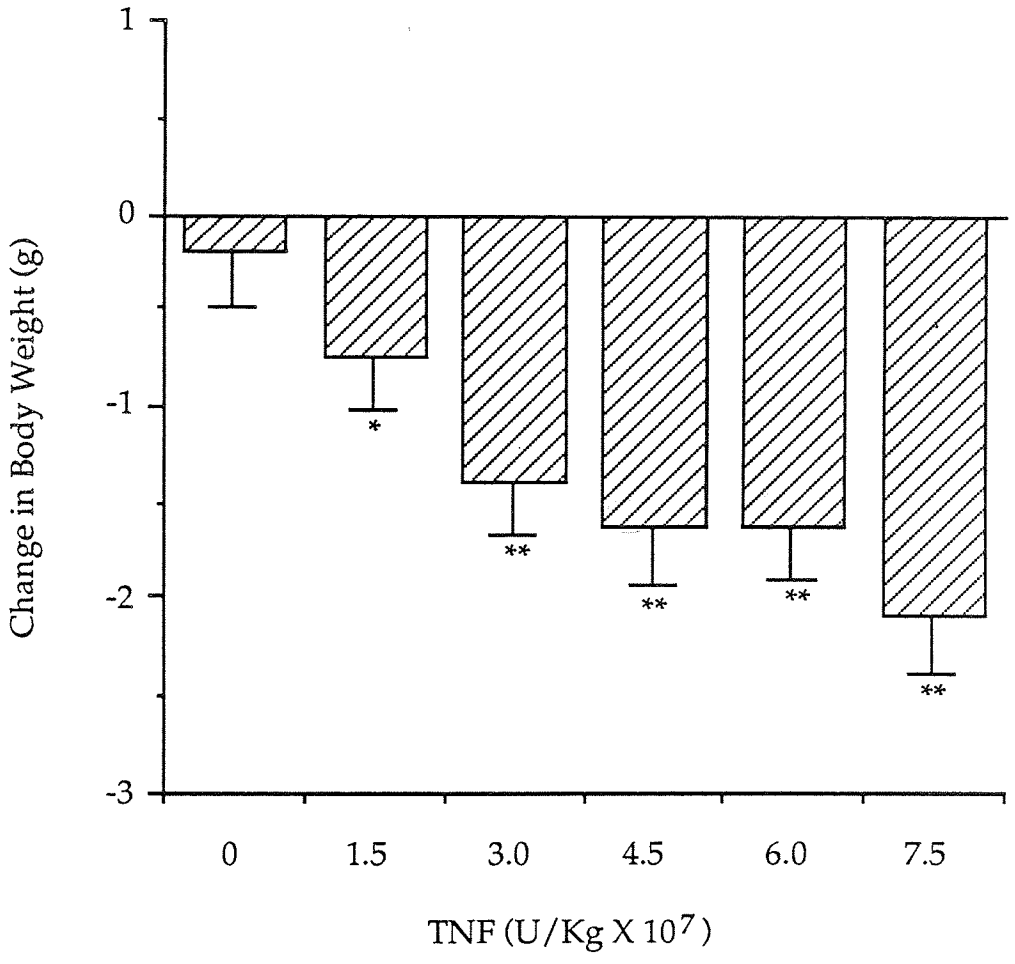


Figure 4.2

The effect of a single injection of TNF on the food and water consumption of female NMRI mice

Recombinant human TNF was administered as a single i.v. injection to female NMRI mice (20±1g) and the food (Kcal) and water (ml) consumption was measured over a 24h period. The values represent the means ± S.E.M. of 4 to 11 animals for each concentration of TNF. *p≤0.05, **p≤0.01 and ***p≤0.005 from saline-injected controls by Student's t-test.

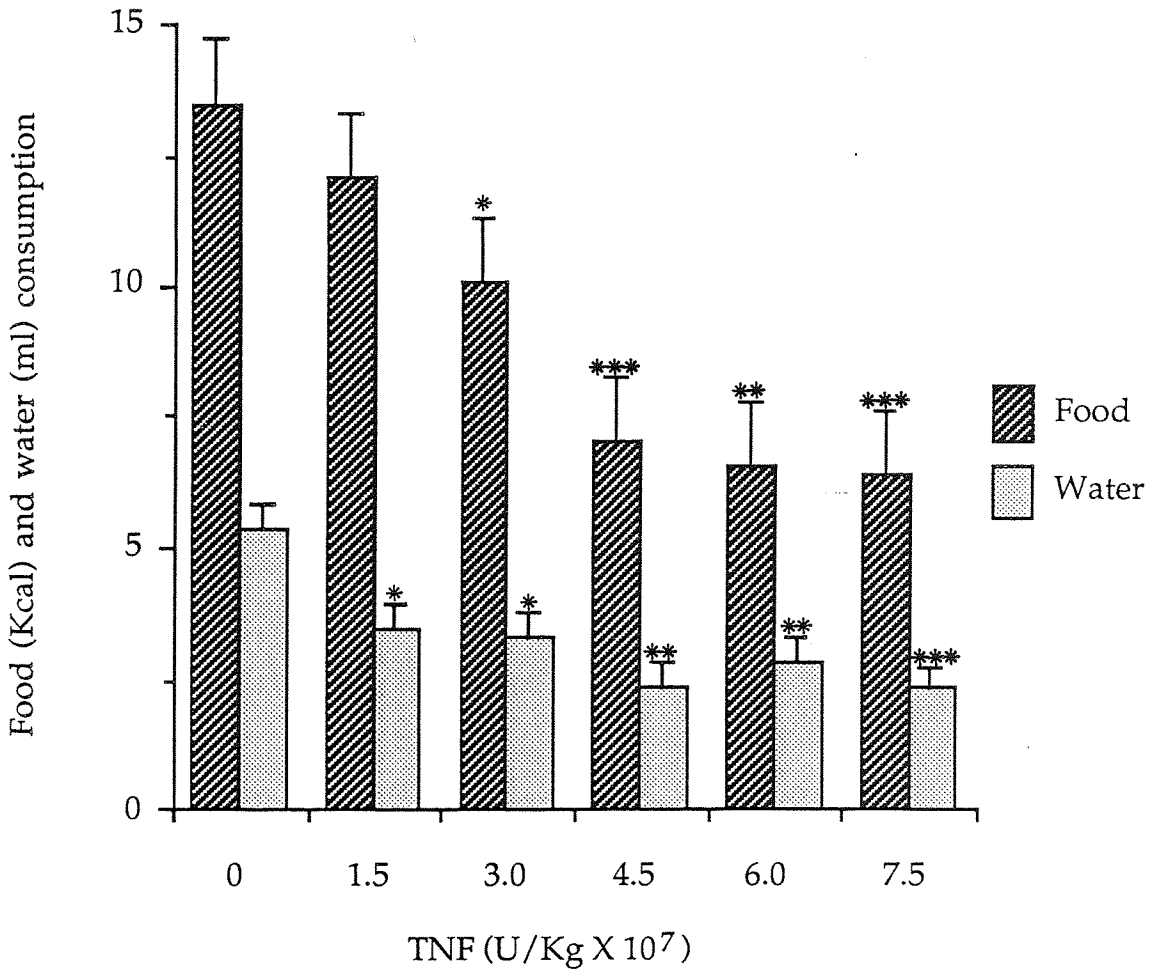
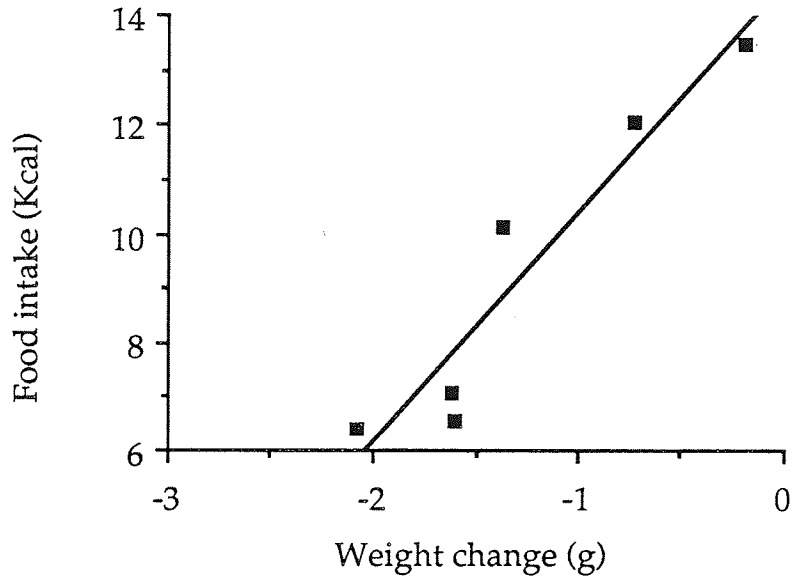
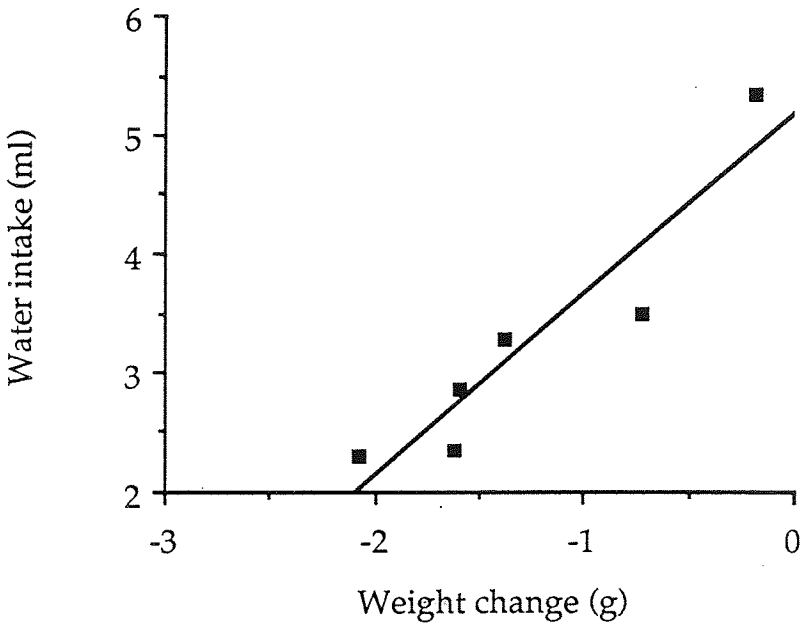


Figure 4.3

The variation of weight change over a 24h period after administration of TNF with the food (Kcal) and water (ml) consumption of female NMRI mice

The results were fitted to a linear model by means of a least squares analysis (r=-0.95 and -0.93 respectively).





4.1.2 Blood metabolite studies

During starvation and weight loss mobilisation of adipose tissue and alterations in blood glucose are observed. Thus, in order to compare the parameters of weight loss produced by TNF with those produced by the MAC 16 adenocarcinoma, the levels of glucose, free fatty acids (FFA) and triglycerides in the blood of mice were investigated (table 4.1).

Animals bearing the MAC 16 tumour displayed a reduced blood glucose level. TNF-treated mice also showed a highly significant hypoglycaemia, which was much more pronounced than observed in the weight-losing tumour-bearing animals. Although the MAC 16 tumour-bearing mice used in this study had an average weight loss of 3.95±0.37g, their blood glucose concentration was more than twice that of mice injected with the highest concentration of TNF, who displayed a weight loss of only 2.08±0.36g.

TNF administration resulted in a dose-related decrease in the plasma FFA concentration of mice, this being of similar magnitude to the decrease in the plasma level of FFA observed in MAC 16 tumour-bearing animals. However, whereas tumour-bearing mice also exhibited a decrease in the plasma level of triglycerides, possibly due to an increased tumour utilization (Beck & Tisdale, 1987), TNF administration resulted in a non-dose-dependent increase in circulatory triglycerides, presumably due to inhibition of lipoprotein lipase (Semb et al, 1987, and section 1.9.4) or to an increased hepatic triglyceride synthesis (Feingold et al, 1987).

Table 4.1

The effect of TNF and the MAC 16 tumour on blood glucose and plasma FFA and triglyceride levels of NMRI mice

Metabolite levels were measured in the blood of mice 90min after the second of 2 i.v. injections of either TNF or 0.9% NaCl over a 24h period, and in tumour-bearing mice when tumour weights were 0.372±0.035g and the average weight loss was 3.95±0.37g. The values represent the means ± S.E.M. of 6 to 13 animals in each group. $^{a}p\leq0.05$, $^{b}p\leq0.005$, $^{c}p\leq0.001$ from non-tumour-bearing controls, and $^{d}p\leq0.001$ from non-tumour-bearing saline-infused controls by Student's t-test.

Treatment	Glucose (mg/100ml)	FFA (mg/100ml)	Triglyceride (mM)
Non-tumour-bearing	136±5	29±2	1.15±0.11
MAC 16 tumour-bearing	108±11[a]	10±1[b]	0.50±0.07[c]
Non-tumour-bearing (saline)	124±5	32±5	0.93±0.31
4.5 X 10^7U/Kg TNF	58±3[d]	16±2[d]	2.43±0.38[d]
6.0 X 10^7U/Kg TNF	56±9[d]	10±3[d]	2.34±0.05[d]
7.5 X 10^7U/Kg TNF	44±4[d]	8±2[d]	2.37±0.15[d]

4.1.3 Body composition

The body compositions, measured in terms of wet and dry carcass weight, total carcass fat and left thigh and gastrocnemius muscle weight, of MAC 16 tumour-bearing mice (a) and of TNF-treated animals (b) are shown in table 4.2.

In MAC 16 tumour-bearing animals both muscle and adipose mass and the total body water content decreased in direct proportion to the weight of the tumour (Beck and Tisdale, 1987, table 4.2). With an average weight loss of 3.95±0.37g the fat and muscle content of MAC 16 tumour-bearing mice decreased by 66% and 22% respectively, and the total body water content decreased by 19% when compared with non-tumour-bearing controls.

The decline in body weight of TNF-treated mice was accompanied by a dose-related decrease in both the body fat and water content of animals, with no alteration in muscle mass. At the highest concentration of TNF employed (7.5 X 10^7 U/Kg), giving an average weight loss of 2.08±0.36g, the body fat and water contents decreased by 30% and 9% respectively, there being no alteration in the muscle mass of these animals.

Table 4.2

The effect of TNF and the MAC 16 tumour on the body compositions of NMRI mice

Body composition analysis was performed 24h after a single i.v. injection of TNF or 0.9% NaCl and when tumour weights were 0.34±0.05g for MAC 16 tumour-bearing animals. The values represent the means ± S.E.M. of 6 to 13 animals in each group. $^{a}p\leq0.001$ from non-tumour-bearing controls, and $^{b}p\leq0.05$, $^{c}p\leq0.01$ from non-tumour-bearing saline-injected controls by Student's t-test. *Weight loss; 3.95±0.37g. **Weight loss; 2.08±0.36g.

(a) MAC 16 TUMOUR-BEARING MALE NMRI MICE (26-28g)

Treatment	Water content (g)	Fat content (g)	Left thigh and gastrocnemius muscle content(g)
Non-tumour-bearing	18.0±0.51	1.70±0.09	0.090±0.003
MAC 16 tumour-bearing*	14.6±0.90[a]	0.58±0.11[a]	0.070±0.002[a]

(b) TNF-TREATED FEMALE NMRI MICE (19-21g)

Treatment	Water content (g)	Fat content (g)	Left thigh and gastrocnemius muscle content (g)
Controls (i.v. saline)	13.11±0.24	2.20±0.11	0.065±0.002
4.5 X 10^7U/Kg TNF	12.00±0.70[b]	1.49±0.26[c]	0.060±0.004
6.0 X 10^7U/Kg TNF	12.17±0.31[b]	1.83±0.32[b]	0.059±0.002
7.5 X 10^7U/Kg TNF**	11.99±0.09[b]	1.54±0.23[c]	0.067±0.023

4.1.4 Serum and tumour TNF levels in non-tumour-bearing and MAC 16 and MAC 13 tumour-bearing mice

The cachexia-inducing MAC 16 adenocarcinoma and the non-cachexia-inducing MAC 13 adenocarcinoma were utilized in this study to determine whether a cachexia-inducing tumour is capable of increased production of TNF and, thus, whether TNF could be implicated in the cachectic properties of this tumour.

No TNF was detected either in the MAC 16 or MAC 13 tumours, or in the serum of tumour-bearing and non-tumour-bearing mice, using the L929 cytotoxicity assay. Mice were thus primed for TNF production by administration of a single i.v. injection of 1.25mg/Kg *E. coli* lipopolysaccharide and the levels of TNF were measured 90min later (Flick & Gifford, 1986). TNF was detected in the serum and tumours of all mice after priming with endotoxin. However, no difference was observed in the extent of response between tumour-bearing and non-tumour-bearing animals and in animals bearing either type of tumour, or in the levels of TNF in the two tumour types (figure 4.4).

4.1.5 The *in vitro* lipolytic activity of TNF and the MAC 16 tumour

The loss of body fat in MAC 16 tumour-bearing animals has been correlated with the presence of a lipolytic factor produced by the tumour (Beck and Tisdale, 1987). This material was quantitated by the extent of release of FFA from mouse epididymal adipose tissue during a 2h incubation period (section 3.1.6). Table 4.3 shows the effect of the MAC 16 tumour and three concentrations of TNF on this assay. While extracts of the MAC 16 tumour caused an enhanced release of FFA, TNF had no effect on the release of FFA, and thus did not possess *in vitro* lipolytic activity under the conditions of the assay, up to a concentration of 4×10^5U/ml.

Figure 4.4

Serum and tumour TNF levels in non-tumour-bearing and MAC 16 and MAC 13 tumour-bearing male NMRI mice

The levels of TNF in the serum and tumours of mice 90min after i.v. administration of 1.25mg/Kg *E. coli* lipopolysaccharide were determined by means of the L929 cytotoxicity assay. The values represent the means ± S.E.M. of 5 to 9 animals in each group.

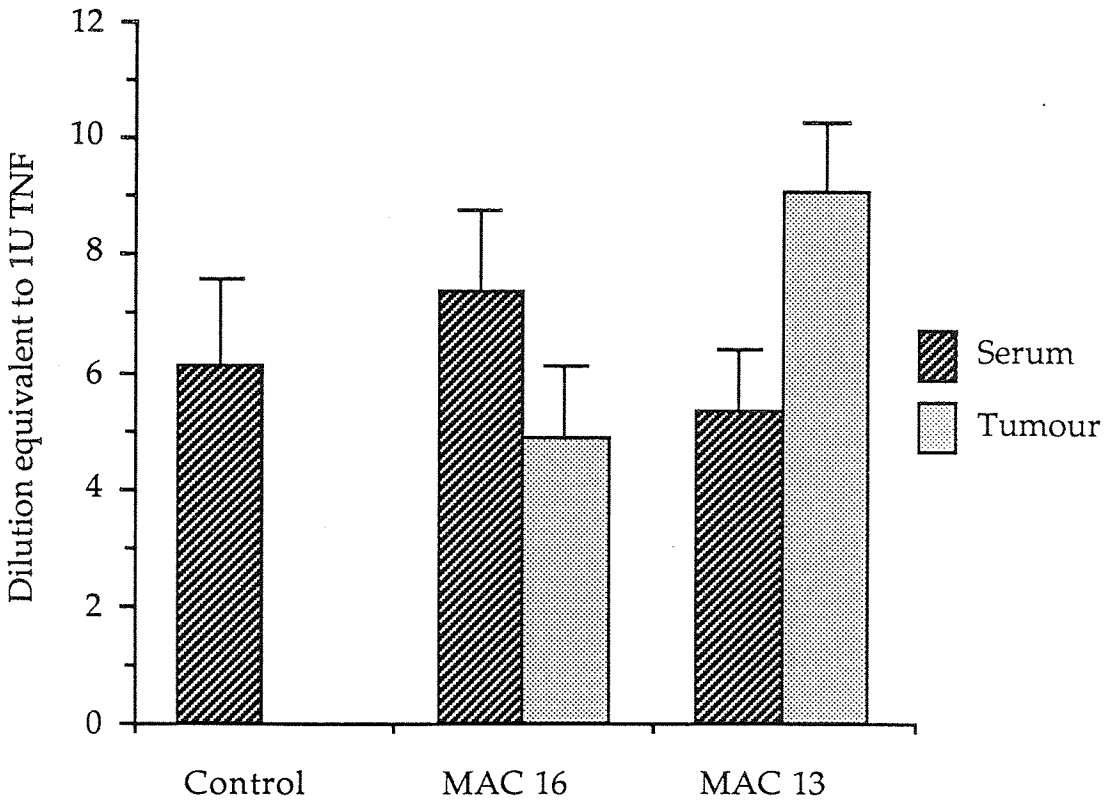


Table 4.3

The effect of TNF and the MAC 16 tumour on the release of FFA from adipocytes

The *in vitro* lipolytic activity of 100µl of the MAC 16 tumour extract (equivalent to 0.32mg of protein) and of TNF (concentrations are expressed as U/ml of the assay mixture) was determined. [a]The values represent the means ± S.E.M. for an average of 11 determinations.

Addition	nmol FFA/mg protein/h
MAC 16 tumour extract	148.1±8.3[a]
4 X 10^5U TNF	0.0±0.0
4 X 10^4U TNF	0.0±0.0
4 X 10^3U TNF	0.0±0.0

4.1.6 The *in vitro* proteolytic activity of TNF and the MAC 16 tumour

The loss of muscle mass in MAC 16 tumour-bearing animals has been correlated with the presence of a proteolytic factor produced by the tumour (Beck and Tisdale, 1987). Using the mouse diaphragm as a model of skeletal muscle, this material was quantitated by the extent of release of amino acids from muscle during a 2h incubation period (section 3.1.7). TNF, at high concentrations (10^4 and 10^5U per assay), also caused an enhanced release of amino acids (figure 4.5), and thus possessed *in vitro* proteolytic activity under the conditions of the assay. This effect was not due to contamination by endotoxin, since the proteolytic activity was completely destroyed if the TNF were heated to 70°C for 15min, which would destroy the TNF but not the endotoxin. The proteolytic activity of TNF was almost completely suppressed by indomethacin and by human α-1 antitrypsin. The proteolytic activity of the MAC 16 tumour extract was also partially suppressed by indomethacin and by α-1 antitrypsin and there was a synergistic inhibition by a combination of indomethacin and antitrypsin. Proteolysis by trypsin was also inhibited by indomethacin.

4.1.7 The *in vitro* proteolytic activity of prostaglandins

Since the TNF-induced and the MAC 16 tumour-induced enhanced release of amino acids from mouse diaphragm was suppressed by indomethacin (4.1.6), this implicated prostaglandins as a possible cause of the *in vitro* proteolytic activity of both TNF and the MAC 16 tumour. Table 4.4 shows the effect of four prostaglandins on the release of amino acids from the mouse diaphragm. An enhanced amino acid release was observed when diaphragms were incubated in the presence of PgE_1 or PgE_2, but not in the presence of PgF_1-α or PgF_2-α. Thus, prostaglandins of the E series may play a role in the proteolytic activity of both TNF and the MAC 16 tumour.

Figure 4.5

Rates of release of amino acids from mouse diaphragms by the MAC 16 tumour homogenate and TNF

The *in vitro* proteolytic activity of the MAC 16 tumour and of TNF was determined. The values represent the means ± S.E.M. for an average of 11 determinations. B and C; $p \leq 0.05$ from Krebs-Ringer buffer alone, D and E; $p \leq 0.05$ from C, C; $p \leq 0.05$ from F, and, H and I; $p \leq 0.001$ from F by Student's t-test.

A = 10^3U TNF, B = 10^4U TNF, C = 10^5U TNF, D = 10^5U TNF + 1mM indomethacin, E = 10^5U TNF + 1mg/ml α-1 antitrypsin, F = 100µl MAC 16 tumour extract (2.9mg protein/ml), G = 100µl MAC 16 tumour extract + 1mM indomethacin, H = 100µl MAC 16 tumour extract + 1mg/ml α-1 antitrypsin, I = 100µl MAC 16 tumour extract + 1mM indomethacin + 1mg/ml α-1 antitrypsin, J = 0.1mg/ml trypsin, K = 0.1mg/ml trypsin + 1mM indomethacin.

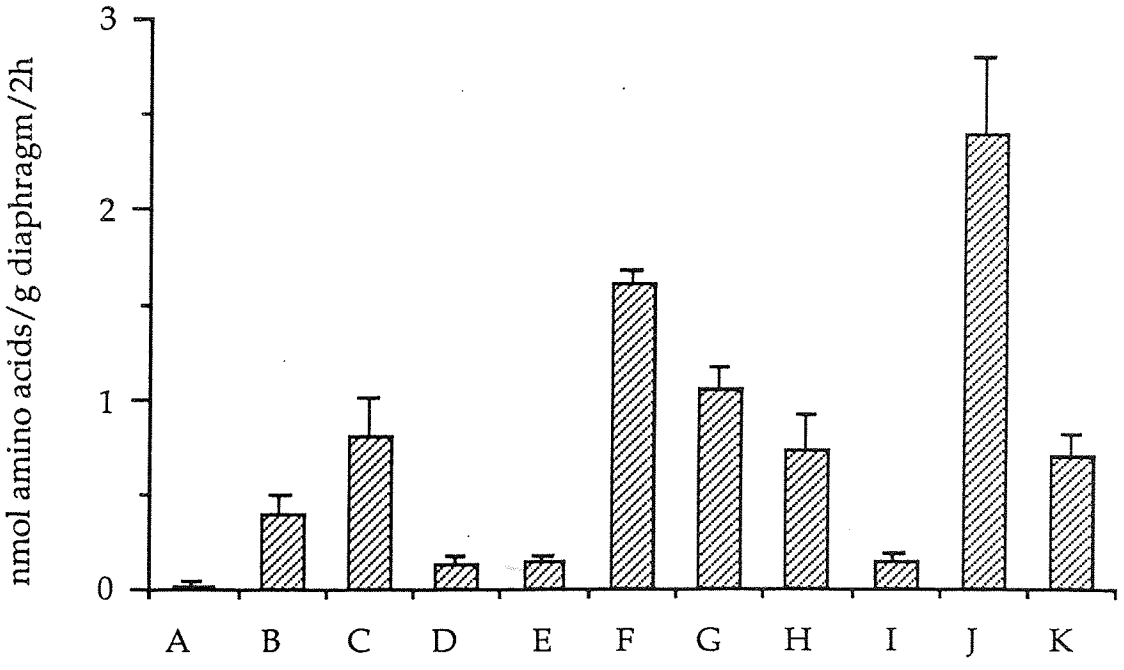


Table 4.4

The effect of prostaglandins on the release of amino acids from mouse diaphragm

The values represent the means ± S.E.M. for an average of 4 determinations. [a]$p \leq 0.05$ and [b]$p \leq 0.005$ from Krebs-Ringer buffer alone by Student's t-test.

	Concentration (μg/ml)	nmoles amino acid released/g diaphragm/2h
PGE_1	5	0.028±0.024
	10	0.085±0.006[b]
	20	0.023±0.066[a]
PGE_2	5	0.069±0.022[a]
	10	0.242±0.079[a]
	20	0.369±0.036[b]
PGF_1-α	5	0.000±0.000
	10	0.000±0.000
	20	0.000±0.000
PGF_2-α	5	0.000±0.000
	10	0.000±0.000
	20	0.000±0.000

4.2 The effect of TNF on the excretion of creatinine and urea nitrogen by female NMRI mice

The effects of the MAC 16 tumour on the excretion of creatinine and urea nitrogen by male NMRI mice have been reported (Beck and Tisdale, in press). Briefly, creatinine and urea nitrogen levels in the urine of tumour-bearing mice were elevated at small tumour masses, when the weight loss did not exceed 3 to 4g. However, above this weight loss, there was a conservation of nitrogen, and the excretion of urea nitrogen and creatinine fell to below that found in non-tumour-bearing animals. In contrast, a single i.v. injection of TNF ($7.5 \times 10^7 U/Kg$) had no effect on the levels of either creatinine or urea nitrogen in the urine of female NMRI mice (table 4.5).

4.3 Comparison of the effects of human TNF with those of murine TNF

As described previously, human TNF (hTNF) and murine TNF (mTNF) possess several structural dissimilarities (section 1.3) and species specificity in some of the actions of this cytokine have been reported (Fransen et al, 1986a, Smith et al, 1986, Fiers et al, 1989, and Zentella et al, 1989). Thus, in order to determine whether the results presented in this thesis, which were obtained with hTNF in mice, could be extrapolated back to the situation with mTNF in the same species, the effects of these two TNFs on the parameters of weight loss discussed so far (sections 3.1.2 and 3.1.3) were compared.

No difference was observed in the effects of a single i.v. injection of the same concentration (0, 0.50, 0.75. 1.0 or 1.25mg/Kg) of either hTNF or mTNF on the weight change and the food and water consumption over a 24h period of female NMRI mice. Both treatments resulted in a similar dose-related decrease in body weight, accompanied by a dose-dependent decrease in the consumption of food and water (figures 4.6 and 4.7). When administered as two i.v. injections over a 24h period, no difference was observed in the effects of hTNF and mTNF on the concentration of blood glucose or plasma FFA, as measured 90min after the second injection; both treatments resulted in a similar hypoglycaemia and decrease in plasma FFA concentration (figure 4.8).

Table 4.5

The effect of TNF on the levels of creatinine and urea nitrogen in the urine of female NMRI mice

Female NMRI mice were administered a single i.v. injection of 7.5 X 10^7U/Kg TNF or 200µl of 0.9% NaCl. The animals were then placed in metabolic cages and urine was collected over a 24h period for the determination of creatinine and urea nitrogen. The values represent the means ± S.E.M. of 5 to 7 animals in each group.

	Creatinine (mg/100ml)	Urea nitrogen (mg/100ml)
Controls (i.v. saline)	78±17	2500±300
TNF	82±20	2600±400

Figure 4.6

Comparison of the effects of human TNF with those of murine TNF on the body weight change of female NMRI mice

Female NMRI mice (19.2±0.5g) were administered a single i.v. injection of either human TNF (hTNF) or murine TNF (mTNF) and the animals were weighed 24h later. The values represent the means ± S.E.M. of 6 to 13 animals for each concentration of TNF. *p≤0.001 from saline-injected controls by analysis of variance.

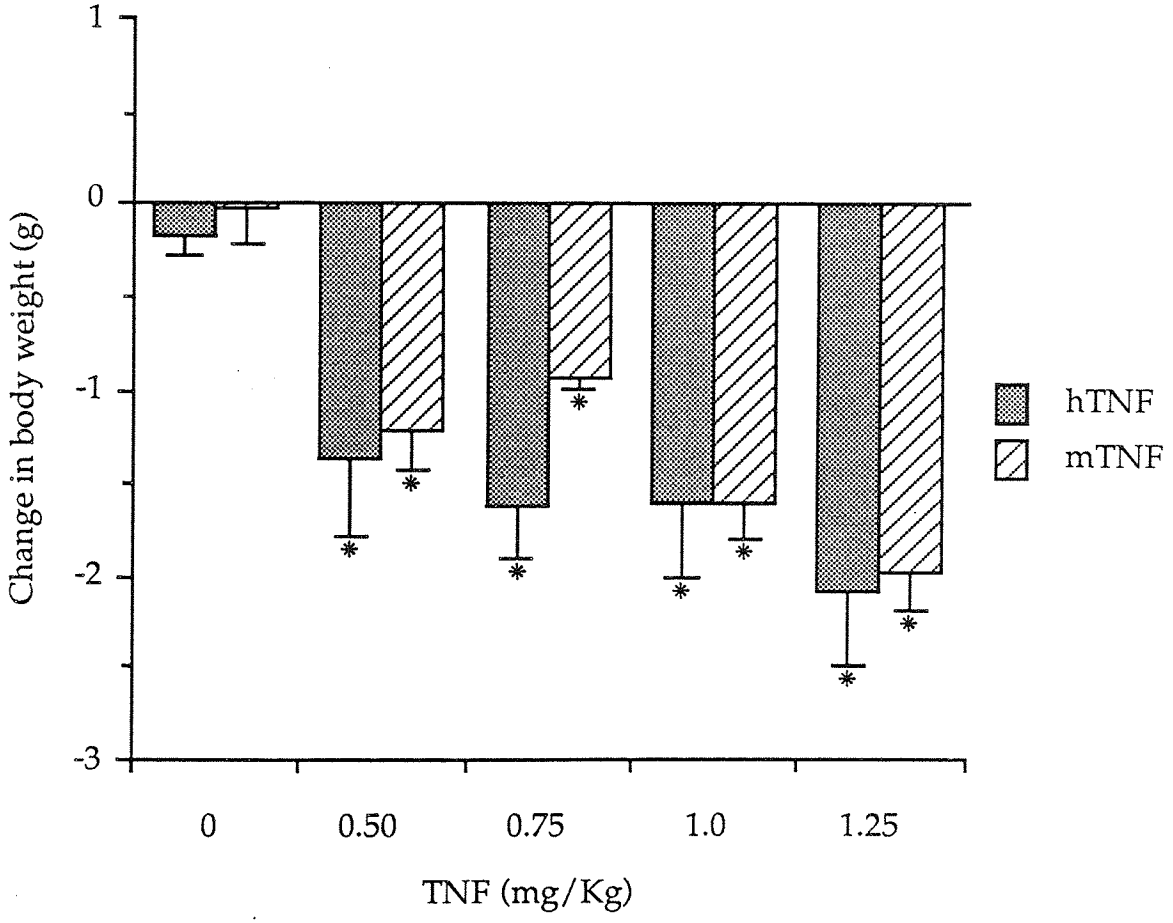


Figure 4.7

Comparison of the effects of human TNF with those of murine TNF on the food and water consumption of female NMRI mice

Female NMRI mice (19.2±0.5g) were administered a single i.v. injection of either human TNF (hTNF) or murine TNF (mTNF) and the food (g) and water (ml) consumption was measured over a 24h period. The values represent the means ± S.E.M. of 6 to 13 animals for each concentration of TNF. *p≤0.05, **p≤0.01 and ***p≤0.005 from saline-injected controls by analysis of variance.

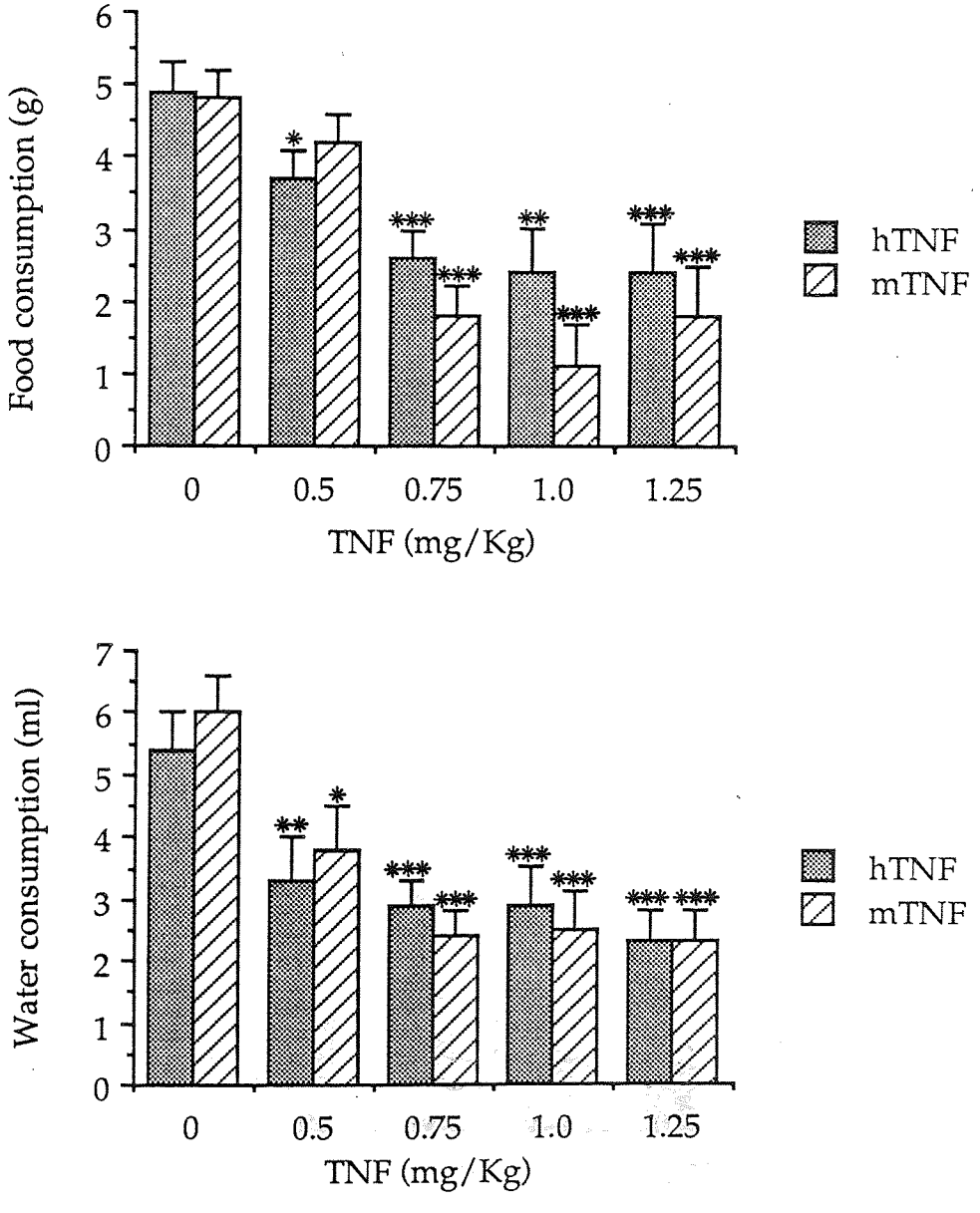
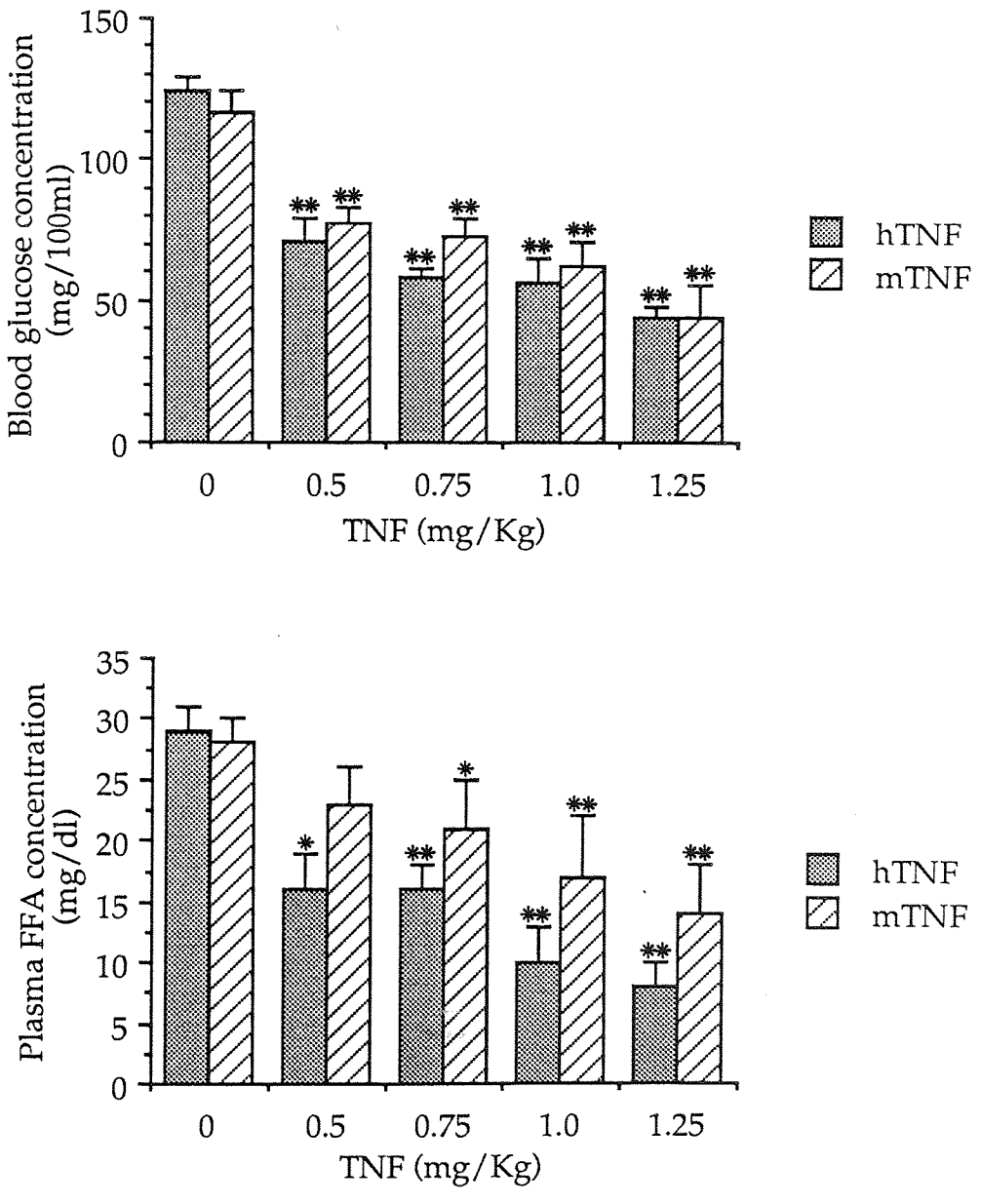


Figure 4.8

Comparison of the effects of human TNF with those of murine TNF on the blood glucose and plasma FFA concentration of female NMRI mice

Blood glucose and plasma FFA levels were determined 90min after the second of 2 i.v. injections over a 24h period of either human TNF (hTNF) or murine TNF (mTNF). The values represent the means ± S.E.M. of 6 to 13 animals for each concentration of TNF. *p≤0.005 and **p≤0.001 from saline-injected controls by analysis of variance.



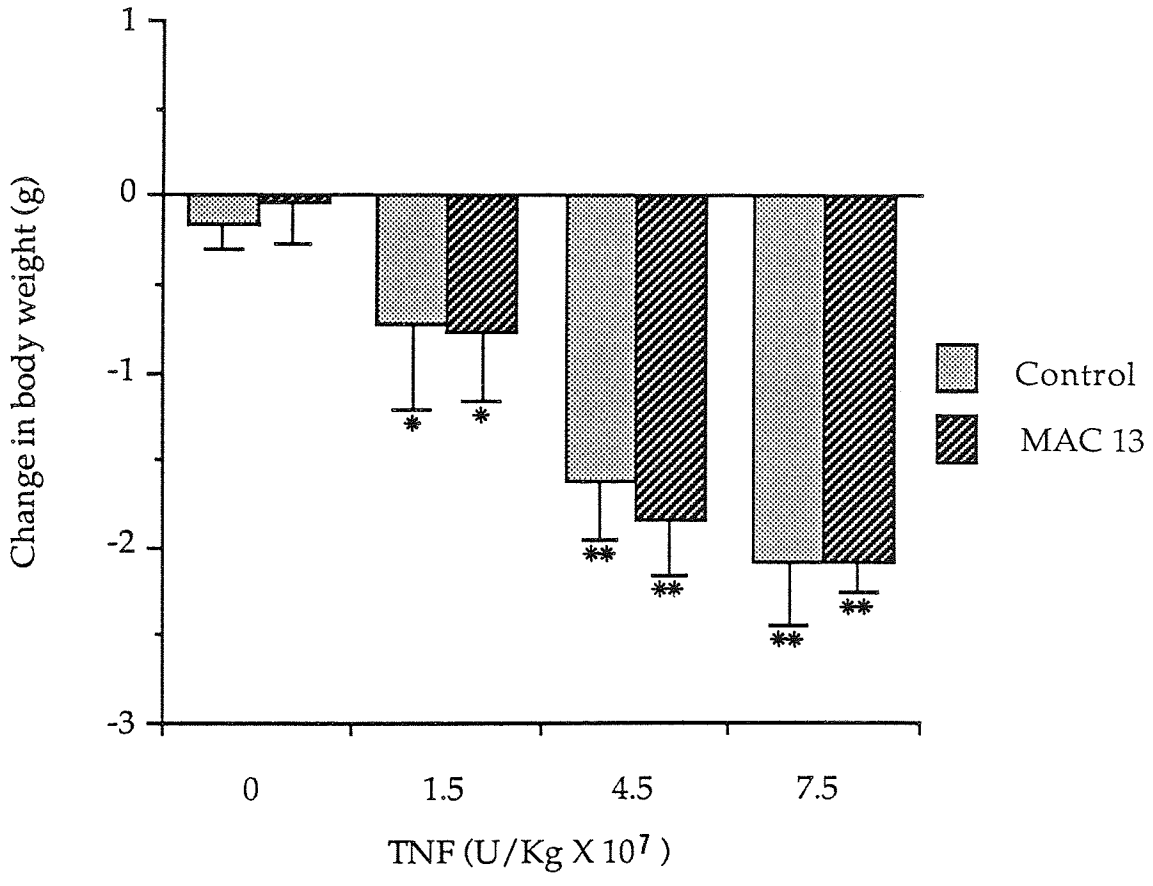
4.4 The effect of TNF on MAC 13 tumour-bearing mice

Animals bearing tumours have previously been shown to be highly sensitive to the toxic effects of TNF (Bartholeyns et al, 1987). In order to determine whether this hypersensitivity included the TNF-induced weight loss, female NMRI mice bearing the non-cachexia-inducing MAC 13 adenocarcinoma were injected i.v. with increasing doses of TNF (0, 1.5, 4.5 and 7.5 X 10^7U/Kg), and the weight change over a 24h period was determined. No difference was observed between the TNF-induced weight loss in the MAC 13 tumour-bearing animals and the TNF-induced weight loss in non-tumour-bearing controls (figure 4.9). Thus, MAC 13 tumour-bearing female NMRI mice were no more sensitive to the weight loss induced by TNF than were non-tumour-bearing control NMRI mice.

Figure 4.9

The effect of TNF on MAC 13 tumour-bearing female NMRI mice

The weight change over a 24h period following a single i.v. injection of TNF or 0.9% NaCl was determined in MAC 13 tumour-bearing and control non-tumour-bearing female NMRI mice (20.6±0.3g). The values represent the means ± S.E.M. of 5 animals for each concentration of TNF. $^{*}p \leq 0.01$ and $^{**}p \leq 0.001$ from saline-injected controls by analysis of variance.



4.5 Comparison of TNF with Mitozolamide

In order to determine whether the TNF-induced weight loss was merely a result of general malaise induced by this agent, some of the parameters contributing to weight loss produced by TNF were compared with those produced by mitozolamide, a drug which in toxic doses induces weight loss with a decrease in nutrient and water intake.

At a concentration of 20mg/Kg mitozolamide caused general malaise and a decrease in food and water consumption equal to that obtained with 7.5 X 10^7U/Kg TNF. There was no significant difference between the weight loss over a 24h period produced by a single i.p. injection of 20mg/Kg mitozolamide and by a single i.v. injection of 7.5 X 10^7U/Kg TNF (figure 4.10), suggesting that the weight loss produced by TNF may have been due to a generalised toxicity.

When administered as two separate i.v. injections over a 24h period, TNF caused marked hypoglycaemia and hypertriglyceridaemia 90min after the second injection. However, no change in blood glucose or plasma triglyceride concentration was noted after similar i.p. administration of mitozolamide (figure 4.11). Thus, these effects were distinct to TNF and were not a direct result of general malaise or hypophagia.

No difference was observed in the total body water content of TNF-treated and mitozolamide-treated animals (table 4.6), both groups exhibiting a decrease in this parameter over a 24h period. Whereas TNF caused a decrease in carcass lipid content, mitozolamide had no effect on the fat content of animals. No change in left thigh and gastrocnemius muscle content was observed after either TNF or mitozolamide administration.

Figure 4.10

Comparison of the effects of TNF with those of mitozolamide on the body weight change and food and water consumption of female NMRI mice

Female NMRI mice (19.3±0.15g) were administered either a single i.v. injection of 7.5 X 10^7U/Kg TNF, or an i.p. injection of 20mg/Kg mitozolamide (2mg/ml) in arachis oil containing 10% DMSO. Controls were injected with 0.9% NaCl i.v. or arachis oil containing 10% DMSO i.p. 24h later mice were weighed and the food and water consumption was determined. The values represent the means ± S.E.M. of 6 to 13 animals in each group. *p≤0.001 from controls (saline), (A), and **p≤0.001 from controls (arachis oil), (C), by analysis of variance.

A = control (saline), B = TNF, C = control (arachis oil), D = mitozolamide.

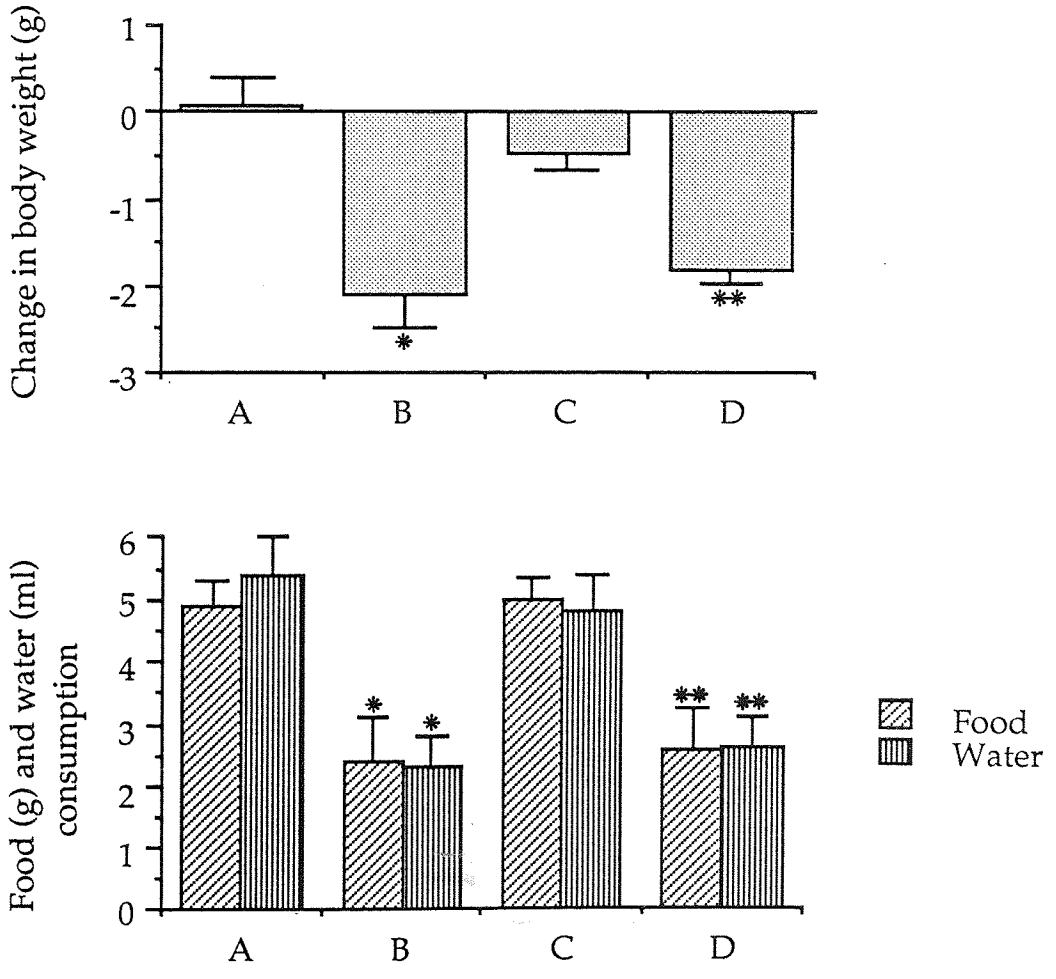


Figure 4.11

Comparison of the effects of TNF with those of mitozolamide on the blood glucose and plasma triglyceride concentration of female NMRI mice

Blood glucose and plasma triglyceride levels were determined in female NMRI mice 90min after the second of 2 i.v. injections of TNF (7.5 X 10^7U/Kg), or 2 i.p. injections of mitozolamide (20mg/Kg) over a 24h period. Controls were injected with 0.9% NaCl i.v. or arachis oil containing 10% DMSO i.p. The values represent the means ± S.E.M. of 6 to 13 animals in each group. *p≤0.001 from controls (saline), (A), by analysis of variance.

A = control (saline), B = TNF, C = control (arachis oil), D = mitozolamide.

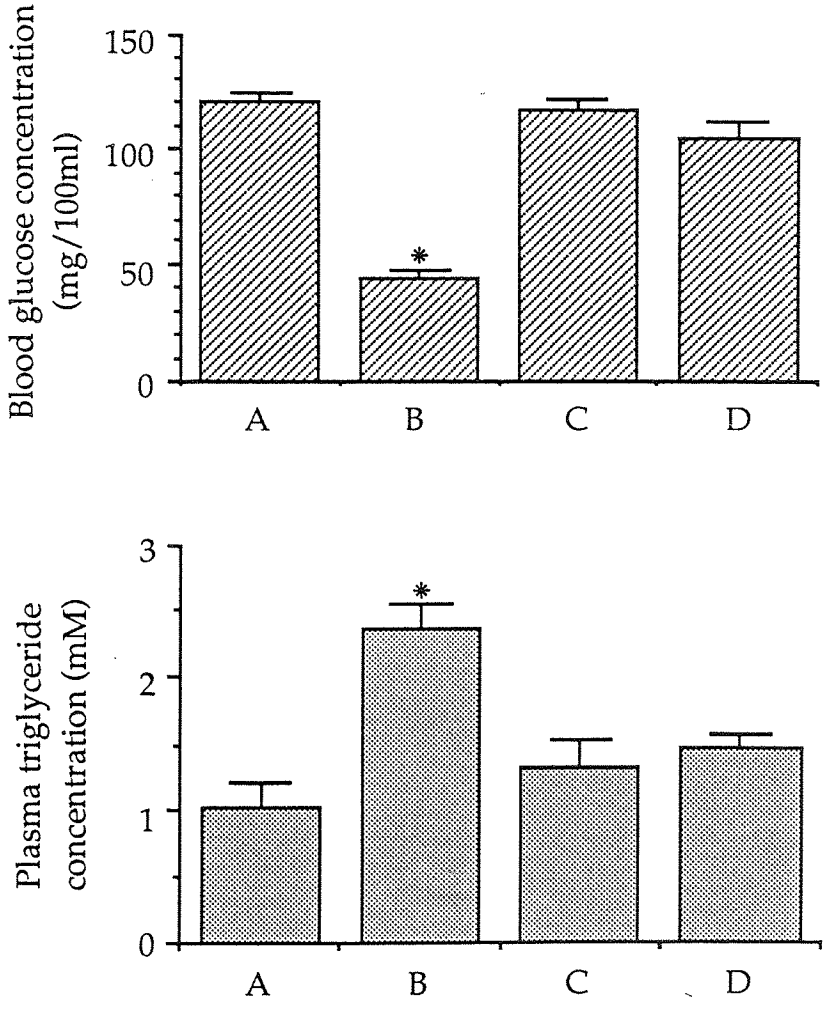


Table 4.6

Comparison of the effects of TNF and mitozolamide on the body compositions of female NMRI mice

Body composition analysis was performed 24h after a single i.v. injection of TNF (7.5 X 10^7U/Kg), or a single i.p. injection of mitozolamide (20mg/Kg) in arachis oil containing 10% DMSO. Controls were injected with 0.9% NaCl i.v. or with arachis oil containing 10% DMSO i.p. Starting weight for animals in all groups was 19.2±0.46g. The values represent the means ± S.E.M. of 6 to 13 animals in each group. [a]$p \leq 0.01$ from controls (arachis oil), [b]$p \leq 0.05$ from controls (saline) and [c]$p < 0.01$ from controls (saline) by analysis of variance.

Treatment	Water content (g)	Fat content (g)	Left thigh and gastrocnemius muscle content (g)
Controls (i.v. saline)	13.11±0.24	2.20±0.11	0.065±0.002
TNF (7.5 X 10^7U/Kg)	11.99±0.09[a,b]	1.54±0.23[c]	0.067±0.023
Controls (i.p. arachis oil + 10% DMSO)	13.25±0.52	1.79±0.10	0.069±0.007
Mitozolamide (20mg/Kg)	11.70±0.168[a,c]	1.84±0.22	0.062±0.001

4.6 Comparison of the effects of acute and chronic administration of TNF to female NMRI mice

4.6.1 Body weight and food and water consumption

Mice receiving single i.v. injections of TNF at the same time each day over a 5 day period exhibited a biphasic dose-related decrease in weight (figure 4.12, table 4.7). All of the weight loss occurred during the first 24h after injection and, thereafter, the body weight change increased toward that of saline-injected controls despite further daily injections of TNF. When the relative body mass of animals for the 3 concentrations of TNF (4.5, 6.0 and 7.5 X 10^7U/Kg) were plotted, it was seen that the weights of mice, relative to the initial weights, gradually returned toward the 100% value over the 5 day period. This biphasic trend was dose-related, the higher the concentration of TNF employed the longer the time to reach the 100% value, due to the initial large decrease in body weight.

Both the food and water consumption of mice receiving daily injections of TNF closely followed the pattern of weight loss, with an initial dose-dependent sharp decrease, followed by a period of 1 to 2 days in an increased consumption, and with the relative consumption gradually increasing toward that of saline-injected controls over the 5-day period (figure 4.13, table 4.8).

4.6.2 Blood metabolite studies

TNF-treated mice showed a highly significant dose-related hypoglycaemia 60-90min after the second of 2 i.v. injections over a 24h period (acute administration), but not after 5 daily injections (chronic administration) of the 3 highest concentrations (4.5, 6.0 and 7.5 X 10^7U/Kg) of TNF (figure 4.1.4). The TNF-induced hypoglycaemia was directly proportional to both the decrease in body weight of animals and to the difference in food and water consumption of TNF-treated animals, as compared to saline-injected controls, over the first 24h following injection (figure 4.15).

The plasma levels of FFA were reduced in a dose-related manner after acute administration of TNF, but not after chronic administration (figure 4.16). Marked hypertriglyceridaemia was also observed after acute administration of TNF, and a slight increase in the plasma level of triglycerides was still apparent after 5 daily injections of the 3 highest doses of TNF (figure 4.17). As described previously, this effect of TNF on FFA and triglycerides may have been due to inhibition of lipoprotein lipase activity (Semb et al, 1987, and section 1.9.4) or to an increased hepatic triglyceride synthesis (Feingold et al, 1987).

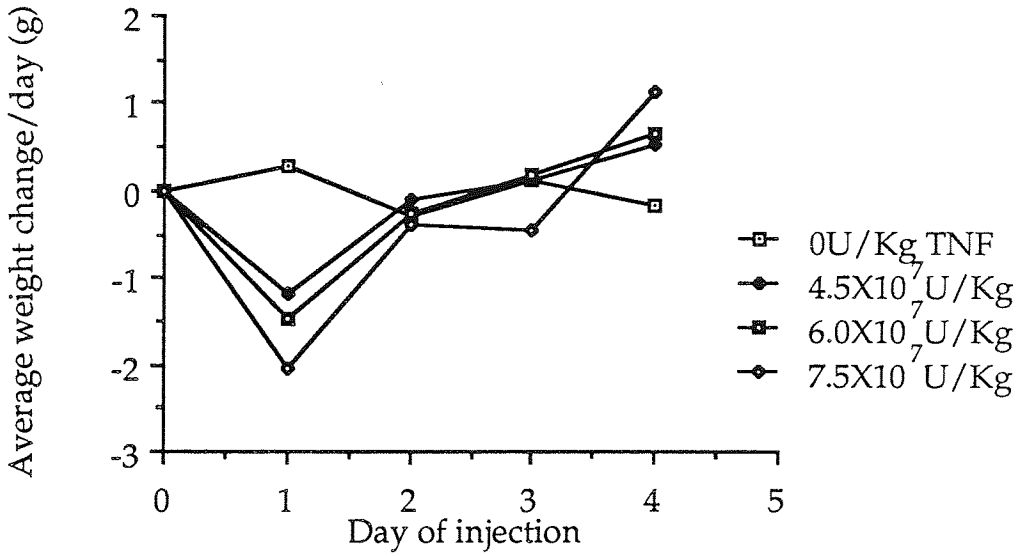
4.6.3 Body Composition (table 4.9)

The decrease in body weight of animals was accompanied by a decrease in total body fat after acute administration of 7.5 X 10^7U/Kg TNF, when compared with saline-injected controls, and also after 5 daily injections of TNF, when mice were no longer losing weight. A decrease in total body water content was observed after acute administration of TNF, when compared with saline-injected controls, but a slight increase was seen after chronic administration, and this probably contributed to the increase in weight of the mice. No change in the left thigh and gastrocnemius muscle content was observed after either acute or chronic administration of TNF.

Figure 4.12

The effect of daily administration of TNF on the weight of female NMRI mice

Recombinant human TNF was administered daily by i.v. injection over a 5 day period to female NMRI mice (19-22g) and the animals were weighed at the same time each day. The values represent the means of 6 to 13 animals for each concentration of TNF.



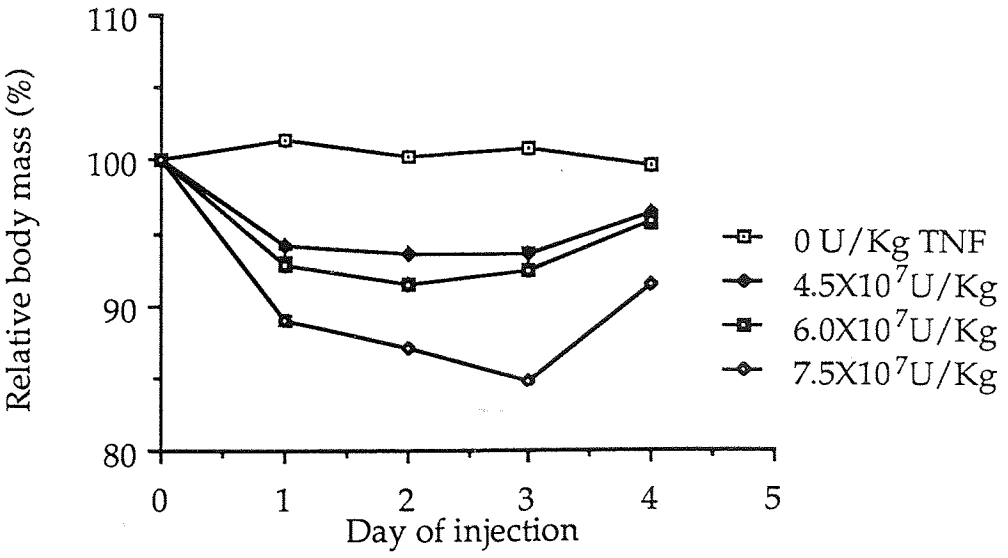


Table 4.7

The effect of daily administration of TNF on the weight of female NMRI mice

Recombinant human TNF was administered daily by i.v. injection over a 5 day period to female NMRI mice (19-22g) and the animals were weighed at the same time each day. The values represent the means ± S.E.M. of 6 to 13 animals for each concentration of TNF. $^{a}p\leq0.01$, $^{b}p\leq0.005$ and $^{c}p\leq0.001$ from saline-injected controls by analysis of variance.

AVERAGE WEIGHT CHANGE/DAY (g)

Day	1	2	3	4
0U/Kg TNF	+0.3±0.19	-0.3±0.14	+0.1±0.08	-0.2±0.07
4.5 X 10^7U/Kg TNF	-1.2±0.13^{c}	-0.1±0.18	+0.1±0.29	+0.6±0.13
6.0 X 10^7U/Kg TNF	-1.5±0.19^{c}	-0.3±0.20	+0.2±0.13	+0.7±0.26^{b}
7.5 X 10^7U/Kg TNF	-2.0±0.13^{c}	-0.4±0.20	-0.4±0.21^{a}	+1.2±0.41^{b}

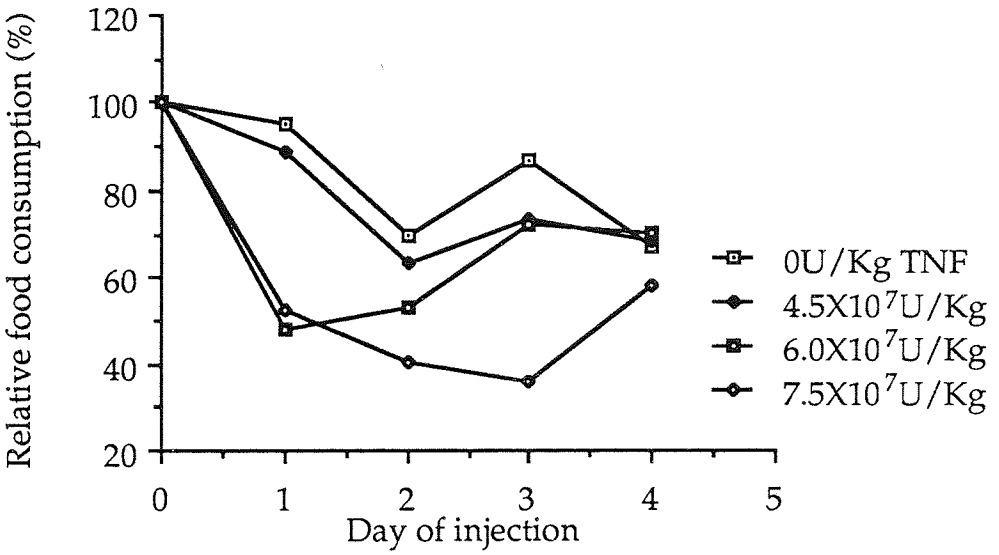
RELATIVE BODY MASS (%)

Day	1	2	3	4
0U/Kg TNF	101.4±0.95	100.3±0.78	100.9±0.78	99.7±1.20
4.5 X 10^7U/Kg TNF	94.2±0.73^{c}	93.7±0.81^{c}	93.6±1.68^{c}	96.4±1.11^{c}
6.0 X 10^7U/Kg TNF	92.8±1.22^{c}	91.5±1.46^{c}	92.4±1.47^{c}	95.9±1.33^{c}
7.5 X 10^7U/Kg TNF	89.0±0.41^{c}	87.1±1.02^{c}	84.9±1.00^{c}	91.5±1.29^{c}

Figure 4.13

The effect of daily administration of TNF on the food and water consumption of female NMRI mice

Recombinant human TNF was administered daily by i.v. injection over a 5 day period to female NMRI mice (19-22g) and the food and water consumption was measured at the same time each day. The values represent the means of 4 to 13 animals for each concentration of TNF.



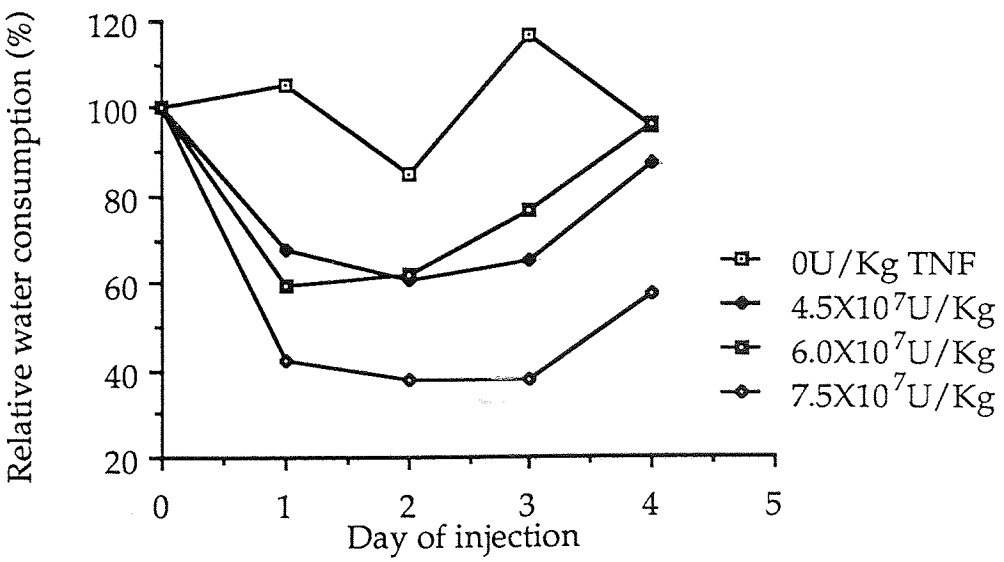


Table 4.8

The effect of daily administration of TNF on the food and water consumption of female NMRI mice

Recombinant human TNF was administered daily by i.v. injection over a 5 day period to female NMRI mice (19-22g) and the food and water consumption was measured at the same time each day. The values represent the means ± S.E.M. of 4 to 13 animals for each concentration of TNF. [a]$p \leq 0.005$ from saline-injected controls by analysis of variance.

RELATIVE FOOD CONSUMPTION (%)

Day	1	2	3	4
0U/Kg TNF	97.3±6.2	69.7±10.7	86.8±8.5	67.4±10.3
4.5 X 10^7U/Kg TNF	60.9±12.1[a]	63.6±6.5	73.6±9.3	68.5±2.6
6.0 X 10^7U/Kg TNF	48.0±11.3[a]	53.4±7.2[a]	72.0±12.0	70.2±3.4
7.5 X 10^7U/Kg TNF	52.4±10.3[a]	40.6±1.0[a]	35.8±7.8[a]	58.5±1.5

RELATIVE WATER CONSUMPTION (%)

Day	1	2	3	4
0U/Kg TNF	105.6±13.0	85.0±7.6	117.0±13.0	96.0±4.0
4.5 X 10^7U/Kg TNF	67.6±12.6[a]	60.6±4.5[a]	65.0±5.0	87.5±7.5
6.0 X 10^7U/Kg TNF	59.6±12.5[a]	61.8±1.7[a]	77.0±7.0[a]	96.8±9.25
7.5 X 10^7U/Kg TNF	42.6±10.7[a]	37.6±10.2[a]	37.6±12.4[a]	57.5±18.6[a]

Figure 4.14

Comparison of the effects of acute and chronic administration of TNF on the blood glucose concentration of female NMRI mice

Glucose was measured in the blood of female NMRI mice 90min after the second of 2 i.v. injections of TNF over a 24h period (acute administration), or 90min after the fifth of 5 daily i.v. injections of TNF (chronic administration). The values represent the means ± S.E.M. of 4 to 11 (acute dosage) or 6 to 13 (chronic dosage) animals for each concentration of TNF. *p≤0.001 from saline-injected controls by analysis of variance.

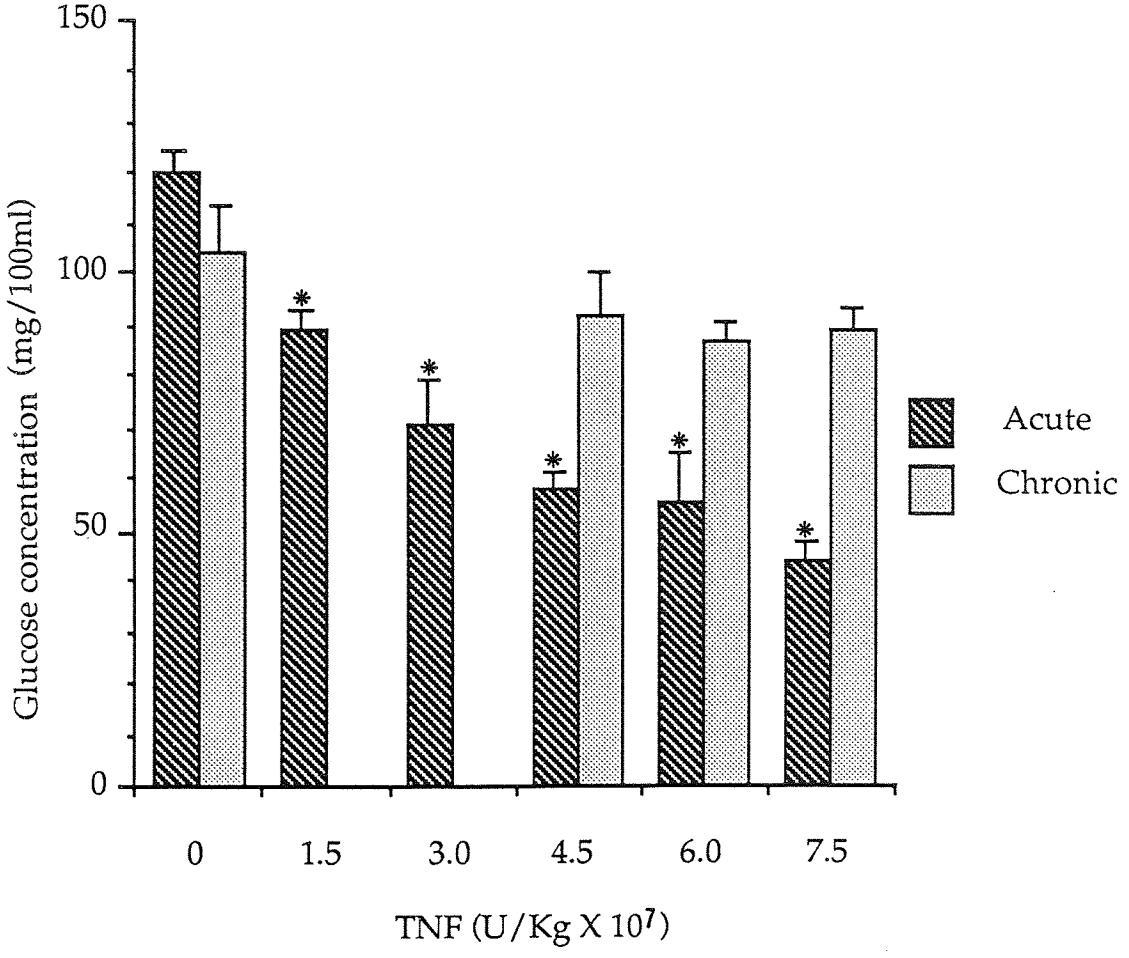
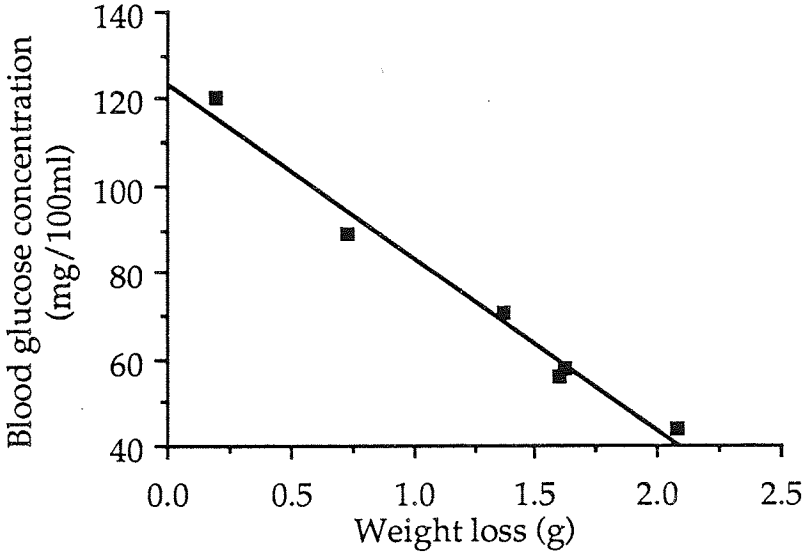


Figure 4.15

The variation of blood glucose concentration with the weight loss during a 24h period after administration of TNF, and with the difference in food and water consumption during a 24h period between a saline-infused group and the TNF-treated groups

The results were fitted to a linear model by means of least squares analysis (r=-0.99 and 0.98 respectively).



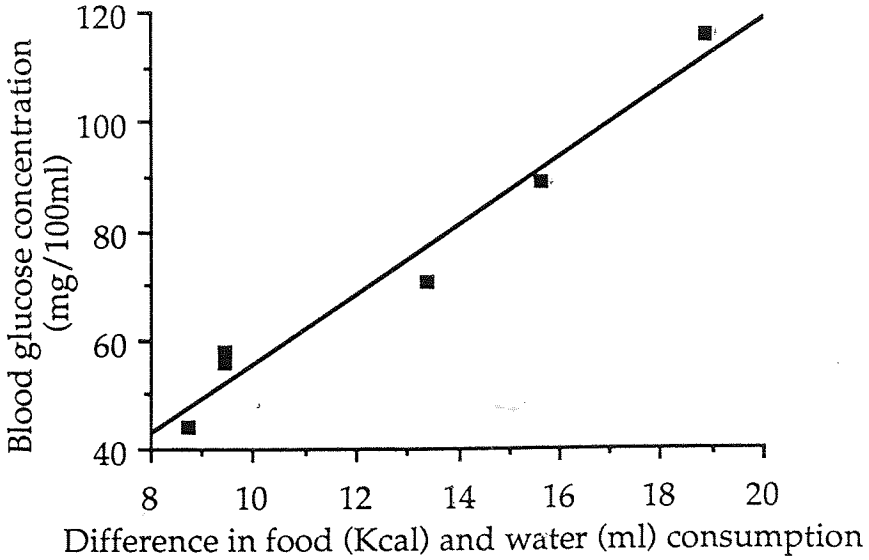


Figure 4.16

Comparison of the effects of acute and chronic administration of TNF on the plasma FFA concentration of female NMRI mice

FFA was measured in the plasma of female NMRI mice 90min after the second of 2 i.v. injections of TNF over a 24h period (acute administration), or 90min after the fifth of 5 daily i.v. injections of TNF (chronic administration). The values represent the means ± S.E.M. of 4 to 7 (acute dosage) or 6 to 13 (chronic dosage) animals for each concentration of TNF. *p≤0.005 and **p≤0.001 from saline-injected controls by analysis of variance.

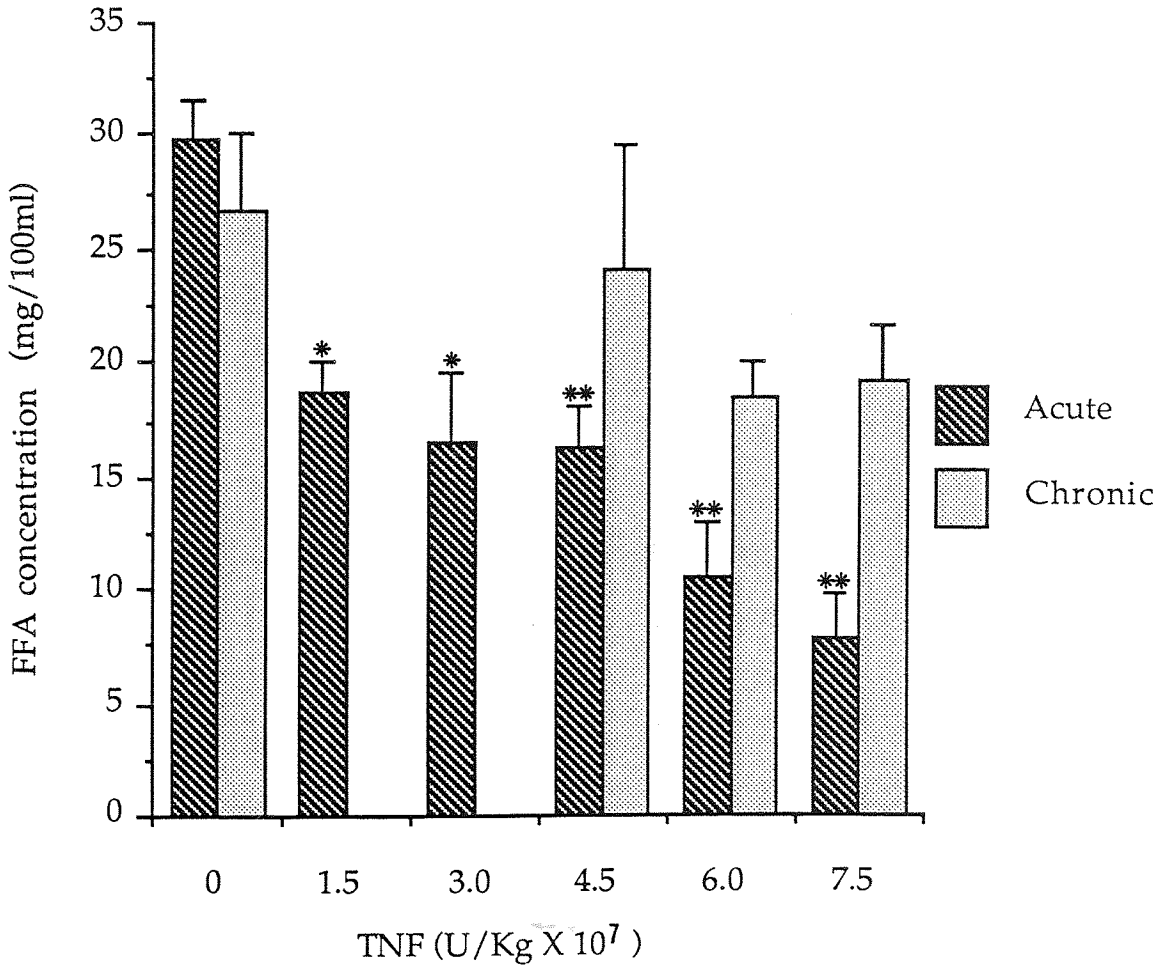


Figure 4.17

Comparison of the effects of acute and chronic administration of TNF on the plasma triglyceride concentration of female NMRI mice

Triglyceride was measured in the plasma of female NMRI mice 90min after the second of 2 i.v. injections of TNF over a 24h period (acute administration), or 90min after the fifth of 5 daily i.v. injections of TNF (chronic administration). The values represent the means ± S.E.M. of 4 to 11 (acute dosage) or 6 to 13 (chronic dosage) animals for each concentration of TNF. *$p \leq 0.05$ and **$p \leq 0.001$ from saline-injected controls by analysis of variance.

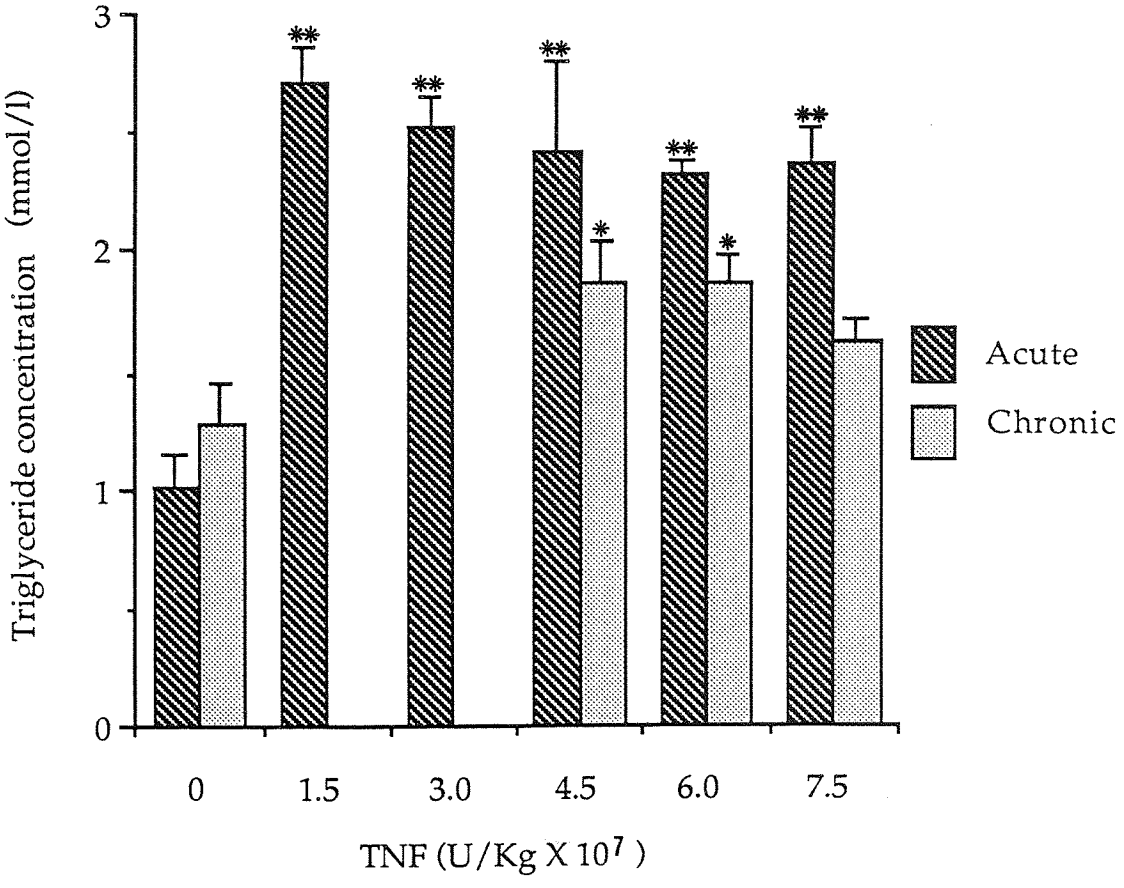


Table 4.9

The effects of acute and chronic administration of TNF on the body compositions of female NMRI mice

Body composition analysis was performed 24h after a single i.v. injection (acute administration) or 4 daily i.v injections (chronic administration) of 7.5 X 10^7U/Kg TNF or 0.9% NaCl. The values represent the means ± S.E.M. of 6 to 13 animals in each group. Starting weight for animals in all groups was 19.2±0.46g. $^a p \leq 0.05$ and $^b p \leq 0.01$ from saline-injected controls by analysis of variance.

Treatment		Water content (g)	Fat content (g)	Left thigh and gastrocnemius muscle content (g)
Acute	Control	13.11±0.24	2.20±0.11	0.065±0.0015
	TNF	11.99±0.09[a]	1.54±0.23[b]	0.061±0.002
Chronic	Control	12.56±0.20	1.79±0.12	0.061±0.002
	TNF	13.35±0.18[a]	0.61±0.08[b]	0.063±0.003

4.7 Comparison of the effects of TNF with those of pair-feeding

The TNF-induced weight loss was accompanied by, and directly proportional to, a decrease in both the food and water consumption of animals (section 4.1.1). To investigate whether this hypophagia was responsible for the weight loss and metabolic effects observed after TNF administration, female NMRI mice were injected i.v. with 0.9% NaCl and were given the same amount of food and water over a 24h period as that consumed by animals following injection of 7.5 X 10^7U/Kg TNF. The pair-fed controls were then utilized in this study to compare the effects of TNF with those of hypophagia on the weight change of female NMRI mice.

4.7.1 Body weight

Pair-fed female NMRI mice showed an identical pattern of weight loss, with an initial large decrease in body weight during the first 24h period, followed by an increase in body weight toward that of saline-injected controls, as that produced by the TNF- treated animals (figure 4.18). The TNF-induced weight loss thus appeared to be solely due to an anorectic effect of this agent.

4.7.2 Blood metabolite studies (figures 4.19 and 4.20)

As previously reported (4.1.2), TNF, when given as two separate i.v. injections over a 24h period, caused a marked hypoglycaemia, a decrease in plasma FFA and a hypertriglyceridaemia 90min after the final injection. Female NMRI mice, given the same amount of food and water as that consumed by mice over a 24h period following injection of 7.5 X 10^7U/Kg TNF, did not show a significant hypoglycaemia or hypertriglyceridaemia, these effects being distinct to TNF. Pair-fed animals did, however, exhibit a decrease in plasma FFA concentration of similar magnitude to that observed in TNF-treated mice, this decrease presumably being due to fat utilization as an energy source in these nutrient-restricted animals.

Figure 4.18

Comparison of the effects of TNF with those of pair-feeding on the body weight change of female NMRI mice over a 5 day period

Female NMRI mice (19.2±0.46g) were administered TNF (7.5 X 10^7U/Kg) or 0.9% NaCl daily by i.v. injection over a 5 day period, or were pair-fed as described in section 3.7 for 5 days. The values represent the means ± S.E.M. of 6 to 13 animals in each group. *p≤0.005 and **p≤0.001 from saline-injected controls by analysis of variance.

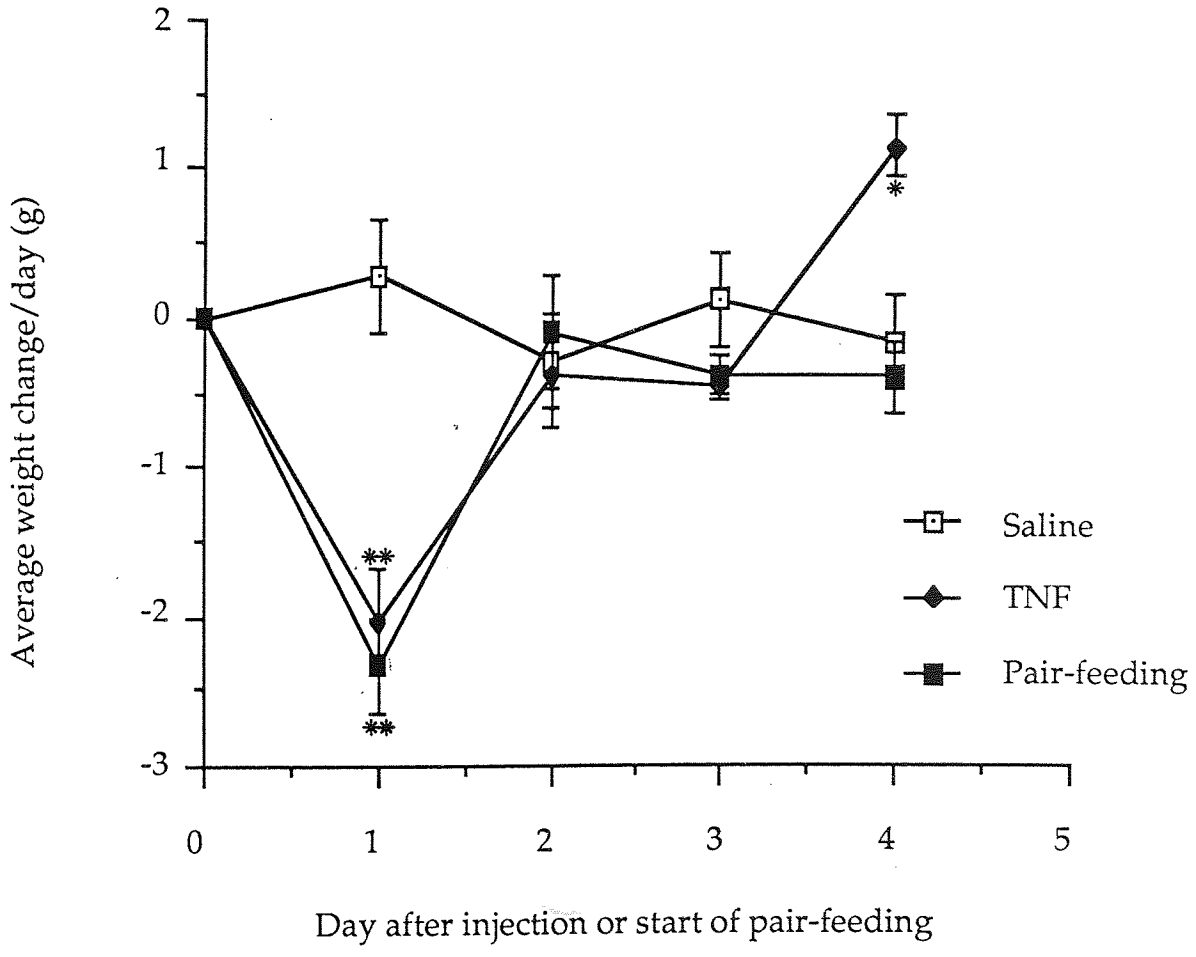


Figure 4.19

Comparison of the effects of TNF with those of pair-feeding on the blood glucose concentration of female NMRI mice

Female NMRI mice were administered two i.v. injections of TNF (7.5 X 10^7U/Kg) or 0.9% NaCl over a 24h period and glucose was measured in the blood of mice 90min after the second injection, or mice were pair-fed as described in section 3.7 and the blood glucose concentration was measured 24h later. The values represent the means ± S.E.M. of 6 to 13 animals in each group. *p≤0.001 from controls (i.v. saline), (A), by analysis of variance.

A = Control (i.v. saline), B = TNF, C = Control (no treatment), D = Pair-feeeding.

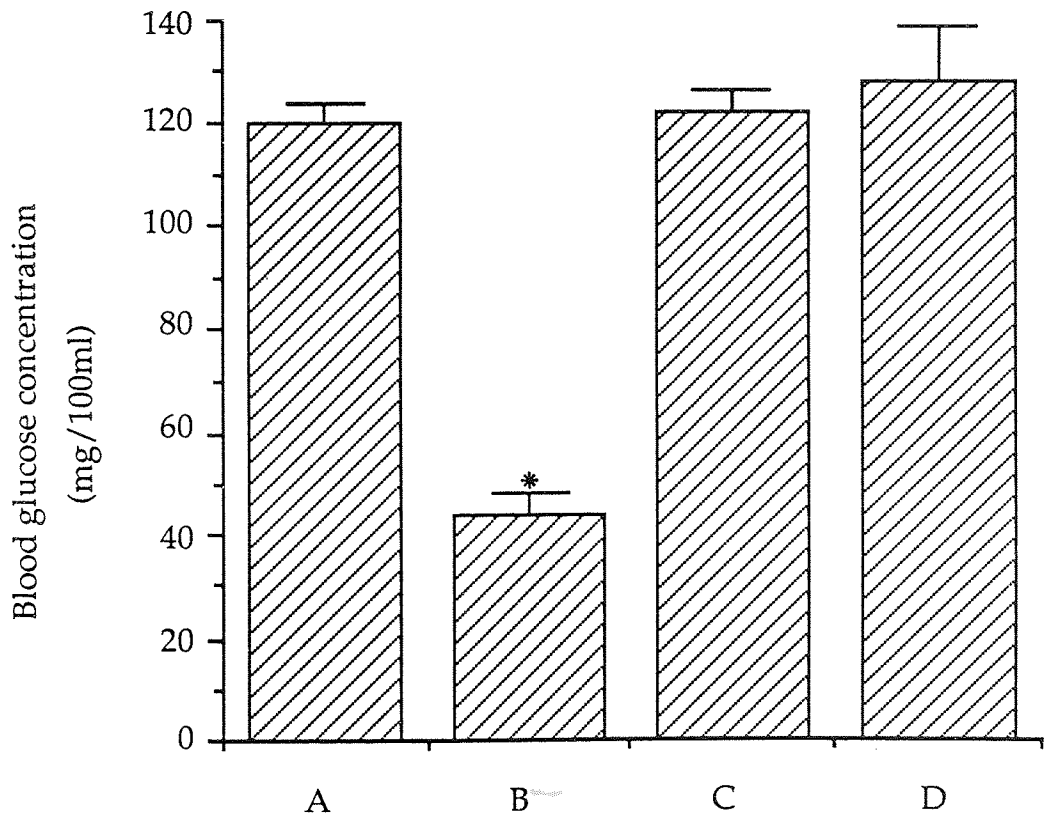
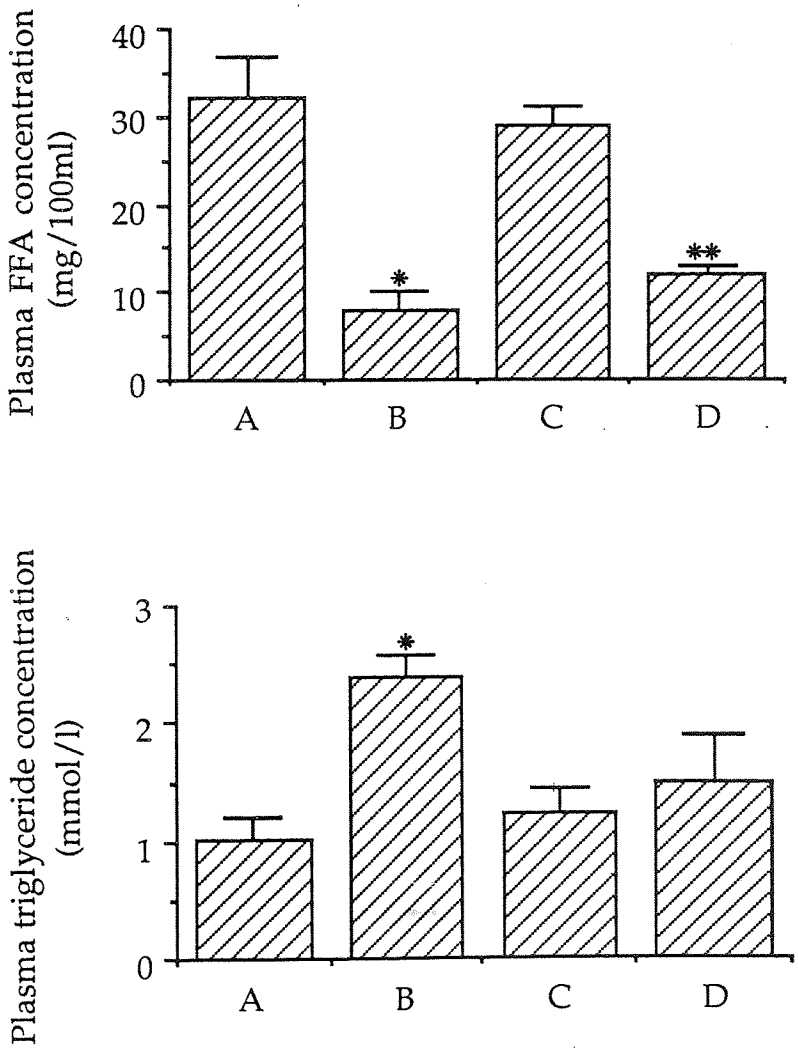


Figure 4.20

Comparison of the effects of TNF with those of pair-feeding on the plasma levels of FFA and triglyceride in female NMRI mice

Mice were administered two i.v. injections of TNF (7.5 X 10^7U/Kg) or 0.9% NaCl over a 24h period and FFA and triglyceride were measured in the plasma of mice 90min after the second injection, or mice were pair-fed as described in section 3.7 and the plasma FFA and triglyceride concentrations were measured 24h later. The values represent the means ± S.E.M. of 6 to 13 animals in each group. *p≤0.001 from controls (i.v. saline), (A), and **p≤0.001 from controls (no treatment), (C), by analysis of variance.

A = Control (i.v. saline), B = TNF, C = Control (no treatment), D = Pair-feeding.



4.7.3 Body Composition (table 4.10)

Pair-fed mice exhibited a decrease in total body water and carcass fat contents after a 24h period of similar magnitude to that observed in TNF-treated animals. Chronic administration of 7.5 X 10^7U/Kg TNF resulted in an increase in total body water content, which probably accounted for the increase in weight observed, and a decrease in body fat. However, chronically pair-fed controls exhibited a decrease in both body water and fat contents, the latter being of similar magnitude to that observed in TNF-treated mice. Neither TNF-treated animals nor pair-fed controls showed a decrease in the left thigh and gastrocnemius muscle content after acute or chronic treatments.

Table 4.10

The effects of TNF and pair-feeding on the body compositions of female NMRI mice

Body composition analysis was performed 24h after either a single i.v. injection (acute administration) or 4 daily i.v. injections (chronic administration) of TNF or 0.9% NaCl, and after both acute and chronic pair-feeding as described in section 3.7. The values represent the means ± S.E.M. of 6 to 13 animals in each group. Starting weight for animals in all groups was 19.2±0.46g. $^{a}p\leq0.01$ from controls (no treatment), $^{b}p\leq0.05$ from controls (i.v. saline), and $^{c}p\leq0.01$ from controls (i.v. saline) by analysis of variance.

(a) ACUTE ADMINISTRATION

Treatment	Water content (g)	Fat content (g)	Left thigh and gastrocnemius muscle content (g)
Controls (i.v. saline)	13.11±0.24	2.20±0.11	0.065±0.0015
TNF (7.5 X 10^7U/Kg)	11.99±0.09^{b}	1.54±0.23^{c}	0.063±0.003
Controls (no treatment)	13.07±0.36	2.30±0.19	0.069±0.005
Pair-feeding	11.80±0.24^{a}	1.62±0.13^{a}	0.062±0.003

(b) CHRONIC ADMINISTRATION

Treatment	Water content (g)	Fat content (g)	Left thigh and gastrocnemius muscle content (g)
Controls (i.v. saline)	12.56±0.20	1.79±0.12	0.061±0.002
TNF (7.5 X 10^7U/Kg)	13.35±0.18^{b}	0.61±0.08^{c}	0.063±0.003
Controls (no treatment)	13.07±0.36	2.30±0.19	0.069±0.005
Pair-feeding	11.54±0.40^{a}	0.92±0.11^{a}	0.053±0.004

4.8 The effect of force-feeding on the TNF-induced weight loss

It has been previously reported that administration of TNF to female NMRI mice resulted in a reduction in the weight of these animals over a 24h period (section 4.1.1, figure 4.1). This weight loss was accompanied by, and directly proportional to, a decrease in water consumption of mice (figures 4.2 and 4.3), and was also accompanied by a decrease in the total body water content of animals (table 4.2). In addition, the observed weight loss was directly proportional to the decrease in blood glucose concentration seen after TNF administration (table 4.1, figures 4.14 and 4.15). The aim of this study was to determine whether the TNF-induced weight loss could be reversed by rehydrating mice and/or by returning the blood glucose concentration of these animals to within the normal range.

TNF-treated female NMRI mice, given increasing quantities of water and a glucose solution orally over an 8h period, exhibited corresponding decreases in the weight loss normally induced by TNF (figure 4.21). There was no difference between the effect of the glucose solution and that of water alone on the reversal of weight loss. Administration of a medium chain triglyceride emulsion (MCT), of equal calorific value to the glucose solution (figure 4.21, I), caused a reversal of weight loss similar to that due to glucose and to water alone, thus indicating that hypophagia was not the main factor leading to the observed weight loss.

The total body water contents of TNF-treated mice given 100µl of water (C) or glucose solution (D), and 800µl of water (G) or glucose solution (H) every 2h are shown in figure 4.22. There was no difference between the water contents of these animals when compared with saline-injected controls. Thus, as the weight change in these animals was similar to that observed in saline-injected mice (figure 4.21), the TNF-induced weight loss was reversed by rehydrating female NMRI mice.

Figure 4.21

The effect of force-feeding on the weight of TNF-treated female NMRI mice

TNF (7.5 X 10^7U/Kg) was administered as a single i.v. injection to female NMRI mice. The animals were force-fed as described below, and the weight change over an 8h period was recorded. Starting weight for animals in all groups was 19.2±0.3g. The values represent the means ± S.E.M. of 4 to 15 animals in each group. *p≤0.005 and **p≤0.001 from TNF-treated mice, (B), and ***p≤0.001 from saline-injected controls, (A), by analysis of variance.

A = saline-injected control; B = TNF (7.5 X 10^7U/Kg); C = TNF plus 100µl of water every 2h; D = TNF plus 100µl of glucose solution (40mg/ml) every 2h; E = TNF plus 100µl of water every 45min; F = TNF plus 100µl of glucose solution (400mg/ml) every 45min; G = TNF plus 800µl of water every 2h; H = TNF plus 800µl of glucose solution (5mg/ml) every 2h; I = TNF plus 800µl of MCT emulsion (23mg lipid/ml, isocaloric with the glucose solution) every 2h.

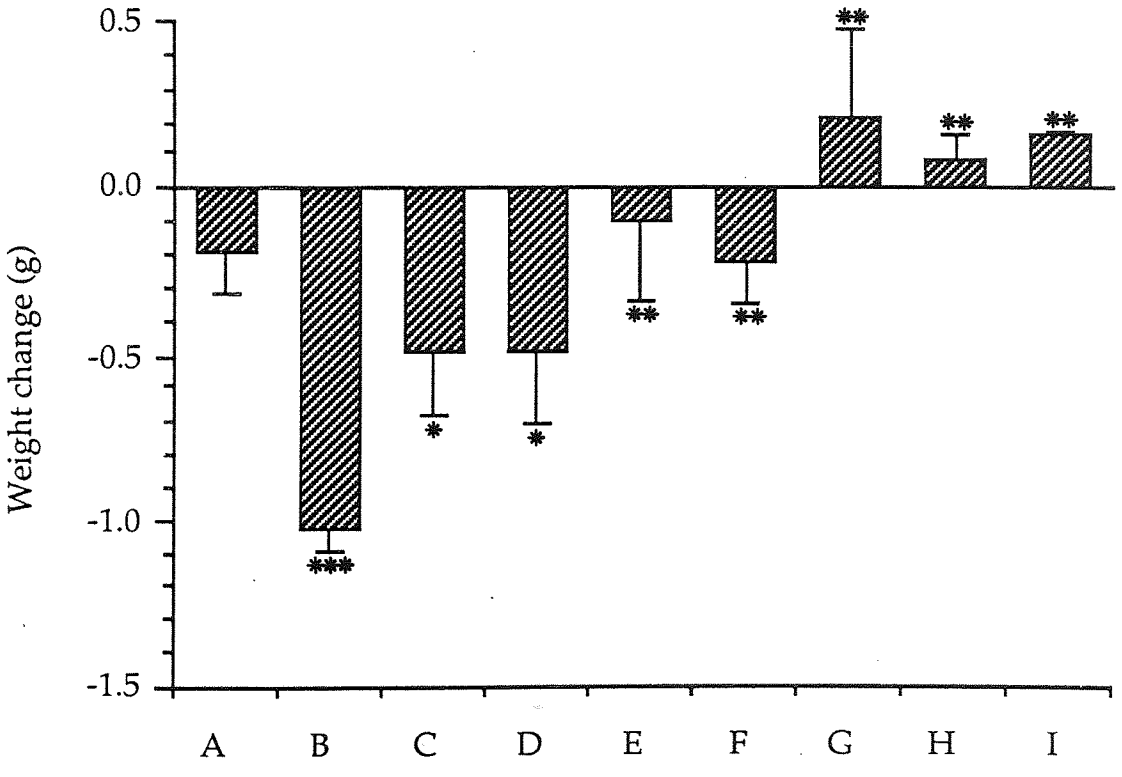
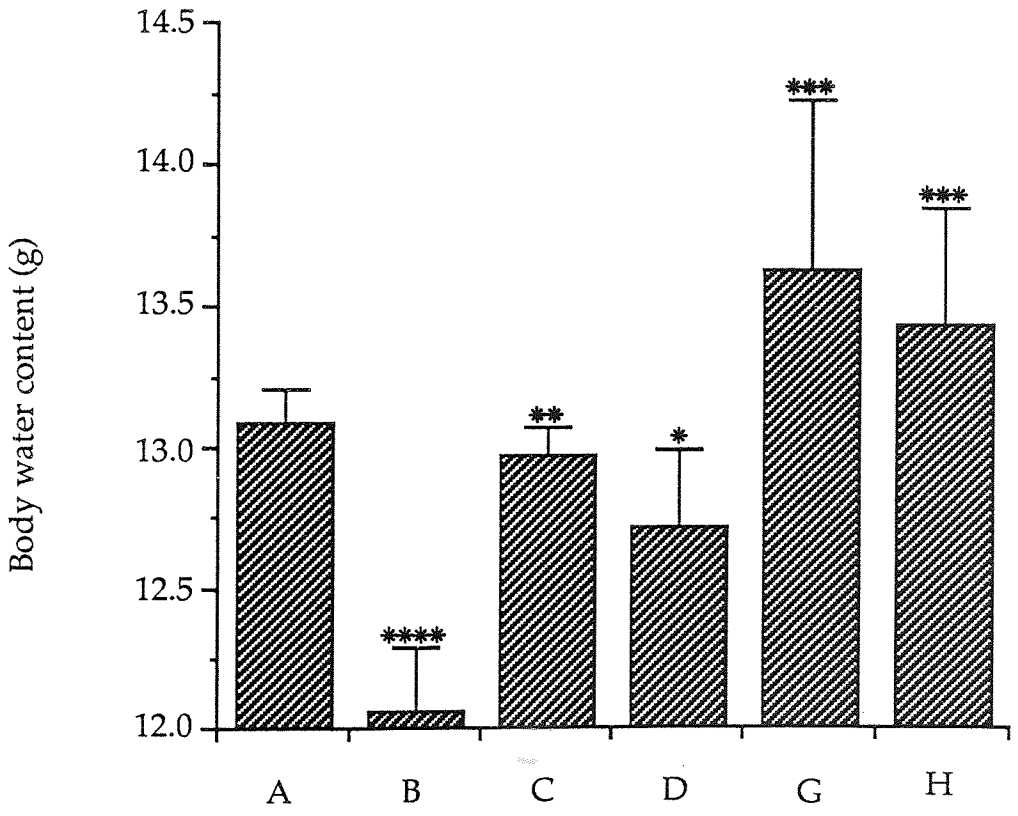


Figure 4.22

The effect of force-feeding on the body water content of TNF-treated female NMRI mice

Female NMRI mice were force-fed as described below over an 8h period. Mice were then killed and the body water content was measured. Starting weight for animals in all groups was 18.7±0.4g. The values represent the means ± S.E.M. of 5 animals in each group. *p≤0.05, **p≤0.005 and ***p≤0.001 from TNF-treated mice, (B), and ****p≤0.001 from saline-injected controls, (A), by analysis of variance.

A = saline-injected control; B = TNF (7.5×10^7 U/Kg); C = TNF plus 100µl of water every 2h; D = TNF plus 100µl of glucose solution (40mg/ml) every 2h; G = TNF plus 800µl of water every 2h; H = TNF plus 800µl of glucose solution (5mg/ml) every 2h.

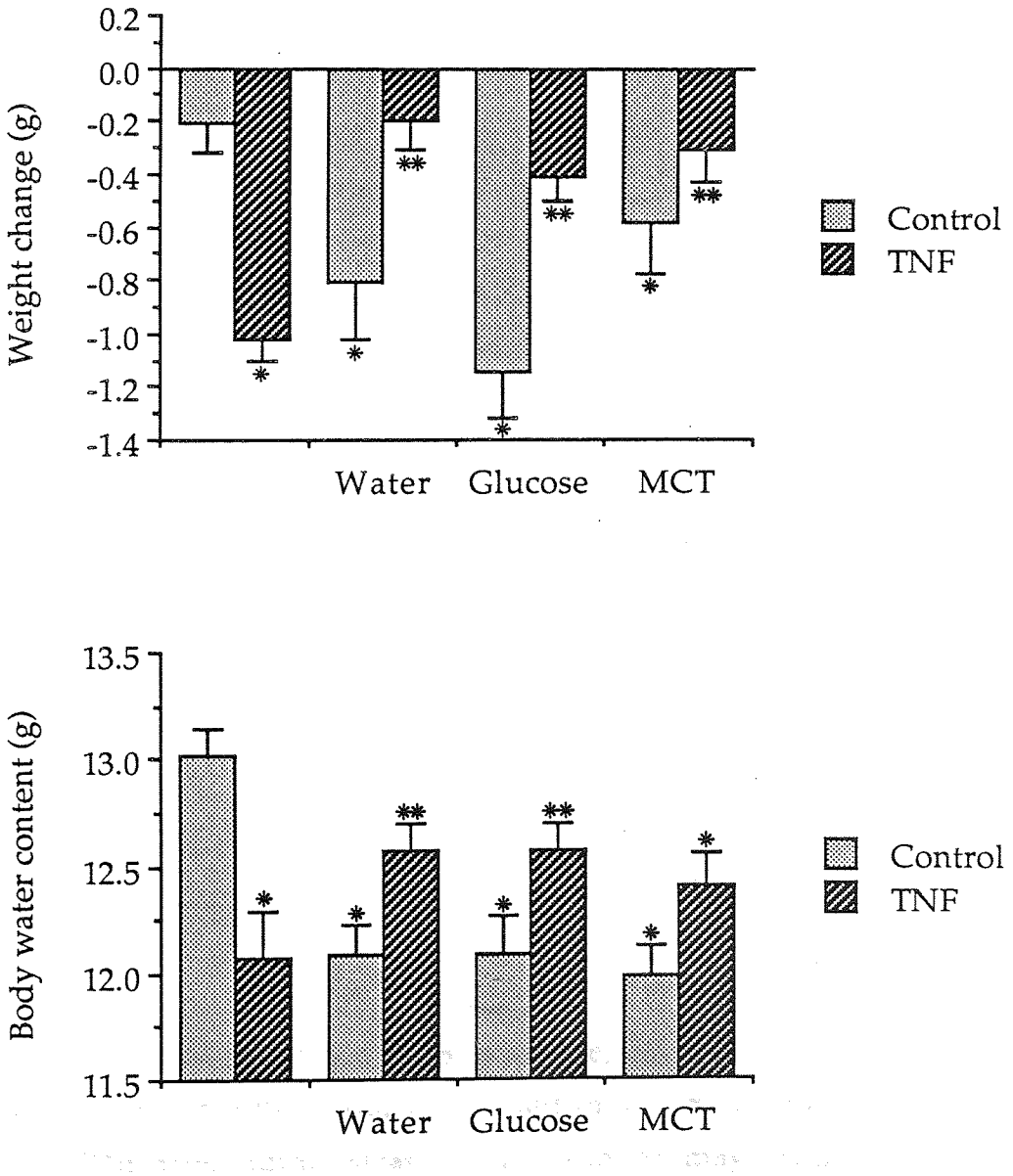


In order to show that this observed reversal of weight loss was not an artifact, saline-injected control female NMRI mice were similarly force-fed, and the weight change was measured and body composition analysis was performed after an 8h period. Administration of water, a glucose solution, or MCT at 45min intervals to TNF-injected mice over an 8h period resulted in identical reductions in the TNF-induced weight loss (figure 4.23). However, similar administration to saline-injected controls resulted in a greater degree of weight loss in these groups (0.8±0.22, 1.14±0.17 and 0.58±0.19g respectively) when compared with saline injection alone (0.19±0.1g). This weight loss was accompanied by a decrease in the total body water content of force-fed saline-injected controls (figure 4.23), and probably arose from the increased manipulation of these animals, resulting in stress and an increased urination. Thus, the replenishment of body water in TNF-treated animals may have arisen from a reduction in fluid output. No change in the fat and muscle contents of mice was observed after any of the treatments over the 8h investigation period (results not shown). These results suggest that the short-term weight loss produced by TNF in mice was principally due to dehydration.

Figure 4.23

Comparison of the effects of force-feeding on the weight change and body water content of TNF-injected female NMRI mice with those of saline-injected controls

Female NMRI mice were injected i.v. with TNF (7.5 X 10^7U/Kg) or 0.9% NaCl (200µl). Mice were fed orally 100µl of water, glucose solution or MCT at 45min intervals over an 8h period and the weight changes were recorded. Mice were then killed and the body water contents were determined. Starting weight for animals in all groups was 18.5±0.3g. The values represent the means ± S.E.M. of 8 to 14 animals in each group. *p≤0.001 from saline alone, and **p≤0.001 from TNF alone by analysis of variance.



4.9 The role of prostaglandins in the TNF-induced weight loss

Kettlehut et al (1987) reported that the toxic and metabolic effects of TNF could be blocked by a single injection of the cyclo-oxygenase inhibitors indomethacin or ibuprofen before the TNF treatment. This suggested that some of the effects of TNF may be mediated through a prostaglandin intermediate in analogy with septic shock, where large increases in circulatory prostaglandins have been reported in a variety of experimental animal models (Cook et al, 1980). Prostaglandins have also been implicated in the *in vitro* proteolytic activity of TNF (section 4.1.7). These experiments were undertaken in order to study the role of prostaglandins in the mechanism of weight loss induced by TNF.

4.9.1 The effect of TNF on PgE_2 production

PgE_2 production by spleen cells, which were rapidly removed from TNF-injected male NMRI mice, was utilized as an indirected method of measuring prostaglandin production because of difficulties in measuring plasma levels of PgE_2 directly. The radioimmunoassay utilized for these measurements had not previously been tested on mouse plasma, and our investigations revealed an inhibition of the binding of PgE_2 from mouse plasma with the antibody used in the assay. The results presented in figure 4.24 show an enhanced production of PgE_2 by spleen cells following i.v. administration of TNF (7.5×10^7 U/Kg) to mice, which was significantly greater than saline-injected controls within 1h after administration and remained elevated up to 6h after treatment. This suggests that the metabolic effects of TNF may be mediated via prostaglandin production.

4.9.2 Comparison of the effects of TNF and 16,16-dimethyl PgE_2 on the body weight, food and water consumption, and body water content of female NMRI mice

Administration of the stable PgE_2 analogue, 16,16-dimethyl PgE_2, at a dose of 0.5mg/Kg i.p. three times daily, resulted in a decrease in body weight within the first 24h after administration of a similar magnitude to that observed

Figure 4.24

The effect of a single i.v. injection of TNF on PgE_2 production by spleen cells

Spleens were rapidly removed from control and TNF-treated (7.5×10^7 U/Kg) male NMRI mice and were utilized for the determination of PgE_2 production by means of a radioimmunoassay. The values represent the means ± S.E.M. of 5 to 6 animals. *$p \leq 0.001$ from saline-injected controls by Student's t-test.

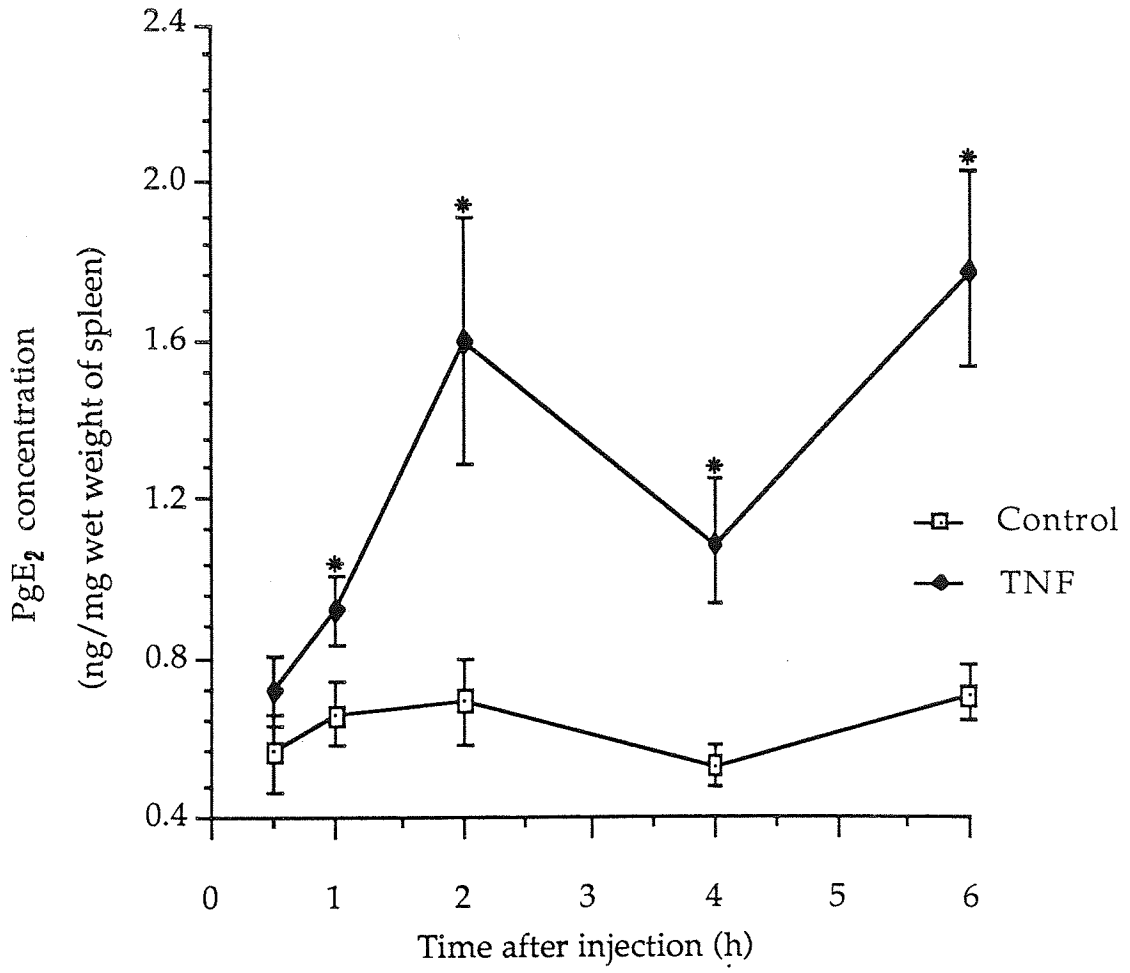


Figure 4.25

The effect of TNF and 16,16-dimethyl PgE_2 administration on the body weight and food and water consumption of female NMRI mice

Female NMRI mice (19±1g) were administered either a single i.v injection of TNF (7.5 X 10^7U/Kg) or 16,16-dimethyl PgE_2 (0.5mg/Kg) injected i.p. at three 6 hourly intervals. 24h after the first injection mice were weighed and the food and water consumption was determined. The values represent the means ± S.E.M. of 9 animals in each group. *p≤0.001 from saline-injected controls by analysis of variance.

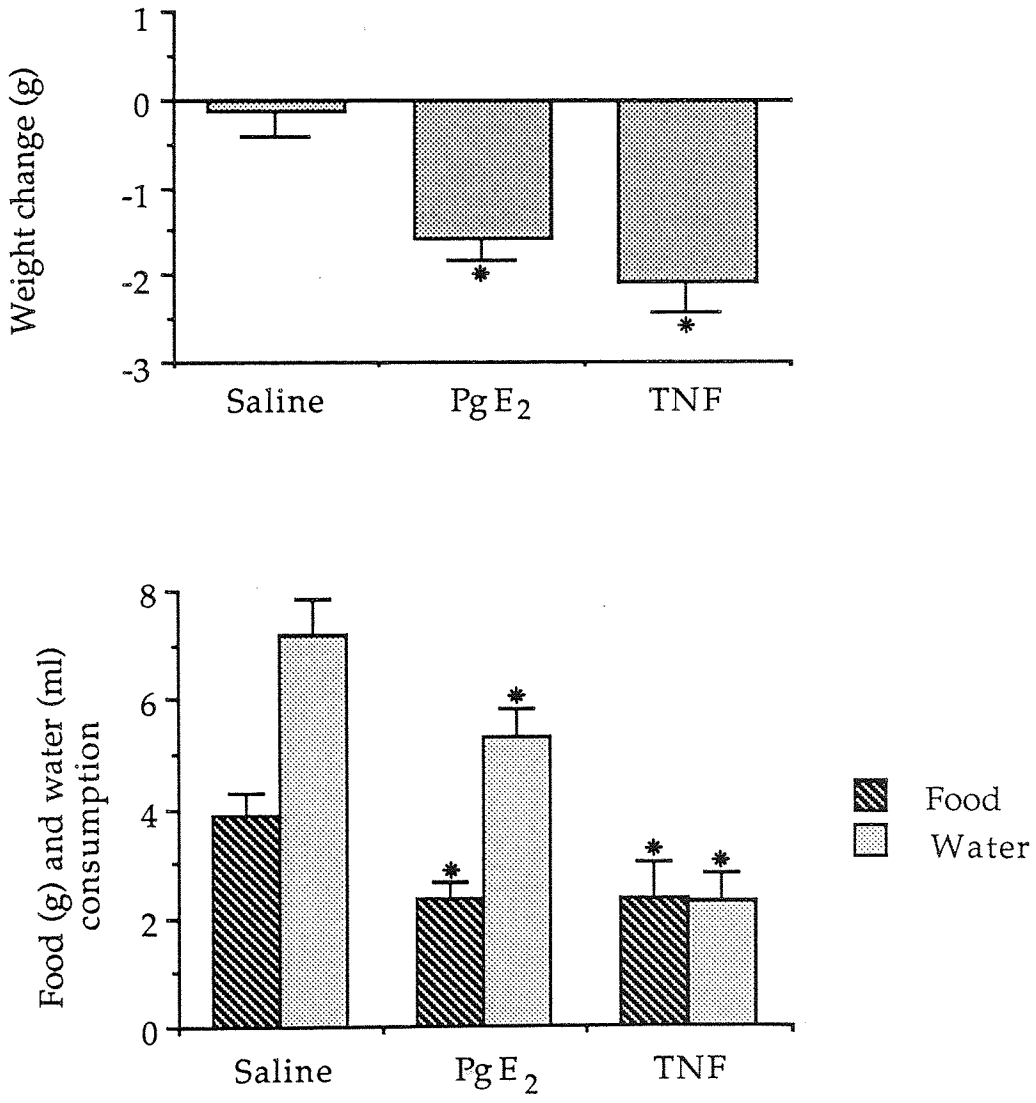
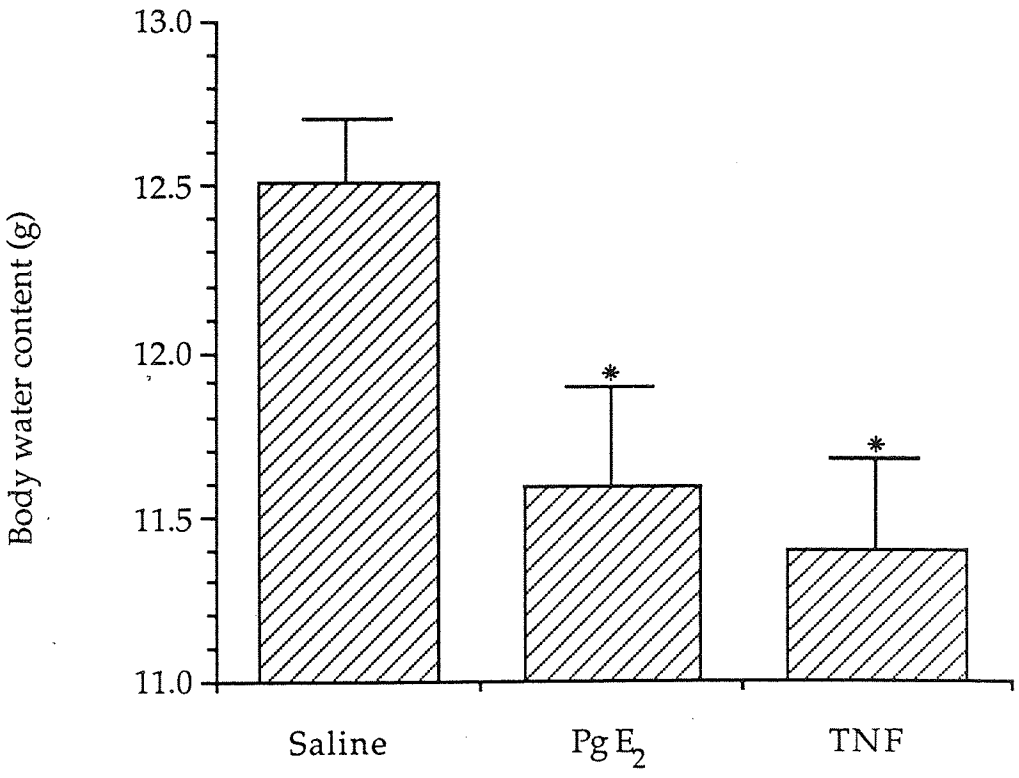


Figure 4.26

The effect of TNF and 16,16-dimethyl PgE_2 administration on the body water content of female NMRI mice

Female NMRI mice (19±1g) were administered either a single i.v. injection of TNF (7.5 X 10^7U/Kg) or 16,16-dimethyl PgE_2 (0.5mg/Kg) injected i.p. at three 6 hourly intervals. 24h after the first injection mice were killed and the body water content was determined. The values represent the means ± S.E.M. of 9 animals in each group. *p≤0.001 from saline-injected controls by analysis of variance.



following a single i.v. injection of 7.5 X 10^7U/Kg TNF (figure 4.25). No effect was seen after a dose of 1.25mg/Kg PgE_2 administered as a single i.v. injection (results not shown). Both the TNF-induced and the PgE_2-induced weight losses were accompanied by a decrease in food and water consumption (figure 4.25) and by a similar decrease in total body water content (figure 4.26).

These results suggest that the effect of TNF on body weight may be mediated via a prostaglandin intermediate, and that it may be possible to reverse the effects with inhibitors of prostaglandin synthesis, such as indomethacin.

4.9.3 The effect of TNF and indomethacin, either alone or in combination, on the body weight change, food and water consumption, and body water content of female NMRI mice

Administration of indomethacin (10mg/Kg, i.p.) 2h prior to a single i.v. injection of 7.5 X 10^7U/Kg TNF caused a significant reduction (63%) in the TNF-induced weight loss (figure 4.27). The time of administration of indomethacin appeared to be critical since no weight reversal was observed 0.5 or 1.5h prior to the TNF. When compared with indomethacin-treated controls (B), the decrease in water intake in the 2h indomethacin/TNF-treated mice (F) (32%) was not as great as in the TNF-treated mice (C) compared with saline-injected controls (A) (52%), although the food intake was reduced to about the same extent in both cases (44% and 48% respectively) compared with the respective controls (figure 4.28). Also there was a significant increase in food consumption of the TNF/indomethacin (2h)-treated mice (F) when compared with TNF treatment alone (C), and body composition analysis showed an increase in the total body water content of the TNF/indomethacin (2h) group when compared with TNF alone (figure 4.29).

4.9.4 The effect of TNF, indomethacin and 16,16-dimethyl PgE_2 on the excretion of urine and faeces by female NMRI mice (table 4.11)

Animals treated with TNF showed a significant reduction in the excretion of both urine (70%) and faeces (72%) when compared with saline-injected

Figure 4.27

The effect of TNF and indomethacin, alone and in combination, on the body weight of female NMRI mice

Female NMRI mice (19-22g) were administered 0.9% NaCl i.v., TNF (7.5 X 10^7U/Kg, i.v.) and indomethacin (10mg/Kg, i.p.), either alone or in combination as described below, and the change in weight over a 24h period was determined. The values represent the means ± S.E.M. of 5 to 16 animals in each group. *p≤0.005 and **p≤0.001 from saline-injected controls, (A), ***p≤0.001 from TNF alone, (C), and ****p≤0.001 from TNF/indomethacin (2h), (F), by analysis of variance.

A = control (i.v. saline); B = i.v. saline + 10mg/Kg indomethacin; C = TNF (7.5 X 10^7 U/Kg i.v.); D = TNF + indomethacin 30min before TNF; E = TNF + indomethacin 1.5h before TNF; F = TNF + indomethacin 2h before TNF.

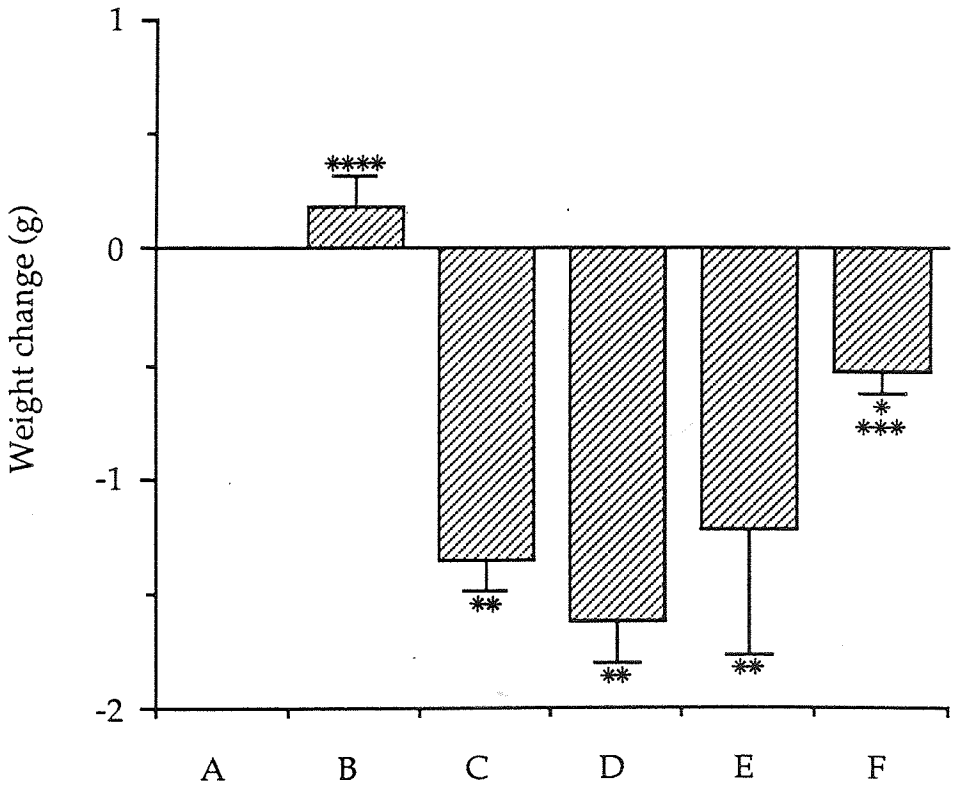


Figure 4.28

The effect of TNF and indomethacin, alone and in combination, on the food and water consumption of female NMRI mice

Female NMRI mice (19-22g) were administered 0.9% NaCl i.v., TNF (7.5 X 10^7U/Kg, i.v.) and indomethacin (10mg/Kg, i.p.), either alone or in combination as described below, and the food and water consumption over a 24h period was determined. The values represent the means ± S.E.M. of 5 to 16 animals in each group. *p≤0.001 from saline-injected controls, (A), **p≤0.05 from TNF alone, (C), and ***p≤0.001 from indomethacin, (B), by analysis of variance.

A = control (i.v. saline); B = i.v. saline + 10mg/Kg indomethacin; C = TNF (7.5 X 10^7 U/Kg i.v.); D = TNF + indomethacin 30min before TNF; E = TNF + indomethacin 1.5h before TNF; F = TNF + indomethacin 2h before TNF.

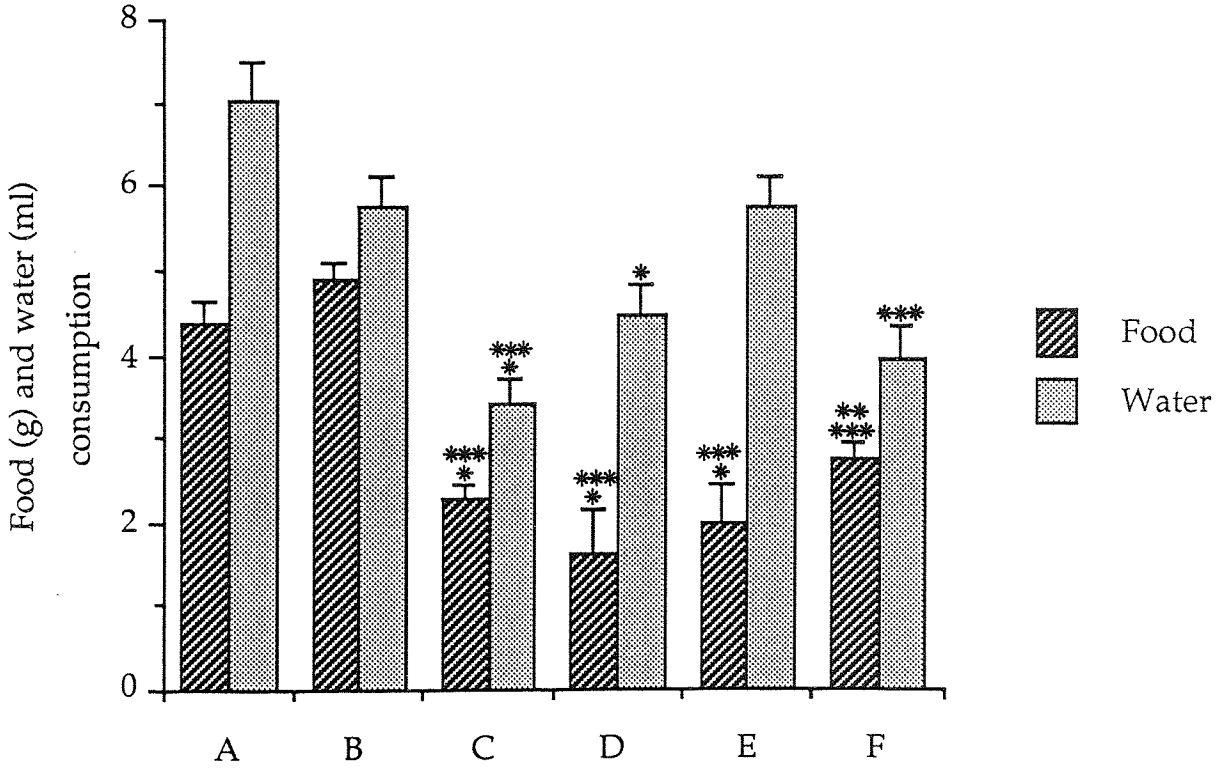


Figure 4.29

The effect of indomethacin administration 2h prior to TNF injection on the body water content of female NMRI mice

Female NMRI mice (19-22g) were administered 0.9% NaCl i.v. (saline), 7.5 X 10^7U/Kg TNF i.v. (TNF), 10mg/Kg indomethacin i.p. 2h prior to 0.9% NaCl i.v. (indo), or 10mg/Kg indomethacin i.p. 2h prior to 7.5 X 10^7U/Kg TNF i.v. (TNF/indo). Mice were killed 24h later and the body water content was determined. The values represent the means ± S.E.M. of 7 animals in each group. *p≤0.001 from TNF-treated animals and **p≤0.001 from saline-injected controls by analysis of variance.

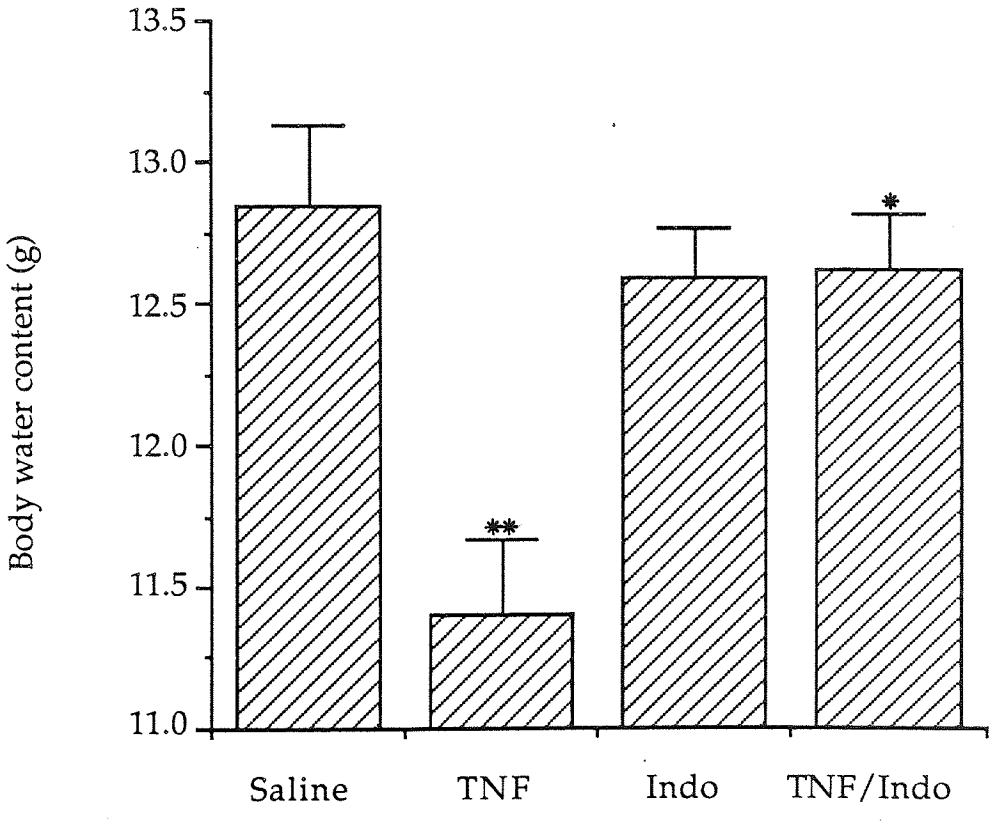


Table 4.11

The effect of TNF, indomethacin and 16,16-dimethyl PgE_2 on the excretion of urine and faeces by female NMRI mice

Mice were administered 0.9% NaCl i.v., TNF (7.5 X 10^7U/Kg, i.v.), indomethacin (10mg/Kg, i.p.) and 16,16-dimethyl PgE_2 (0.5mg/Kg, i.p. 3 x daily), either alone or in combination, as described below. Mice were then placed in metabolic cages and the amount of urine and faeces excreted over a 24h period was determined. Results are expressed as the means ± S.E.M. of 6 to 7 animals in each group. $^a p \leq 0.05$, $^b p \leq 0.005$ and $^c p \leq 0.001$ from saline controls, $^d p \leq 0.001$ from indomethacin controls, and $^e p \leq 0.005$ from TNF alone by analysis of variance.

Treatment	Urine volume (ml)	Wet faeces weight (g)	Dry faeces weight (g)	Total fluid excretion (ml)
Controls - i.v. saline	1.28±0.3	2.13±0.9	1.15±0.4	2.05±0.34
10mg/Kg indomethacin 2h before i.v. saline	0.97±0.2	2.24±0.3	1.18±0.2	2.00±0.30
TNF (7.5 X 10^7U/Kg)	0.44±0.2^b	0.50±0.08^a	0.32±0.06^b	0.62±0.16^c
TNF (7.5 X 10^7U/Kg) + 10mg/Kg indomethacin 2h before TNF	0.66±0.2	0.80±0.13^{d,e}	0.51±0.08^{d,e}	0.95±0.26^d
Dimethyl PgE_2, (0.5mg/Kg) 3 X daily	0.75±0.2	0.97±0.2^a	0.34±0.11^b	1.38±0.12^a

controls, indicating that the decrease in body water content did not arise from a diuretic effect of TNF. 16,16-dimethyl PgE_2 also caused a reduction in urine (33%) and faeces (70%) production. Indomethacin had no effect on urine or faeces production in control animals, although there was a small increase in faeces production (37%) in the TNF/indomethacin-treated animals as compared to TNF alone.

4.9.5 The effect of indomethacin on the TNF-induced production of PgE_2

The effect of indomethacin on PgE_2 production by spleen cells from TNF-treated animals is shown in figure 4.30. All values were measured 2h after TNF administration since PgE_2 production in response to TNF was significantly elevated at this point (figure 4.24). Indomethacin inhibited PgE_2 production in response to TNF irrespective of the time of administration with respect to TNF (D, E and F), although the reversal of body weight loss was highly dependent on the time of administration (figure 4.27). This suggests that indomethacin did not reverse the TNF-induced weight loss as a result of inhibition of prostaglandin synthesis, and that prostaglandin production was not necessary for weight loss to occur.

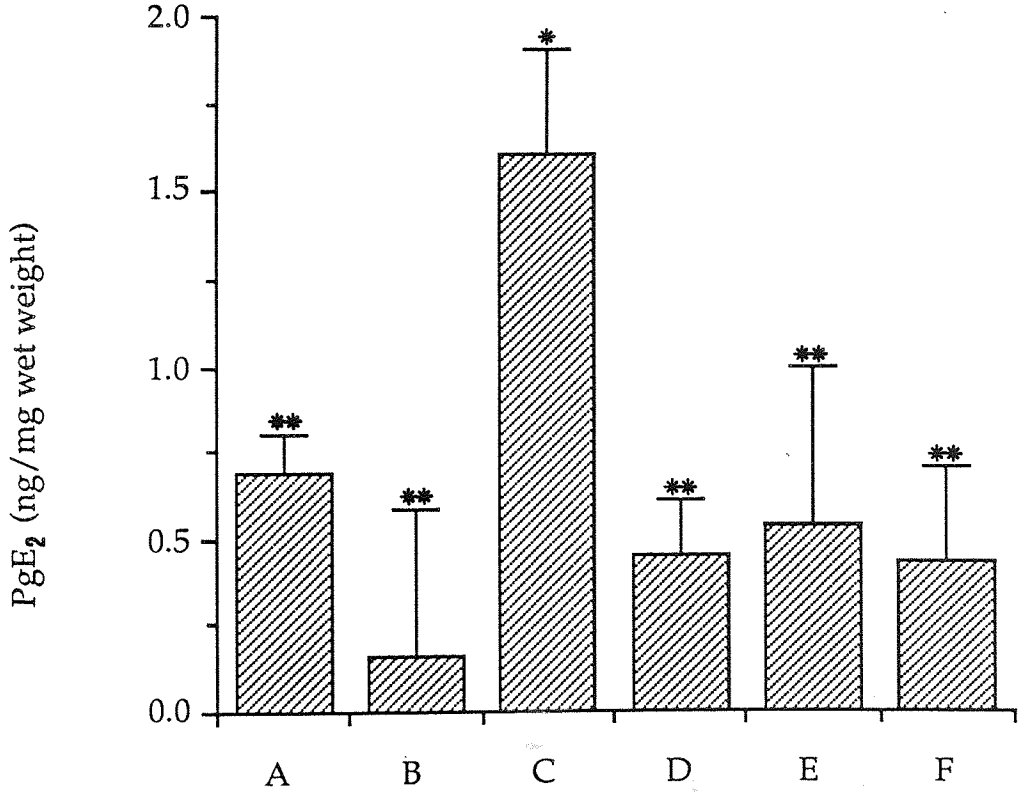
This conclusion was substantiated by measurement of PgE_2 production by spleen cells when TNF was administered chronically. As previously reported (Mahony and Tisdale, 1988, figure 4.12, table 4.7) animals became resistant to subsequent injections of TNF after the first 24h, with the body weight increasing towards that of controls. At 24h PgE_2 production by spleen cells was 0.51±0.04ng/mg wet weight in controls and 1.98±0.16 ng/mg wet weight in TNF-treated animals, ie 3.9 times the control value. This difference was maintained up to 5 days of TNF administration (3.7 times the control value) despite the fact that the animals were gaining weight. This suggests that PgE_2 production was not involved in the weight loss produced by TNF.

Figure 4.30

The effect of TNF and indomethacin, alone and in combination, on PgE_2 production by spleen cells

Animals were administered 0.9% NaCl i.v., TNF (7.5 X 10^7U/Kg, i.v.) and indomethacin (10mg/Kg, i.p.), either alone or in combination, as described below. 2h later mice were killed and the spleens were rapidly removed and used for the determination of PgE_2. The values represent the means ± S.E.M. of 5 to 16 animals in each group. *p≤0.001 from saline-injected controls, (A), and **p≤0.001 from TNF alone, (C), by analysis of variance.

A = control (i.v. saline); B = i.v. saline + 10mg/Kg indomethacin; C = TNF (7.5 X 10^7U/Kg i.v.); D = TNF + indomethacin 30min before TNF; E = TNF + indomethacin 1.5h before TNF; F = TNF + indomethacin 2h before TNF.



4.10 The effect of TNF on glucose metabolism in female NMRI mice

Acute administration of TNF to female NMRI mice has previously been shown to cause a dose-dependent decrease in body weight, accompanied by a decrease in the consumption of food and water over a 24h period (section 4.1.1). TNF administration also resulted in a marked hypoglycaemia 90min after the second of two injections over a 24h period, which was directly proportional to both the decrease in body weight of animals and to the decrease in food and water consumption (sections 4.1.2 and 4.6.2). The TNF-induced weight loss was reversed by force-feeding female NMRI mice with a concentrated glucose solution (section 4.8). However, it appears that this effect was due to replacement of body water following TNF-induced dehydration of animals. The direct effect of hypoglycaemia on the observed weight loss has not yet been investigated.

The aim of this study was to determine the cause of the TNF-induced hypoglycaemia in female NMRI mice in order to discover the role of the decrease in blood glucose, if any, in the weight loss induced by TNF. In order to achieve this objective, the levels of various metabolites of glucose were measured following a single i.v. injection of TNF, to determine which of the biochemical pathways are favoured for the metabolism of glucose following TNF administration. A concentration of TNF of 7.5 X 10^7U/Kg was employed as this produced a weight loss over a 24h period of about 10% (figure 4.1), and a decrease in blood glucose concentration of about 65% (table 4.1) 90min after the second of two injections over a 24h period, when compared with saline-injected controls.

4.10.1 The effect of TNF on blood glucose, liver glycogen and rectal body temperature of female NMRI mice (figures 4.31 and 4.32)

TNF-injected mice were compared with saline-injected pair-fed controls, to eliminate any effects of the injection and hypophagia on the levels of blood glucose. A single i.v. injection of TNF caused an initial hyperglycaemia, within 0.5h after injection, followed by a marked hypoglycaemia. This decrease in blood glucose concentration (about 32%) was evident by 2h after

the TNF injection and was maintained for the 24h period of study.

The hypoglycaemia observed following TNF administration was accompanied by a marked reduction in liver glycogen (about 80%), which was evident by 2h following injection and was maintained for 4h, but thereafter there was an increase in glycogen levels such that by 8h the level was not significantly different from pair-fed controls.

Administration of a single i.v. injection of TNF also caused a marked dose-dependent hypothermia which was evident 0.5h after the injection and persisted for up to 4h.

4.10.2 The effect of TNF on the blood levels of L-(+)-lactate, L-alanine and pyruvate of female NMRI mice (table 4.12)

The blood levels of L-(+)-lactacte, L-alanine and pyruvate did not change after TNF administration over an 8h period, when compared with saline-injected pair-fed controls. Thus, it would appear that the glucose was not being metabolised anaerobically to form L-(+)-lactate or undergoing transamination to form L-alanine. The glucose must therefore have been metabolised via acetyl Co A. However, by 24h after TNF administration, lactate levels were significantly higher than in the saline-injected pair-fed controls, indicating that, at this later time-point, some anaerobic metabolism was occuring.

Figure 4.31

The effect of a single injection of TNF on the blood glucose and liver glycogen concentration of female NMRI mice

Mice were injected i.v. with 7.5 X 10^7U/Kg TNF. Controls were pair-fed and injected i.v. with 0.9% NaCl. At various time points after injection blood was removed by cardiac puncture (glucose) or livers were dissected out (glycogen) and the levels of blood glucose and liver glycogen were determined. Each point represents the mean ± S.E.M. of 5 to 10 animals. *p≤0.001 from controls by Student's t-test.

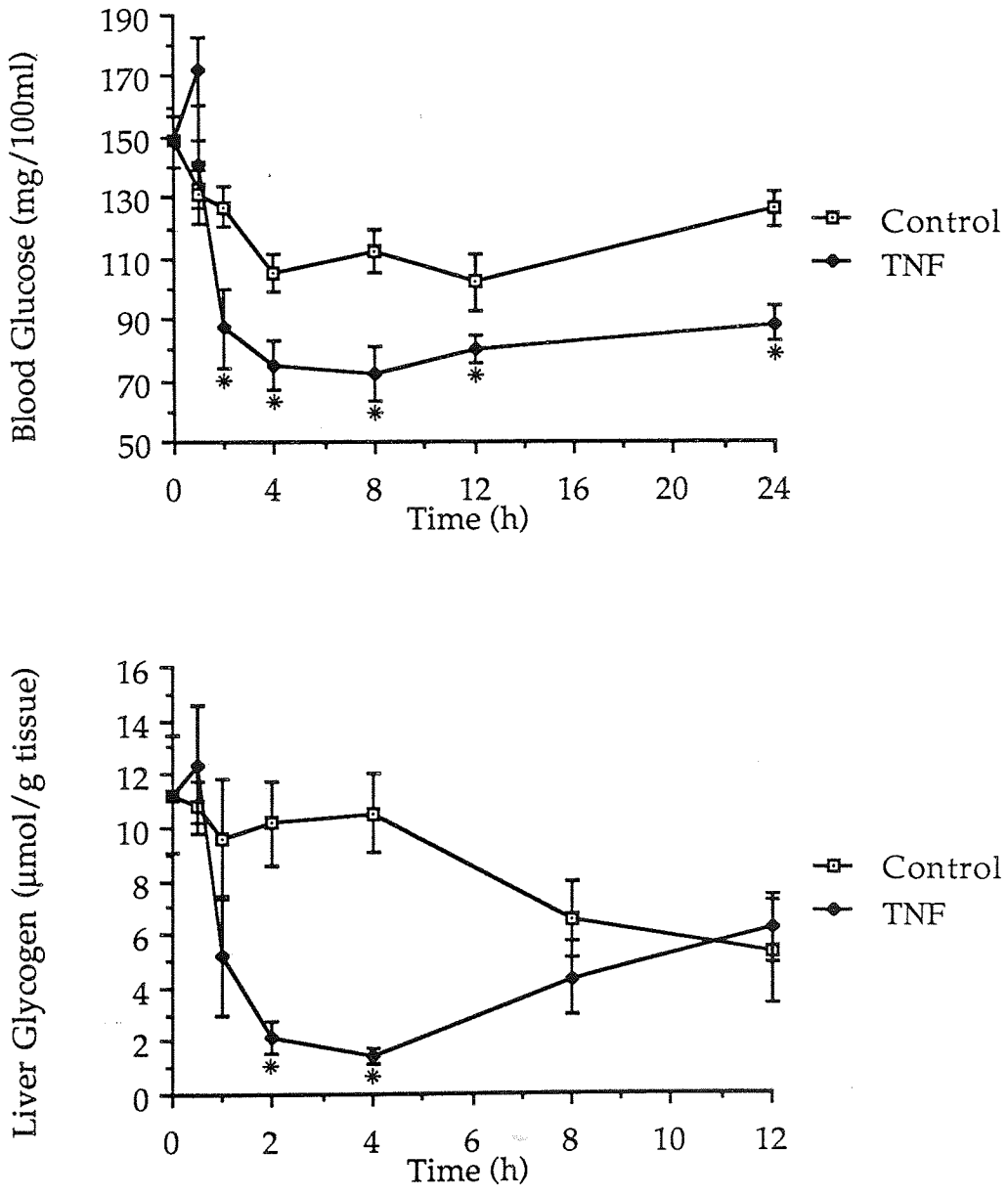


Figure 4.32

The effect of a single injection of TNF on the rectal body temperature of female NMRI mice

Mice were injected i.v. with 3.0 X 10^7U/Kg or 7.5 X 10^7U/Kg TNF, or with 0.9% NaCl (controls). At various time points after injection the rectal body temperature of mice was measured using a thermocouple. Each point represents the mean ± S.E.M. of 5 to 10 animals. *p≤0.001 from controls by Student's t-test.

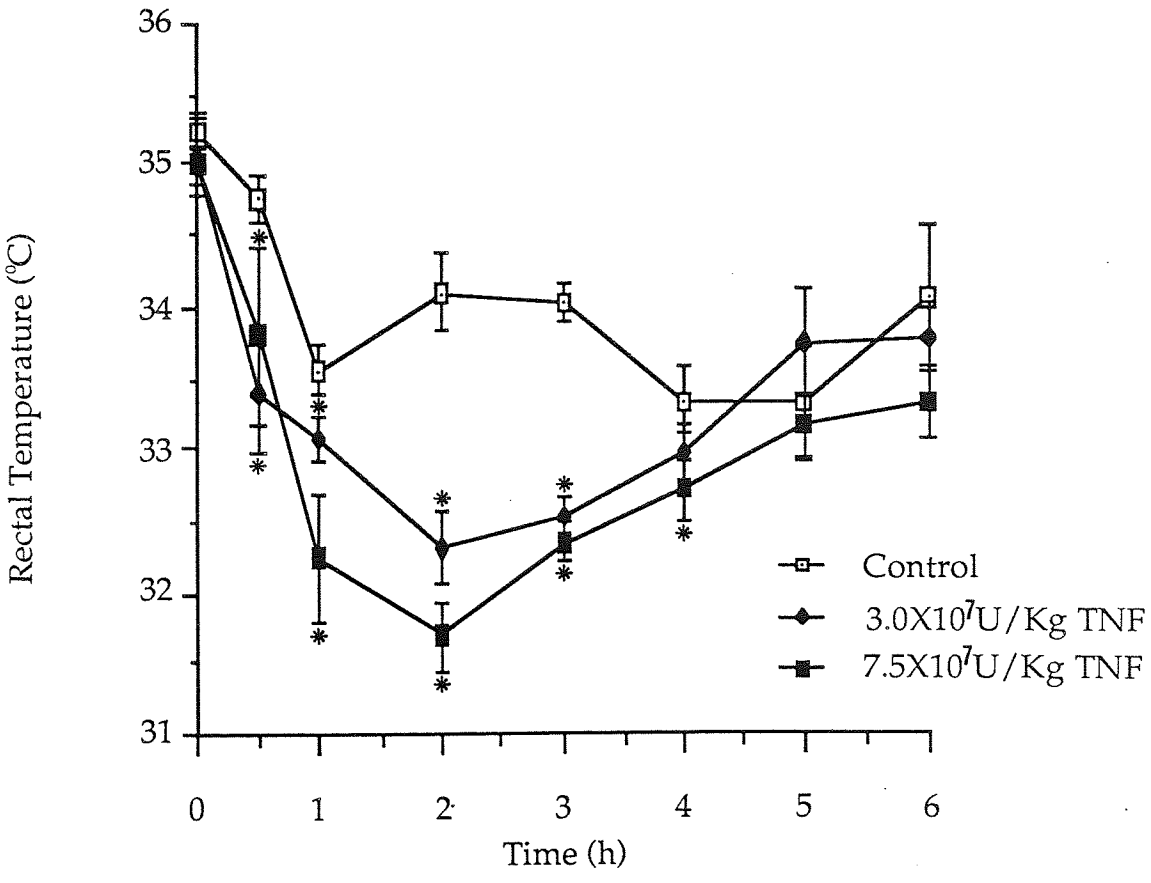


Table 4.12

The effect of a single injection of TNF on the blood levels of L-(+)-lactate, L-alanine and pyruvate of female NMRI mice

Mice were injected i.v. with 7.5 X 10^7U/Kg TNF. Controls were pair-fed and injected i.v. with 0.9% NaCl. At various time points after injection blood was removed by cardiac puncture and the levels of L-(+)-lactate, L-alanine and pyruvate were determined. Values represent the means ± S.E.M. of 5 to 7 animals in each group. [a]$p \leq 0.05$ from controls by Student's t-test.

Treatment	Time (h)	L(+)-Lactate (mM)	L-Alanine (mM)	Pyruvate (mM)
Controls	0	2.0±0.3	0.33±0.04	
	0.5	1.9±0.1	0.37±0.06	
	1.0	2.0±0.4	0.35±0.06	
	2.0	1.7±0.3	0.33±0.10	0.011±0.002
	4.0	1.8±0.1	----	
	8.0	2.4±0.4	0.29±0.06	
	24.0	1.9±0.4	0.33±0.10	
TNF	0	2.0±0.3	0.33±0.04	
	0.5	1.8±0.2	0.34±0.07	
	1.0	1.6±0.2	0.40±0.05	
	2.0	1.9±0.7	0.43±0.04	0.011±0.003
	4.0	1.7±0.3	0.36±0.06	
	8.0	2.4±0.3	0.29±0.06	
	24.0	2.6±0.1[a]	0.34±0.06	

4.10.3 The effect of TNF on the production of $^{14}CO_2$ from D-[U-^{14}C]-glucose and [U-^{14}C]-palmitate (figure 4.33)

If, due to a TNF-induced increase in metabolic activity, glucose were being oxidised via the tricarboxylic acid (TCA) cycle, an increased production of CO_2 would be expected in TNF-injected animals. However, when D-[U-^{14}C]-glucose was injected into female NMRI mice and the exhaled CO_2 was trapped in a mixture of ethanolamine : ethoxyethanol (1 : 4) and the radioactivity counted (section 3.12), it was found that the rate of production of $^{14}CO_2$ from the [U-^{14}C]-glucose in the TNF-injected animals was significantly lower than in saline-injected pair-fed controls. In contrast, production of $^{14}CO_2$ from [U-^{14}C]-palmitic acid in TNF-treated animals did not differ from controls over a 24h period. This suggests that TNF did not inhibit the enzymes of the TCA cycle, since fats could be adequately metabolised via this pathway, but that the glucose was possibly being shunted down the other biochemical pathways to form ketone bodies and/or fatty acids.

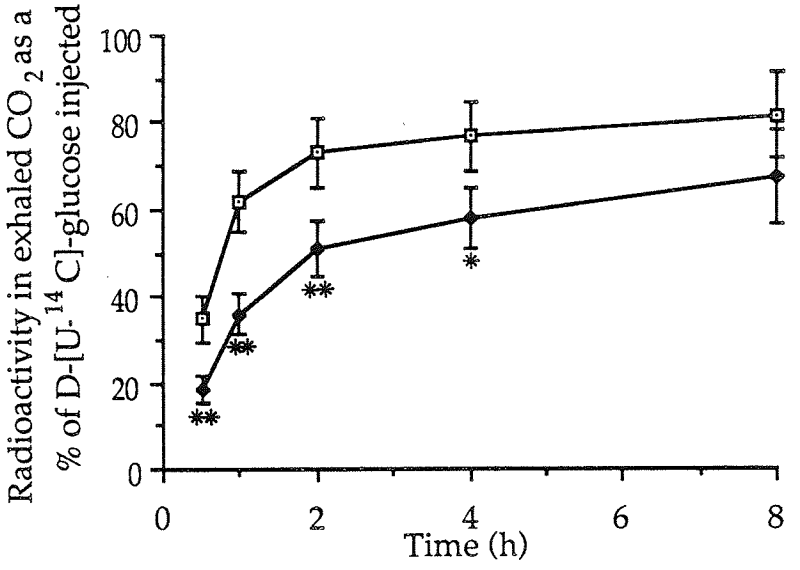
4.10.4 The effect of TNF on the resting oxygen consumption (VO_2) of female NMRI mice (figure 4.34)

The resting oxygen consumption (VO_2) of female NMRI mice, as determined by indirect closed circuit calorimetry (section 3.13), decreased significantly (about 30%) 2h after a single i.v. injection of 7.5 X 10^7U/Kg TNF. However, by 24h after treatment, the value was not significantly different from saline-injected controls. Thus, TNF administration resulted in a short-term decrease in the metabolic rate of female NMRI mice. This result is in agreement with the findings that TNF caused hypothermia and a decreased $^{14}CO_2$ production from ^{14}C glucose.

Figure 4.33

The effect of TNF on the production of $^{14}CO_2$ from D-[U-^{14}C]-glucose and [U-^{14}C]-palmitate

Both TNF-injected (7.5 X 10^7U/Kg) female NMRI mice and 0.9% NaCl-injected pair-fed controls were administered 50μCi/Kg of either D-[U-^{14}C]-glucose or [U-^{14}C]-palmitate and the production of $^{14}CO_2$ was determined. Each point represents the mean ± S.E.M. of 5 animals. *p≤0.05 and **p≤0.001 from controls by Student's t-test.



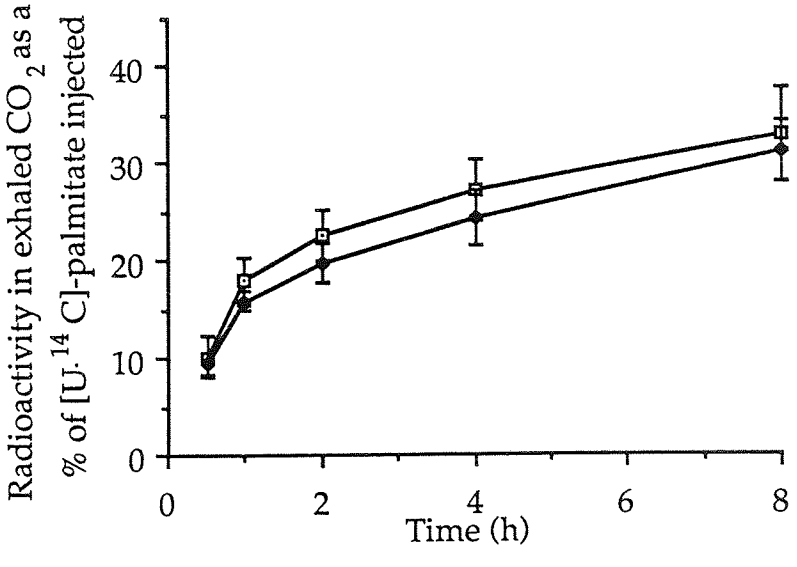
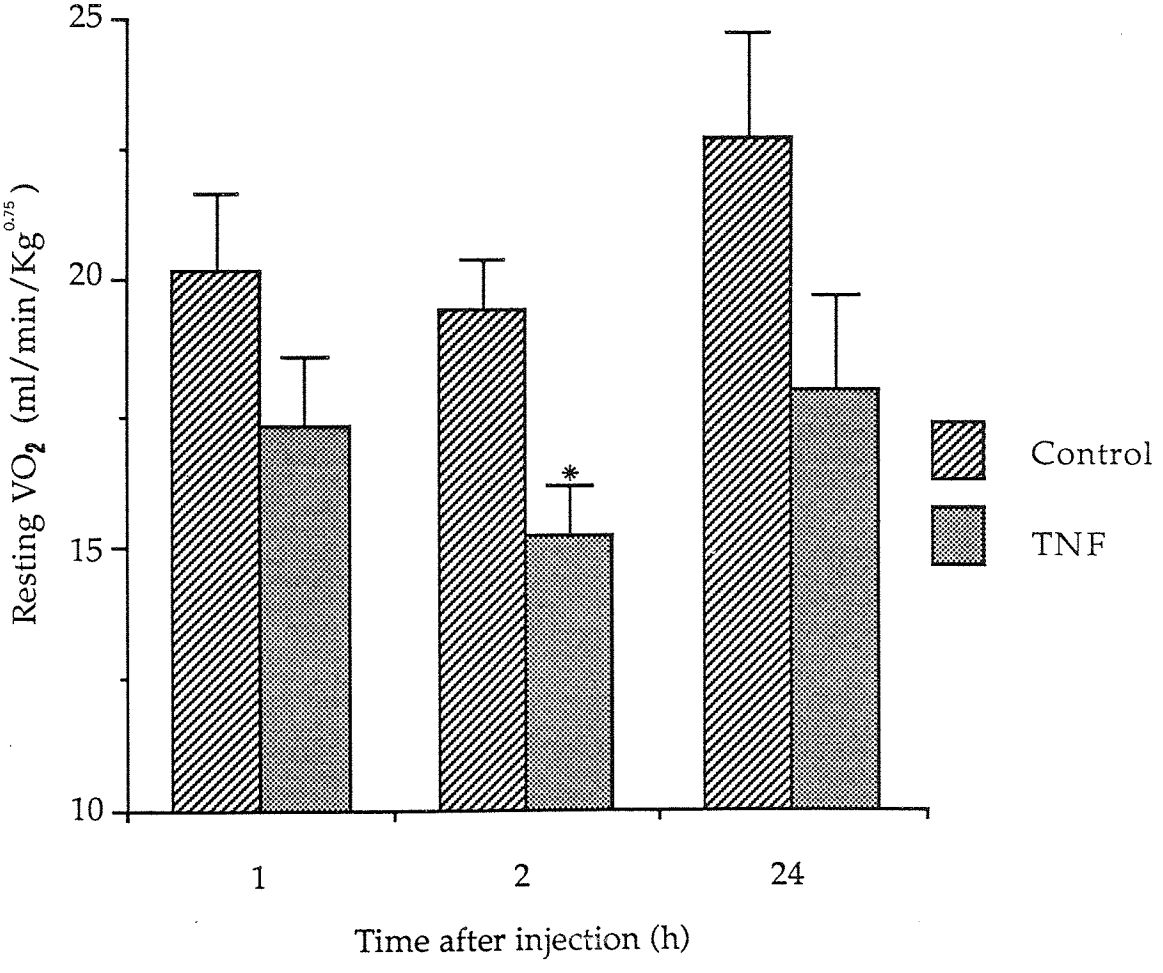


Figure 4.34

The effect of TNF on the resting oxygen consumption (VO_2) of female NMRI mice

The resting oxygen consumption (VO_2) of female NMRI mice 1, 2 and 24h after a single i.v. injection of TNF (7.5 X 10^7U/Kg) or 0.9% NaCl was determined by indirect closed circuit calorimetry. The values represent the means ± S.E.M. of 8 animals in each group. *p≤0.001 from controls by Student's t-test.



4.10.5 The effect of TNF on the glucose utilization of various tissues in female NMRI mice

In order to determine which tissues are the major utilizers of glucose in TNF-treated animals glucose utilization by different tissues was investigated *in vivo* by the 2-deoxyglucose tracer method (section 3.14).

Blood glucose levels in TNF-treated female NMRI mice, that had previously been starved overnight, were significantly lower than saline-injected pair-fed controls up to 45min after treatment (figure 4.35), although there was no difference in the rate of disappearance of the label from 2-deoxy-D-[2,6-^{3}H]-glucose ([^{3}H] 2DG) or of 2-[1-^{14}C]-deoxy-D-glucose ([^{14}C] 2DG) between the two groups.

To quantitate the discrimination against 2-deoxyglucose in pathways of glucose metabolism the lumped constant, i.e. the ratio of the rates of 2-deoxyglucose phosphorylation and glucose utilization (section 3.14.4), for liver and muscle were calculated (table 4.13). Both of these values were close to the values previously reported for other tissues (Ferre et al, 1985, Meszaros et al, 1987a, and Meszaros et al, 1987b) and so the average of the two, i.e. 0.46, was utilized to calculate the Rg values for all tissues.

The tissue glucose metabolic rates (Rg values), given as nmol glucose/g/min, of control and TNF-injected animals, as measured 2h after treatment, are shown in figure 4.36. The Rg values in colon, liver, kidney and spleen were increased by 520, 340, 36 and 25% respectively in TNF-injected animals, while the Rg values in thigh and gastocnemius muscles were decreased by 29 and 31% respectively. TNF had no effect on the glucose metabolic rate in brain, pancreas, diaphragm, lung or stomach.

Figure 4.35

Measurement of blood glucose concentration and the disappearance of [^{3}H] 2DG and [^{14}C] 2DG from the blood of TNF-injected and 0.9% NaCl-injected female NMRI mice

Mice were injected i.v. with 7.5 X 10^{7}U/Kg TNF or 0.9% NaCl. At various time points, up to 60min after injection, blood was removed by cardiac puncture and the level of blood glucose determined. 1h after treatment mice were injected with 50µCi/Kg of [^{3}H] 2DG and 35min later with 5µCi/Kg [^{14}C] 2DG. Serial blood samples were removed at specified time intervals and the radioactivity was counted. Each point represents the mean ± S.E.M. of 5 to 10 animals. *p≤0.01 and **p≤0.001 from controls by Student's t-test.

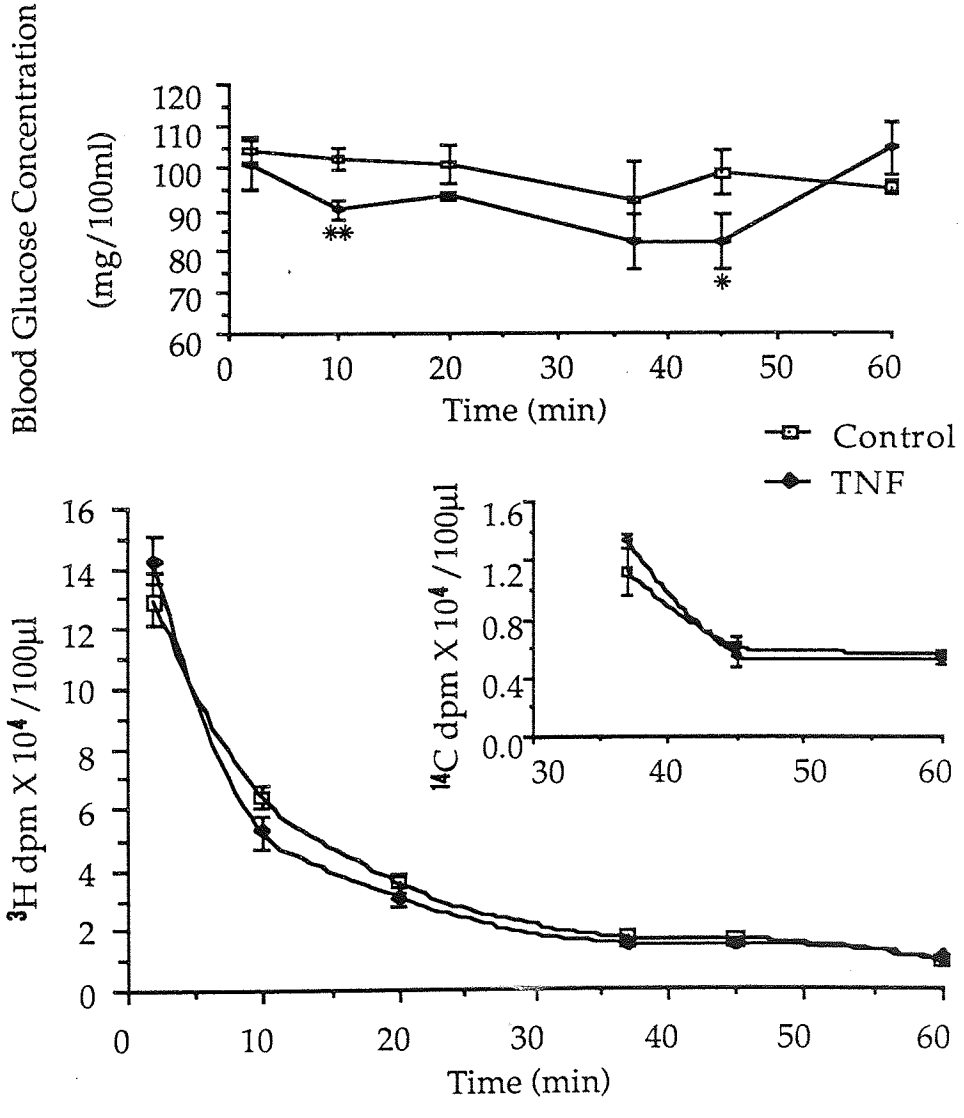


Table 4.13

Determination of the correction factor for the discrimination against 2-deoxyglucose (lumped constant) in the liver and muscle of female NMRI mice

Female NMRI mice were killed by cervical dislocation and the livers, thigh muscles and gastrocnemius muscles were carefully dissected out. They were then weighed and incubated for 1h at 37°C in buffer containing 5mM glucose, 1μCi D-[U-^{14}C]-glucose and 2μCi [^{3}H] 2DG, and the lumped constant was determined. The values represent the means ± S.E.M. of 5 or 6 determinations.

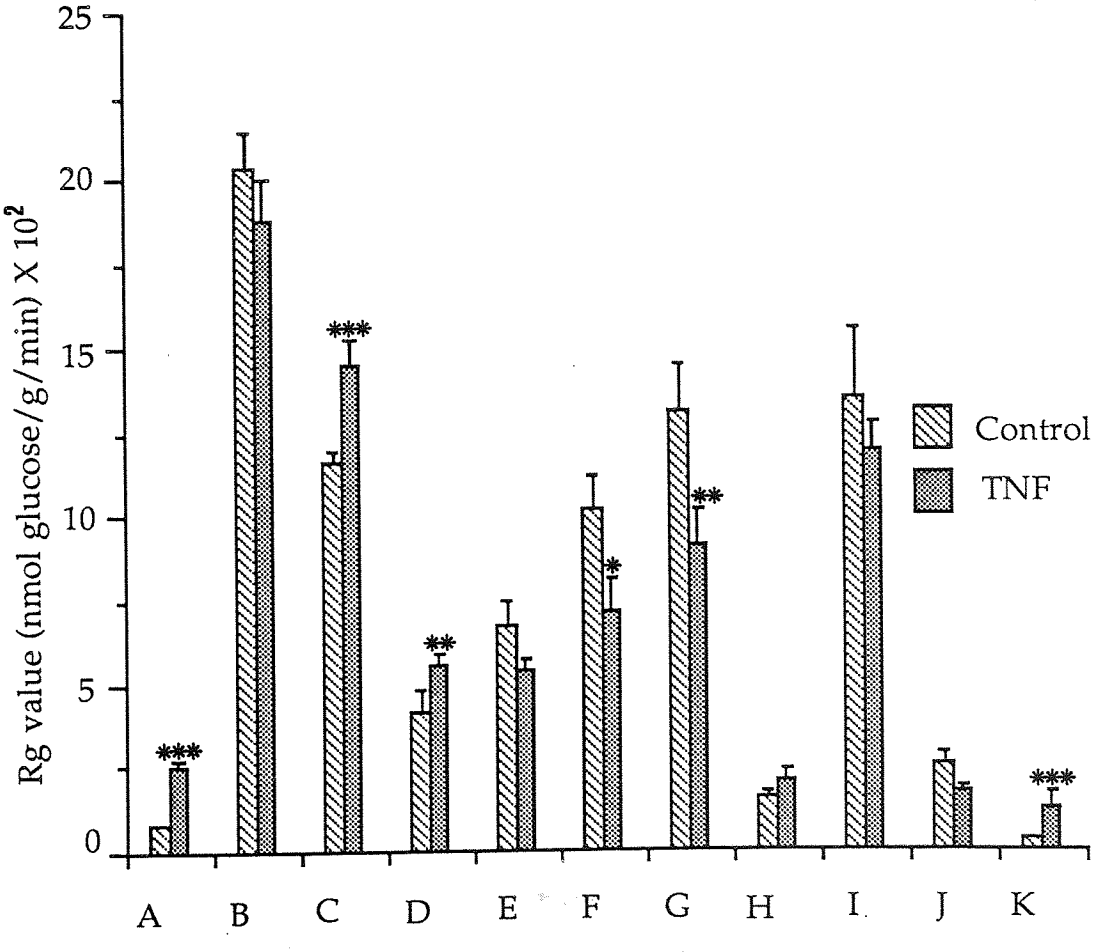
	Lumped constant for 2DG (LC)	Average
Liver	0.45±0.05	0.46
Muscle	0.47±0.03	

Figure 4.36

The effect of TNF on tissue glucose metabolic rate (Rg) in female NMRI mice

Glucose metabolic rates (Rg) of various tissues were determined 2h after a single i.v. injection of either 0.9% NaCl or 7.5 X 10^7U/Kg TNF. Rg values are expressed as nmol glucose/g/min and are given as the means ± S.E.M. of 6 animals in each group. *p≤0.01, **p≤0.005 and ***p≤0.001 from saline-injected controls by Student's t-test.

A = Liver, B = Brain, C = Spleen, D = Kidney, E = Pancreas, F = Thigh muscle, G = Gastrocnemius muscle, H = Diaphragm, I = Lung, J = Stomach, K = Colon.



When calculated on a whole organ basis (figure 4.37), the increase in mean glucose metabolic rate (nmol glucose/organ/min) of TNF-injected animals, as compared to saline-injected controls, in liver, kidney, spleen and colon were 111, 22, 17 and 7nmol/min respectively, while there was a decrease of 37, 24 and 22nmol/min in gastrocnemius muscle, thigh muscle and pancreas respectively. Since the magnitude of the contribution to glucose utilization by the various organs depends on both the increase in Rg value and the size of a particular organ, the contribution of the colon to the total body increase in glucose consumption was modest, although this organ showed the largest increase in Rg value after TNF administration, because of its low contribution to the total body mass. It can thus be seen that the major contributor to an increased utilization, which may have been responsible for the decrease in blood glucose levels after TNF administration, was the liver.

4.10.6 The effect of TNF on the retention of 2DGP by tissues in female NMRI mice

To investigate whether TNF administration altered the retention of 2DGP in the various tissues a sequential double labelling technique was applied, followed by analysis of the two labels in 2DGP (section 3.14.3). Since there was a marked initial decay of the precursor in the blood (figure 4.35), the bulk of the 2-[^{3}H]-DGP was synthesized in the tissues during the initial 35min of the labelling period, while 2-[^{14}C]-DGP was formed during the second 25min period, and the ^{3}H/^{14}C ratio of tissue 2DGP was measured at the end of the experiment. Loss of 2DGP from the tissue would affect the ^{3}H component of the ratio more than the ^{14}C component and, therefore, the ^{3}H/^{14}C ratio of 2DGP in the tissues was a measure of the retention of 2DGP, i.e., a low ratio indicated a high rate of loss. In these experiments the amount of ^{3}H radioactivity administered was 10-times higher than the ^{14}C and thus the ^{3}H/^{14}C ratio would be expected to be near 10. This was true for all control tissues except the brain, kidney and colon which were much lower (7.1±0.3, 5.9±0.5 and 6.6±2.1 respectively) and the diaphragm which was much higher (18.4±4.1) (figure 4.38). The lower ratio for brain has been previously reported (Meszaros et al, 1987a) and arose from an increased rate of loss of 2DGP from

Figure 4.37

The effect of TNF on glucose utilization rates of different organs in female NMRI mice.

Glucose utilization rates of different organs were determined 2h after a single i.v. injection of either 0.9% NaCl or 7.5 X 10^7U/Kg of TNF. The results are expressed as the means ± S.E.M. of 6 animals in each group and are based on organ weights and Rg values as described in section 3.13. *p≤0.05, **p≤0.01 and ***p≤0.001 from saline- injected controls by Student's t-test.

A = Liver, B = Brain, C = Spleen, D = Kidney, E = Pancreas, F = Thigh muscle. G = Gastrocnemius muscle, H = Diaphragm, I = Lung, J = Stomach, K = Colon.

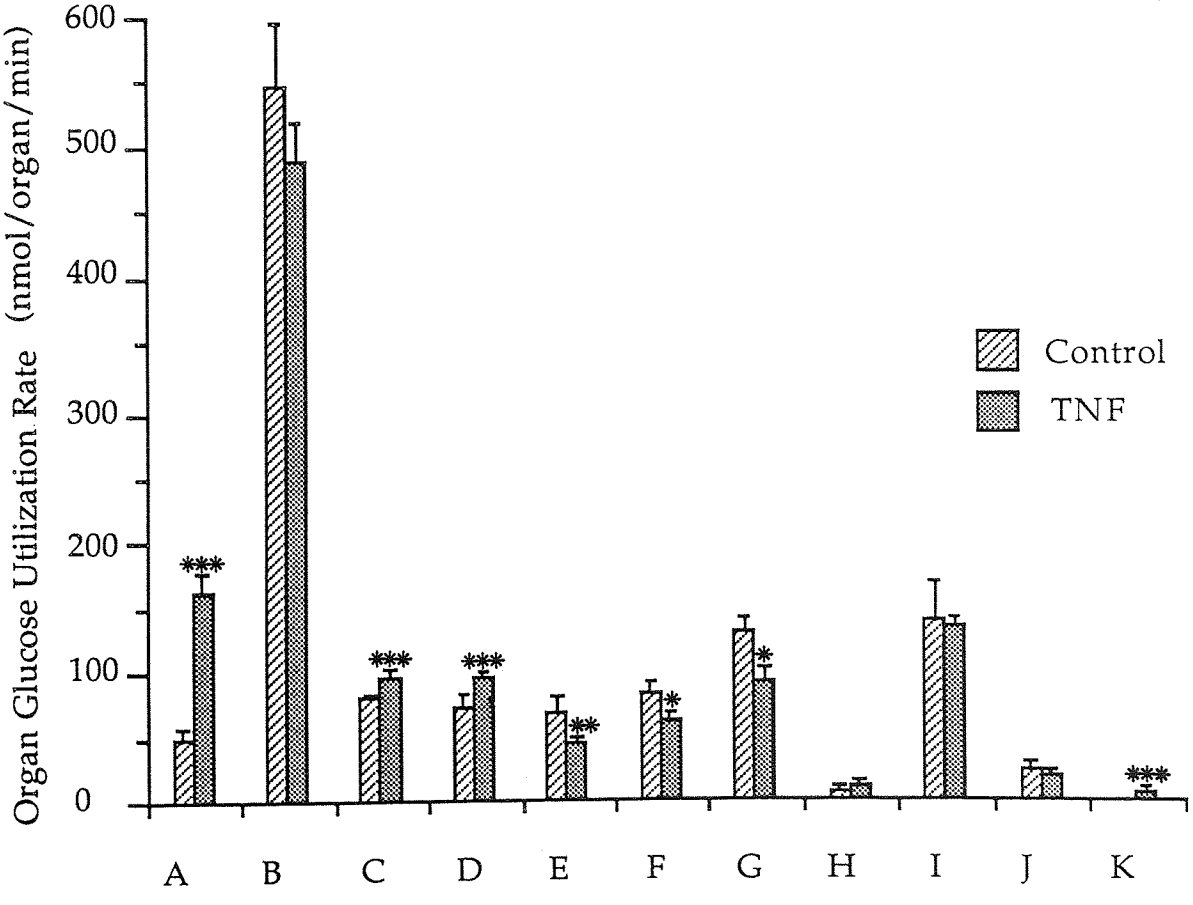
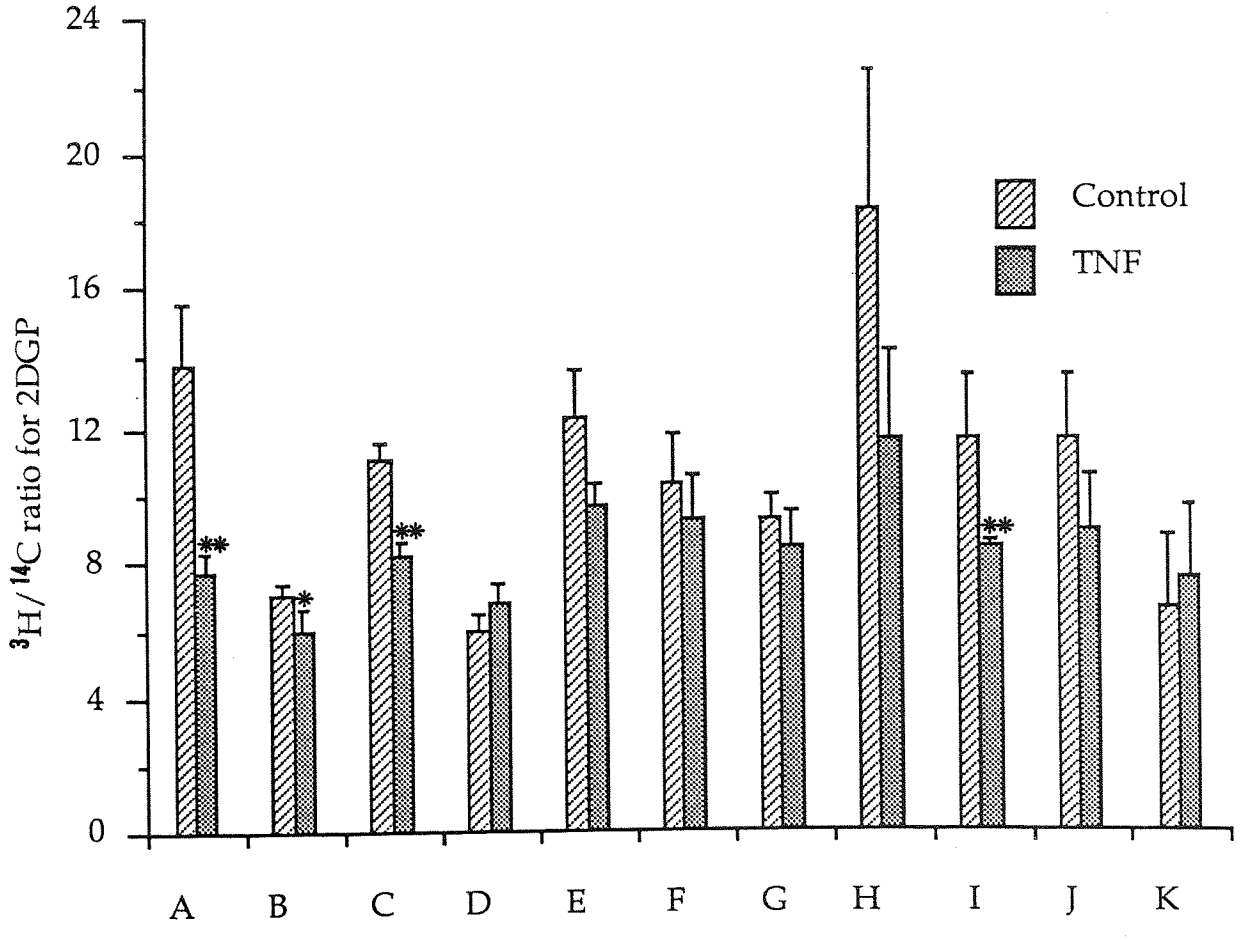


Figure 4.38

The effect of TNF on the ^{3}H / ^{14}C ratio of 2DGP in the tissues of female NMRI mice.

The labelling of 2DGP in tissues after sequential administration of 2-[^{3}H] DG and 2-[^{14}C] DG was determined 2h after a single injection of TNF (7.5 X 10^{7}U/Kg) or 0.9% NaCl and the $^{3}H/^{14}C$ ratio was calculated. The results represent the means ± S.E.M. of 5 to 10 animals in each group. *p≤0.05 and **p≤0001 from saline-injected controls by Student's t-test.

A = Liver, B = Brain, C = Spleen, D = Kidney, E = Pancreas, F = Thigh muscle, G = Gastrocnemius muscle, H = Diaphragm, I = Lungs, J = Stomach, K = Colon.



this tissue. The higher ratio for diaphragm indicates that the concentration of 2-[^{3}H]-DGP was still increasing over the second period. However, after TNF administration, the $^3H/^{14}C$ ratios decreased by 82, 38, 36 and 20% in liver, lungs, spleen and brain respectively, indicating an increased rate of loss of 2DGP from these tissues.

4.10.7 The effect of TNF on the levels of plasma FFA and triglyceride, and blood acetoacetate and 3-hydroxybutyrate of female NMRI mice. (figures 4.39 and 4.40)

The plasma levels of both FFA and triglycerides were increased markedly 2 to 8 h after a single injection of TNF (7.5 X 10^7U/Kg), while the blood levels of both acetoacetate and 3-hydroxybutyrate were increased by 2h and remained elevated for the 24h period of study. This suggests that the increased glucose consumption by the liver in TNF-treated animals may have been due to glucose utilization for the biosynthesis of lipids.

4.10.8 The effect of TNF on lipogenesis from glucose in female NMRI mice.

The results obtained so far suggest that, following TNF administration, glucose was being utilized by the liver for the biosynthesis of lipids. In order to test this hypothesis the effect of TNF on lipogenesis from ^{14}C glucose was investigated (section 3.15). Two hours after a single i.v. injection of TNF the conversion of ^{14}C glucose into lipids in the liver was increased by about 160% and was still evident 3h after TNF administration (figure 4.41). This increase in lipid synthesis in liver was accompanied by an increase of 185, 119 and 30% in fat, blood and spleen respectively 2h after TNF administration, and 554, 321, 94 and 64% in blood, fat, colon and spleen respectively 3h after TNF administration.

These results suggest that TNF administration to female NMRI mice produced severe hypoglycaemia in order to serve an increased lipogenesis, mainly in liver and adipose tissue.

Figure 4.39

The effect of a single injection of TNF on the plasma levels of FFA and triglyceride in female NMRI mice.

Mice were injected i.v. with 7.5 X 10^7U/Kg TNF. Controls were pair-fed and injected i.v. with 0.9% NaCl. At various time points after injection blood was removed by cardiac puncture and the plasma levels of FFA and triglyceride were determined. Each point represents the mean ± S.E.M. of 5 to 6 animals. *p≤0.05, **p≤0.01 and ***p≤0.001 from controls by Student's t-test.

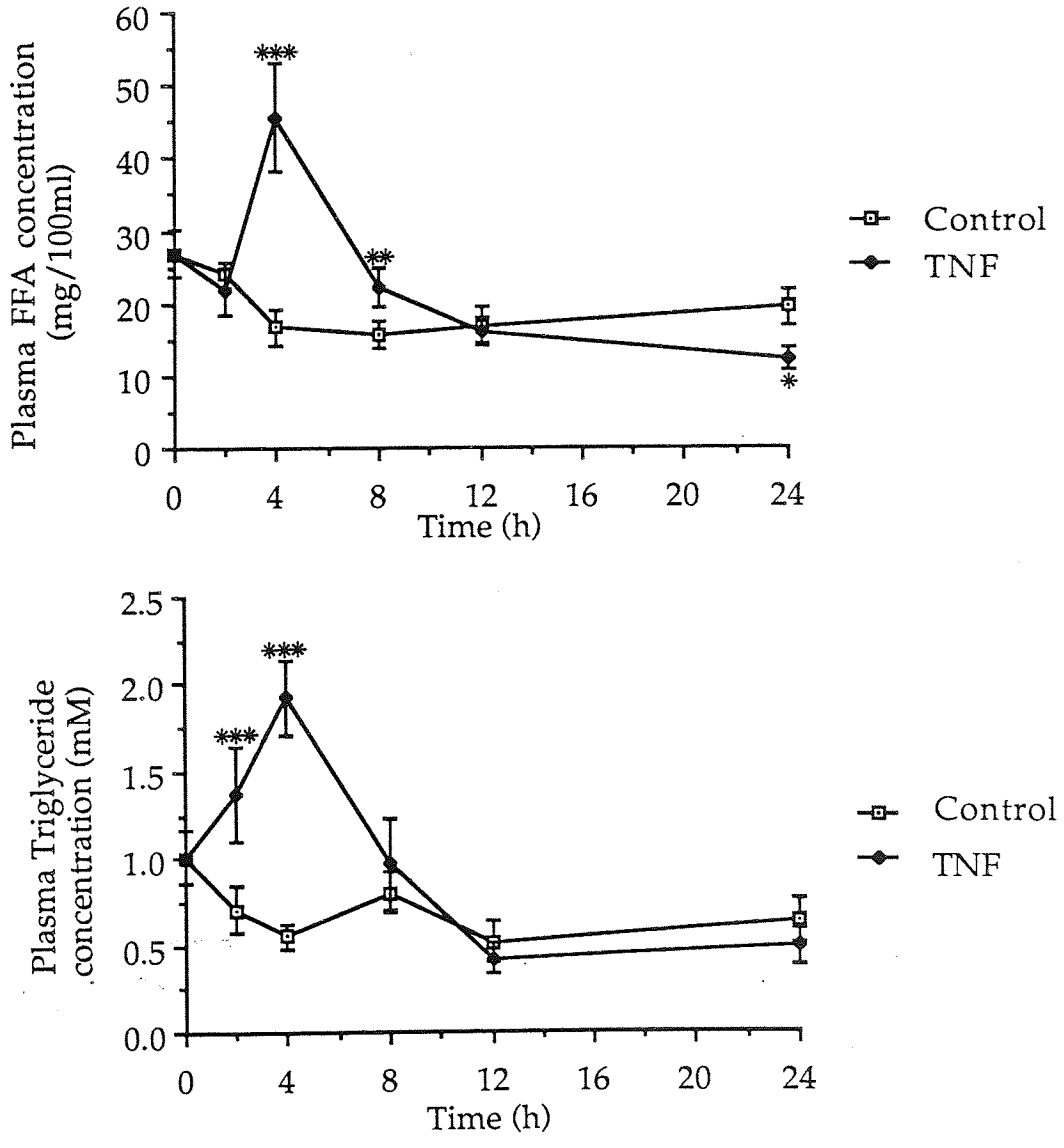


Figure 4.40

The effect of a single injection of TNF on blood acetoacetate and 3-hydroxybutyrate concentrations in female NMRI mice.

Mice were injected i.v. with 7.5 X 10^7U/Kg TNF. Controls were pair-fed and injected i.v. with 0.9% NaCl. At various time points after injection blood was removed by cardiac puncture and the blood levels of acetoacetate and 3-hydroxybutyrate were determined. Each point represents the mean ± S.E.M. of 5 to 6 animals. *p≤0.01 and **p≤0.001 from controls by Student's t-test.

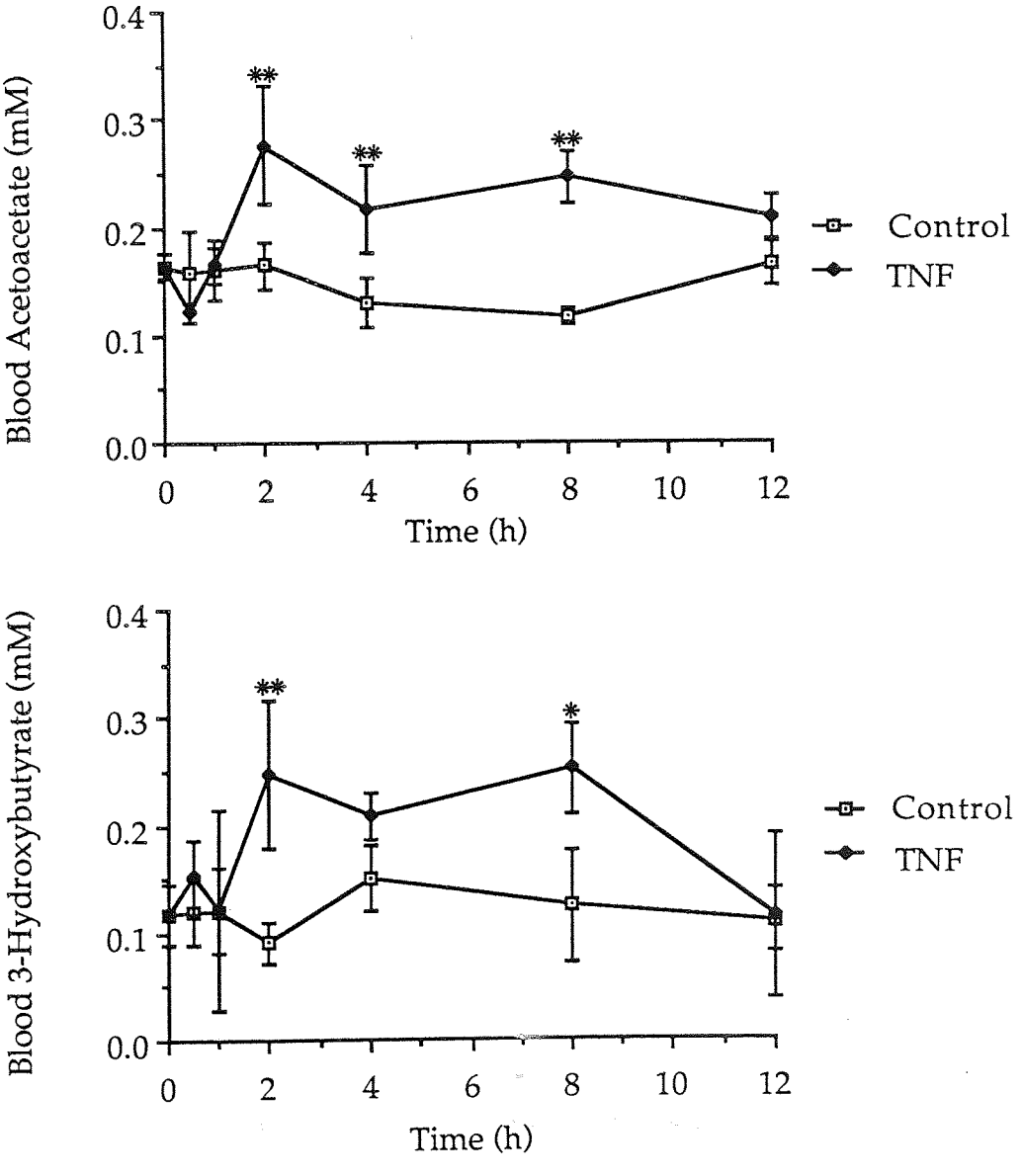
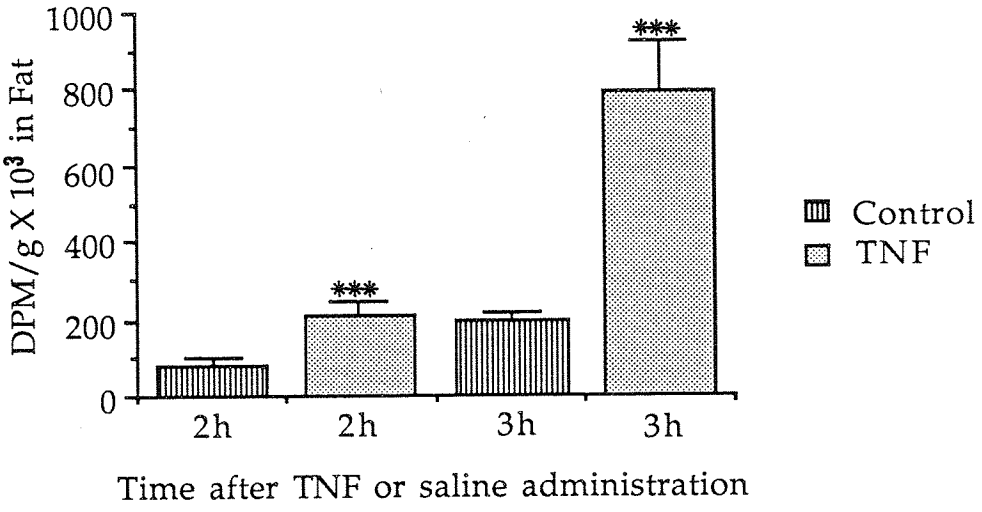
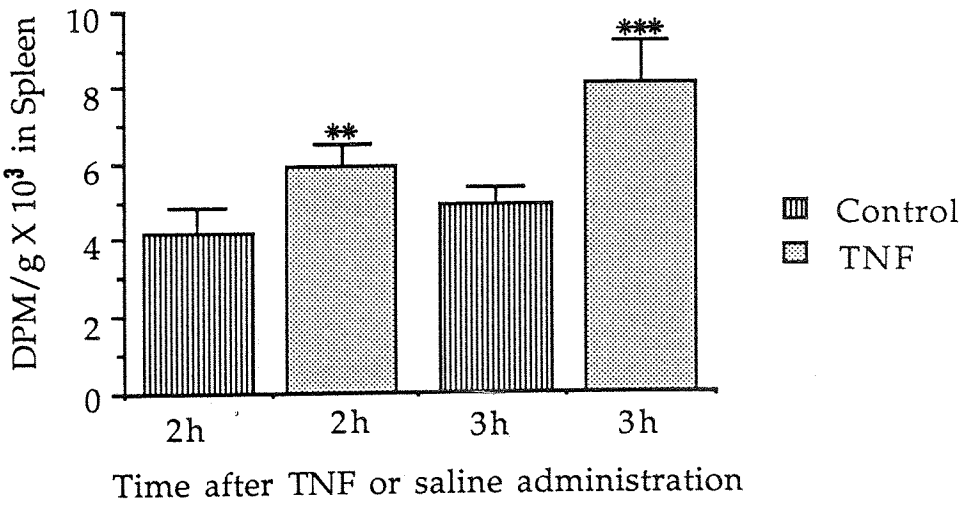


Figure 4.41

The effect of a single injection of TNF on the conversion of [U-^{14}C] glucose into lipids in adipose tissue, spleen, liver, blood and colon.

Female NMRI mice were administered [U-^{14}C] glucose (250μCi/Kg) at the same time or 1h after TNF (7.5 X 10^7U/Kg) administration. 2h later mice were killed and the conversion to ^{14}C lipids was determined. Values represent the means ± S.E.M. of 6 animals in each group. *p≤0.05, **p≤0.01 and ***p≤0.001 from saline-injected controls by Student's t-test.





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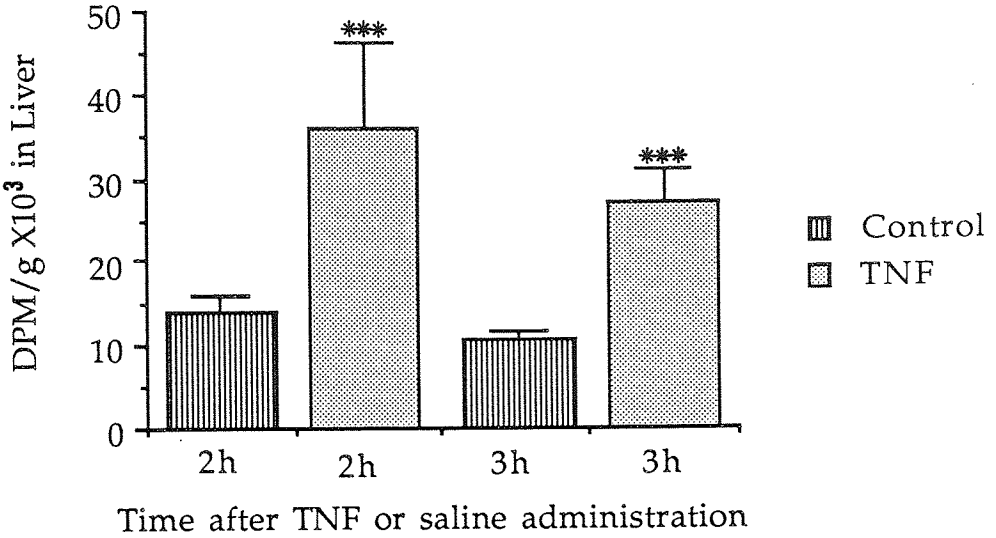
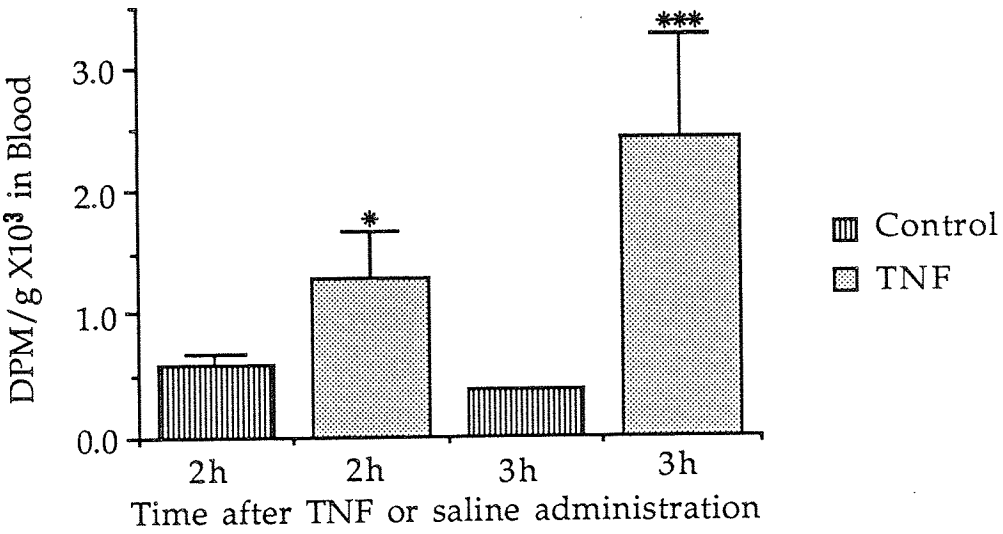
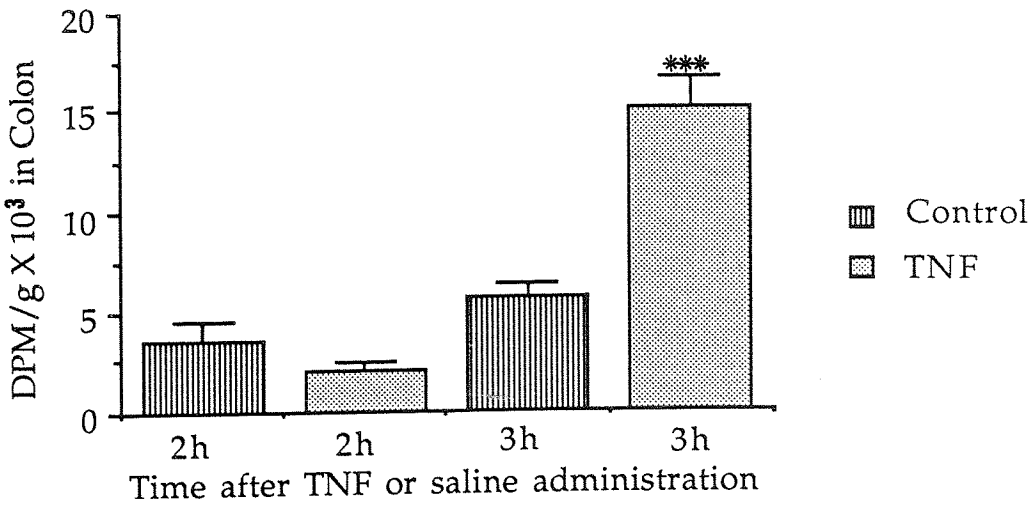






5. DISCUSSION

5.1 The anorectic/cachectic effects of TNF

Administration of dialysed conditioned medium from lipopolysaccharide (LPS) induced peritoneal macrophages caused weight loss and anorexia in mice. (Cerami et al, 1985). Cachectin/TNF was thought to play a central role in this phenomenon and it was suggested, on the basis of this observation, and the lipoprotein lipase (LPL) suppressing action of this cytokine (Beutler et al, 1985a), that TNF was the mediator of cachexia, including that associated with cancer. The above investigation raised two important questions; could purified TNF cause a similar weight loss in animals? and; did the observed anorexia contribute to, or even cause, the weight loss? The work in this thesis was prompted by, and aimed at answering these questions. The first question was tackled by the use of recombinant TNF, and the second, by comparing the effects of TNF with the MAC 16 adenocarcinoma, a cachexia-inducing tumour which does not cause anorexia (Beck and Tisdale, 1987), and with pair-fed controls. The mechanisms involved in the TNF-induced weight loss were also investigated.

In accordance with the results of Cerami et al. (1985) using impure cachectin/TNF, a single intravenous injection of human recombinant TNF caused weight loss in female NMRI mice over a 24h period. This reduction in the weight of animals was dose-related and was accompanied by, and directly proportional to, a decline in the consumption of both food and water. In contrast, the MAC 16 adenocarcinoma caused weight loss in recipient animals without any effect on the consumption of food and water (Beck and Tisdale, 1987), thus exerting a true cachectic rather than an anorectic effect. Differences in the levels of various metabolites following TNF administration or MAC 16 tumour implantation were also seen.

Both TNF-treated and MAC 16 tumour-bearing animals exhibited a decrease in blood glucose concentration, although TNF induced a more marked and possibly life-threatening hypoglycaemia. Satomi et al. (1985) reported that LPS, but not highly purified TNF, caused hypoglycaemia in mice. However, other investigators (Tracey et al, 1986, Bauss et al, 1987, and Kettlehut et al, 1987) have demonstrated large biphasic changes in blood glucose concentrations

after recombinant TNF administration, with an initial hyperglycaemia followed by a sharp decrease in blood glucose levels after about 2h. In contrast, Michie et al. (1988) did not observe a change in the blood glucose concentration of humans following TNF administration and there have been no reports of changes in blood glucose levels in any of the clinical trials undertaken to date.

It has been suggested that TNF may stimulate glucose uptake and oxidation (Kettlehut et al, 1987), contributing to the severe hypoglycaemia observed in rodents. Our results have demonstrated that the glucose was utilized to sustain an increased lipogenesis (discussed in detail later). The hypoglycaemia in MAC 16 tumour-bearing animals probably arose from an increased consumption of glucose by the tumour, an effect which may be seen in clinical cancer cachexia (Tisdale and Brennan, 1986).

Whereas TNF-treated and MAC 16 tumour-bearing mice both exhibited a decrease in plasma free fatty acid (FFA) levels, TNF administration resulted in a marked hypertriglyceridaemia 90min after the second of 2 intravenous injections over a 24h period, and MAC 16 tumour-bearing mice exhibited a decrease in the level of plasma triglycerides. Adipocyte LPL suppression (Beutler et al, 1985a, and Semb et al, 1987), resulting in a decrease in hydrolysis of triglycerides to FFA, may explain these effects of TNF on lipid metabolism. However, Grunfeld et al. (in press, a) and Feingold et al. (in press, a) have recently demonstrated that increased lipogenesis by the liver is the primary cause of the initial TNF-induced hypertriglyceridaemia in rats, and our data on the TNF-induced metabolism of glucose would seem to corroborate these findings (discussed later, section 5.8). It is probable that, with the dosage regimen used in this investigation, adipocyte LPL suppression and increased lipogenesis by the liver both played a role in the observed effects of TNF on plasma lipids. The decrease in plasma FFA and triglyceride levels in MAC 16 tumour-bearing mice probably arose from increased energy demands by the tumour (Beck and Tisdale, 1987).

The decrease in total body water and carcass fat content accompanying the TNF-induced weight loss of NMRI mice over a 24h period was similar to the

effect that one would expect if mice were semi-starved for the same period of time. In fact, pair-fed mice exhibited identical decreases in these two body compartments, with no change in muscle weight, indicating that the acute effects of TNF on the body composition of mice were merely due to an anorectic effect of this agent. MAC 16 tumour-bearing animals also exhibited a decrease in the total body water and carcass fat contents, but, in addition, a 22% reduction in the left thigh and gastrocnemius muscle content of animals was observed. Although differences in the size and sex of the TNF-treated and tumour-bearing animals precluded quantitative comparisons between these two groups, the large decrease in muscle content in MAC 16 tumour-bearing mice, and its absence in TNF-treated animals, suggests that the MAC 16 tumour possesses *in vivo* proteolytic activity, whereas TNF possesses no such activity.

The MAC 16 adenocarcinoma has, in fact, been demonstrated to possess both lipolytic and proteolytic activities *in vitro* (Beck and Tisdale, 1987). Under the conditions of the assay system used in the present investigation TNF did not demonstrate *in vitro* lipolytic activity up to a concentration of 4 X 10^5U/ml of assay mixture. While crude preparations of TNF have previously been reported to suppress the activity of key lipogenic enzymes and to stimulate lipolysis (Pekala et al, 1983, Pekala et al, 1984 and Torti et al, 1985), recombinant TNF was demonstrated to have no effect on either the ability of adipocytes to synthesize and store or to mobilise triacylglycerols (Price et al, 1986b). It was suggested that IL-1, which both suppresses LPL activity and stimulates lipolysis (Price et al, 1986a), may have been responsible for the lipolytic activity of the crude preparations. However, Kawakami et al. (1987) have reported that recombinant TNF increases the lipolysis of stored fat in 3T3-L1 adipocytes, even in the presence of insulin. The difference between these results and the present study may be related to the time of incubation of adipocytes; the assay system used in the present investigation employed a 2h incubation period, whereas Kawakami et al. did not observe an increase in glycerol production in 3T3-L1 cells until 12h after the addition of TNF, after which there was a linear increase in production up to 24h. In contrast, Patton et al. (1986) demonstrated lipolysis in 3T3-L1 cells after only a brief (1h) pre-incubation with TNF, and thus discrepancies in results may be attributable to

the use of this cell type. Other investigations utilizing rat adipocytes and epididymal adipose tissue have demonstrated no stimulation of lipolysis by TNF (Rofe et al, 1987, and Kettlehut and Goldberg, 1988).

In vitro proteolytic activity, as determined by the release of amino acids from mouse` diaphragms, was associated with large concentrations of TNF. This activity was not due to the presence of endogenous proteases in the TNF preparation (Boehringer Ingelheim, personal communication), nor to endotoxin contamination (the activity was destroyed by heating). The proteolysis induced by both TNF and the MAC 16 tumour extract was suppressed by indomethacin, suggesting the possibility of a prostaglandin intermediate. Prostaglandins of the E series, but not of the F series, exhibited proteolytic activity in this assay system. Since prostaglandin E_2 (PgE_2) is believed to be an important stimulus for the production of intracellular proteases (Rodeman and Goldberg, 1982), and TNF has been reported to stimulate PgE_2 production from a variety of cell types in culture (Dayer et al, 1985, Bachwich et al, 1986, Sato et al, 1987, and Baud et al, 1988), this suggests that the enhanced release of amino acids from mouse diaphragm in the presence of TNF was due to an elevation of PgE_2 levels. However, an attempt to measure TNF-induced PgE_2 production from mouse diaphragms in the present investigation was unsuccessful, due to a high degree of variability in the readings, as a result of physical trauma-induced PgE_2 production by the diaphragms.

A similar investigation (Moldawer et al, 1987) demonstrated that, although partially purified supernatants from *Staphlococcus albus*-activated blood monocytes caused an initial increase in the rate of skeletal protein degradation in EDL preparations, recombinant human TNF had no effect on protein synthesis or degradation, despite an increase in PgE_2 release at the concentration of TNF (2200ng/ml) employed. This concentration was equivalent to the smallest concentration of TNF (10^4U) with which proteolysis was observed in the present investigation. Kettlehut and Goldberg (1988) also failed to demonstrate increased proteolysis in soleus and EDL muscles 4h after injection of human TNF to rats. Other workers have failed

to show an alteration in nitrogen balance after TNF administration to humans (Michie et al, 1987). However, Warren et al. (1987b) and Starnes et al. (1988) have reported an increase in total peripheral amino acid efflux after intravenous administration of TNF to humans.

Both TNF-induced growth inhibition and TNF-induced cytolysis were decreased when cells were co-treated with particular protease inhibitors (Suffys et al, 1988). It was suggested that these activities of TNF were associated with serine proteases, as inhibitors of both trypsin-like and chymotrypsin-like proteases interfered with the TNF cytotoxic action. This is in agreement with the present study where the *in vitro* proteolytic activity of TNF was inhibited by α-1 antitrypsin.

The absence of an elevated TNF production following LPS administration in MAC 16 and MAC 13 tumour-bearing animals, compared with non-tumour-bearing controls, negates against a synergistic influence of the presence of a tumour on TNF production in response to LPS. In fact, except for one investigation where elevated levels of a 'TNF-like activity' were found in approximately half of all randomly selected cancer patients (Balkwill et al, 1987a), all other workers have failed to detect TNF in the serum of patients with cancer-associated cachexia (Waage et al, 1986, Socher et al, 1988, and Oliff, 1989). Cerami et al. (personal communication) have recently suggested that local tissue levels, and not serum levels, of TNF may be elevated in cancer cachexia, leading to the wasting observed in this syndrome. This would seem unlikely, however, as it has been demonstrated (Norton et al, 1985) that anorexia/cachexia in tumour-bearing rats is transmitted via the circulation. Thus, if TNF were involved in the cachexia of cancer, elevated levels of this cytokine would be expected to be present in the serum of animals and patients with cachexia. Using the L929 cell cytotoxicity assay, we did not detect TNF in either the MAC 16 adenocarcinoma or the non-cachectic MAC 13 tumour, nor was there evidence of the cytokine in the serum of these animals.

Thus, human recombinant TNF, when administered to female NMRI mice, appeared to exert an anorectic effect, rather than the complex manifestations of cancer cachexia. No lipolytic activity was demonstrated and, although *in*

vitro proteolytic activity was associated with large concentrations of TNF, the application of this to the *in vivo* situation has not been elucidated. In contrast, the MAC 16 adenocarcinoma caused cachexia in recipient animals, without any associated anorexia, and has been shown to possess potent lipolytic and proteolytic activity both *in vitro* and *in vivo* (Beck and Tisdale, 1987).

5.2 Species and strain specificity of TNF

Species specificity in the TNF-receptor interaction (Smith et al, 1986) and in the *in vitro* TNF-induced cytotoxicity (Fransen et al, 1986a) have been demonstrated. Fiers et al (1989) reported that human TNF was 50-fold less toxic to mice than murine TNF, although their specific activity *in vitro* was only 3-fold different. Zentella et al (1989) have also recently demonstrated species specificity in the ability of TNF to trigger metabolic changes *in vitro* and to induce fever *in vivo*. It was proposed that murine TNF induces much more IL-1 *in vivo* than human TNF and that this could explain the higher toxicity of murine TNF in mice (Fiers et al, 1989). Since IL-1 has also been reported to induce anorexia in animals (Plata-Salaman et al, 1988) one would expect, on the basis of the hypothesis of Fiers et al. (1989), that murine TNF would be more potent than human TNF in inducing weight loss and anorexia in mice. However, no species specificity was observed in this investigation in the effects on weight change and food and water consumption, and on blood glucose and plasma FFA concentrations of female NMRI mice, when using human and murine TNF. Thus, it can be assumed that the results obtained with human TNF can be correlated to the situation involving murine TNF in mice.

NMRI mice were used in this study to investigate the mechanism of weight reduction produced by TNF since this strain of mouse was used for transplanting the cachexia-inducing MAC 16 tumour (Bibby et al, 1987), and it was considered that it might be more sensitive to potential cachectic agents. Females were chosen since they display a less aggressive behaviour than males, which may result in selective individuals being deprived of food, and

also because it is easier to induce weight loss in female mice than in males. However, the results presented here on weight reduction with human recombinant TNF show that NMRI mice were much more resistant to TNF than other strains, such as, CBA/J, C3H/HeJ or CD (Remick et al, 1987) where TNF of comparable specific activity was used. Even when NMRI mice were transplanted with the MAC 13 adenocarcinoma, they were no more sensitive to the weight-reducing effects of TNF than non-tumour-bearing controls. This result is surprising as animals bearing tumours have been reported to be highly sensitive to the toxic effects of TNF (Bartholeyns et al, 1987). In fact, in the present investigation, an increased morbidity was observed following TNF administration to tumour-bearing mice, but no increase in weight loss was seen in these animals.

5.3 Comparison of the effects of TNF with those of mitozolamide

Mitozolamide is a member of the group of imidazotetrazines which were suggested to be novel anti-cancer agents (Horgan and Tisdale, 1985). However, phase II clinical trials of mitozolamide were terminated due to the marked toxicity of this compound, and thus its future as a chemotherapeutic agent came to a sudden end. Mitozolamide administration (20mg/Kg) to female NMRI mice caused general malaise, accompanied by a decline in the consumption of food and water and a decrease in the weight of animals. These effects were quantitatively very similar to those observed following a single injection of 7.5 X 10^7U/Kg TNF to NMRI mice. This indicated that the weight loss associated with TNF was due to a generalised cytotoxicity of this agent, causing anorexia which resulted in weight loss. However, TNF has been reported to act directly in the central nervous system to suppress feeding by inhibiting the activity of glucose-sensitive neurons in the lateral hypothalamic area (Plata-Salaman et al, 1988). Thus, the anorectic effect of TNF appears to be a direct effect rather than part of a generalised cytotoxicity induced by this agent. Mitozolamide did not induce the hypoglycaemia and hypertriglyceridaemia that were observed following TNF administration, thus these effects were distinct to TNF and not an effect of the general malaise or hypophagia. Body composition analysis of mitozolamide-treated and TNF-

treated mice demonstrated similar decreases in the total body water content of animals, thus providing further evidence that the acute effects of TNF were due to an anorectic, and possibly dehydrating, effect of this agent.

5.4 Chronic administration of TNF

Cerami et al. (1985) demonstrated that, if the dialysed conditioned medium from LPS-induced macrophages was administered intraperitoneally to mice twice daily over a 5 day period, a biphasic decrease in food and water intake was observed. An initial sharp decrease was seen, followed 2 days later by a second phase in which mice either increased their food and water consumption to the levels of controls (low dose), or continued to limit their food and water intake (high dose). The body mass of mice also decreased in a similar dose-dependent biphasic fashion. Mice receiving the smallest dose of medium continued to lose weight, despite similar food and water intake to control animals, whereas, mice receiving higher doses lost up to 15% of their original body mass.

The present investigation demonstrated that daily administration of recombinant human TNF, as a single intravenous injection, to female NMRI mice also induced a biphasic dose-dependent decrease in food and water consumption. However, whereas mice receiving macromolecules produced by endotoxin-activated macrophages continued to lose weight (Cerami et al, 1985), the weight loss in human recombinant TNF-treated mice only occured over the first 24h, thereafter the weight of treated mice returned towards that of controls, as did the food and water consumption. Thus, the anorectic effects of TNF were confined to the initial exposure, and thereafter the animals became resistant to subsequent dosing. This probably explains the lack of weight loss in cancer patients administered TNF in phase I studies (Blick et al, 1987), although transient anorexia has been reported (Kimura et al, 1987, and Sherman et al, 1988). Even when TNF was administered as a 5-day continuous infusion (Sherman et al, 1988), no clinical evidence of weight loss or accelerated cachexia was observed. Other investigators have recently

reported a similar tolerance to the TNF-induced decrease in food intake and weight loss in rodents (Patton et al, 1987, and Stovroff et al, 1988), although this tachyphylaxis has been overcome by increasing the dose of TNF to maintain a constant food intake (Tracey et al, 1988). Progressive weight loss has also been demonstrated in mice bearing CHO cells transfected with the human TNF/cachectin gene (Oliff et al, 1987). Nevertheless, this weight loss was accompanied by marked anorexia and, since no pair-fed controls were used in the study, the extent of the contribution of anorexia to the observed weight loss is not known.

As described previously, the initial weight loss induced by TNF was associated with a marked and possibly life-threatening hypoglycaemia. Since this decrease in blood glucose was directly proportional to both the decrease in food and water consumption and to the decrease in body weight of mice, and since no hypoglycaemia was observed after 5 daily injections of TNF, when the mice were regaining weight, it was thought that this may be an important feature in the metabolic perturbations induced by TNF, and led to investigations into the mechanisms of the hypoglycaemia which will be discussed later (section 5.8).

Although an initial decrease in plasma FFA levels and a hypertriglyceridaemia were observed 60 to 90min after the second of 2 injections of TNF over a 24h period, no effect of TNF on FFA levels was observed after 5 daily injections of TNF, a tolerance to this effect of TNF also being seen. However, a slight increase in plasma triglyceride levels was still apparent after 5 daily injections of TNF, when the mice were regaining weight. Since the hypertriglyceridaemia induced by TNF was originally attributed to suppression of adipocyte lipoprotein lipase (Beutler et al, 1985a), this result suggested that LPL was still suppressed following 5 daily injections of TNF and that inhibition of this enzyme bore no relation to the weight loss associated with TNF. However, Grunfeld et al. (in press, a) have recently suggested that increased hepatic lipogenesis, and not LPL suppression, was responsible for the hypertriglyceridaemia in rats following TNF administration. These workers also demonstrated that, although the hypertriglyceridaemia persisted for up to 10 days of daily injections of TNF in

rats, and despite tachyphylaxis to the anorectic effects, no decrease in LPL activity was present in a wide variety of tissues after only 3 daily injections of TNF (Grunfeld et al, in press, b). *De novo* hepatic lipogenesis remained elevated in TNF-treated animals after 4 daily injections, but by the fifth day hepatic lipogenesis returned to normal. However, after 5 days of TNF treatment the acute incorporation of labelled glycerol into serum triglycerides remained elevated. Thus, Grunfeld et al. (in press, a and b) concluded that the hypertriglyceridaemia associated with chronic administration of TNF to rats could be dissociated from the induction of anorexia/cachexia and weight loss, and that the TNF-induced increase in triglyceride levels in rats was due to an increased hepatic lipogenesis rather than to adipocyte LPL suppression.

Since TNF was originally suggested to be the factor responsible for cachexia due to its inhibitory effects on adipocyte LPL (Beutler et al, 1986a, and Beutler and Cerami, 1986), these findings place serious doubts on the role of this cytokine in the metabolic manifestations of cachexia.

It has already been mentioned that proteolytic activity associated with high levels of TNF has been detected *in vitro* (section 5.1). However, no breakdown in the left thigh and gastrocnemius muscles of female NMRI mice was observed *in vivo*, even after 5 daily injections of TNF at all of the concentrations employed in this investigation. In fact, preliminary results have demonstrated that administration of increasing doses of TNF, up to a concentration of 3 X 10^8U/Kg, over a 5 day period to female NMRI mice resulted in sustained anorexia and weight loss, but no muscle breakdown, as determined by labelling with [^{14}C]-bicarbonate, was observed (Appendix A1). Under similar conditions, the MAC 16 adenocarcinoma induced extensive proteolysis in recipient animals at weight losses greater than those readily available with TNF. In addition, no alteration in the excretion of creatinine and urea nitrogen in female NMRI mice has been observed in the present investigation following a single injection of TNF, although marked changes in the urinary nitrogen excretion of MAC 16 tumour-bearing animals have been reported (Beck and Tisdale, in press). Thus, with the dosage regimens used in this study, TNF was not demonstrated to cause protein breakdown in female NMRI mice.

A decrease in the carcass fat content of mice was observed after both a single injection of 7.5 X 10^7U/Kg TNF and also after 5 daily injections. This effect could either have been due to the suppression of LPL by TNF, causing fat depletion, or merely to the anorectic effect of this agent. Although, after the initial 24h period following TNF injection, mice began to regain weight, the relative body mass of animals injected with the highest dose of TNF did not reach the 100% value by the fifth day of TNF administration. Thus, it is not surprising that the carcass fat contents of these mice were still below those of controls after 5 daily injections of TNF, even though the animals were regaining weight at this time.

Although the initial weight loss was associated with a decrease in the total body water content of mice, an increase in the water content of animals was seen following 5 daily injections of TNF, and this may have contributed to the weight gain observed at this time. At lower doses of TNF no change in the body water content was observed following 5 days of treatment (results not shown).

5.5 Comparison of the effects of TNF with those of pair-feeding

The results presented thus far would seem to implicate anorexia as the main cause of the TNF-induced weight loss in female NMRI mice. In order to justify this assumption, mice were given the same amount of food and water as that consumed by animals injected with 7.5 X 10^7U/Kg TNF, and the weight loss and metabolic alterations induced by these mice were compared with TNF-injected animals. The pair-fed female NMRI mice exhibited an identical pattern of weight loss to TNF-injected animals, with an initial decrease over the first 24h, followed by an increase towards the values of saline-injected controls. Pair-feeding over a 24h period also resulted in an identical change in the body compositions of mice, with quantitatively similar decreases in the total body water and carcass fat contents of animals as that observed following TNF administration. In addition, after 5 days of pair-feeding or TNF administration, similar decreases in the carcass fat contents were observed. However, the increase in body water content of mice

following chronic administration of TNF was not observed in pair-fed controls and this may explain the large increase in weight of the former group after 5 days of treatment.

The absence of hypoglycaemia and hypertriglyceridaemia in mice after 24h of pair-feeding indicates that these effects of TNF were not due to hypophagia, but were due to distinct actions of TNF. However, the pair-feeding-induced decrease in plasma FFA levels was of similar magnitude to that observed following a single injection of TNF, indicating that this effect may have been due to fat utilization as an energy source in these nutrient-restricted animals. Since the blood glucose, plasma FFA and plasma triglyceride levels of mice were determined 90min after the second of 2 injections over a 24h period, the changes observed may not have been the same as those seen 24h after a single injection of TNF. Thus, it may not have been appropriate to compare these values with those obtained by pair-feeding animals over a 24h period. However, hypoglycaemia and a decrease in plasma FFA levels were still observed 24h after a single injection of TNF (figures 4.35 and 4.43) and thus the above statements would appear to be well-founded.

5.6 **Reversal of the weight loss induced by TNF**

As described previously, the weight reduction produced by TNF in female NMRI mice was accompanied by hypophagia and dehydration, and also by a marked hypoglycaemia, which was directly proportional to both the decrease in food and water consumption and to the decrease in weight of mice. In order to test the possibility that either the hypophagia or the hypoglycaemia was responsible for the weight loss, animals were force-fed a glucose solution or an isocaloric amount of lipid in the form of medium chain triglycerides (MCT). Both glucose and MCT were equally effective in preventing the TNF-induced weight loss, suggesting that hypophagia may have been most important. However, the decline in total body water content of mice, which was normally seen following TNF administration, was not observed in those mice orally fed glucose or MCT, suggesting that the solvent may have been

more important than the additional calories. In fact, when equal volumes of water alone were administered orally to TNF-treated mice, both the body weight and the total body water content were restored to control values, suggesting that neither the glucose nor the MCT were important in weight reversal.

Although oral administration of water, a glucose solution, or MCT to TNF-injected mice resulted in an increase in the weight of these animals, a decline in the body weight of force-fed saline-injected controls, compared to saline injection alone, was observed. This weight loss was accompanied by a decrease in the total body water content of force-fed saline-injected controls and probably arose from the increased manipulation of these animals, resulting in stress and an increased urination. In fact, increased urination was observed in this group when the mice were picked up, but it was not measurable since the small quantities were rapidly lost in the fur of the animals. Thus, the replenishment of body water in TNF-treated female NMRI mice may have arisen from a reduction in fluid output. However, Grunfeld et al. (in press, b) have recently reported that the TNF-induced weight loss in rats was accompanied by an increase in the excretion of urine. This increased urination, in conjunction with a decrease in water intake, by rodents may explain the dehydration observed following administration of TNF to animals, and this may have contributed to, or even caused, the observed weight loss. Unfortunately, it proved impossible to measure the excretion of urine in this study over the 8h period, due to difficulties encountered in collecting and accurately measuring such small volumes of fluid. However, visual signs of an increased urination of force-fed saline-injected controls, but not TNF-treated mice, were observed and this may have caused the dehydation seen in the control animals, although the possible mechanism involved has not been elucidated.

These results demonstrate that the short-term weight loss produced by TNF in female NMRI mice can be reversed by rehydrating the animals and, thus, it appears to be principally due to dehydration. The preliminary results presented in Appendix A2, showing inhibition of the TNF-induced weight loss by megestrol acetate (Megace), an appetite-stimulating agent which also

causes water retention, collaborate the findings of this study.

5.7 The role of prostaglandins in the TNF-induced weight loss

There is some evidence to suggest that prostaglandins may be involved in the metabolic effects of TNF. Kettlehut et al. (1987) demonstrated a 10-fold increase in the blood content of PgE_2 in rats within 1h of a single intravenous injection of TNF. This increase in PgE_2 levels was accompanied by a drop in body temperature, hyperglycaemia followed by hypoglycaemia, acidosis, diarrhoea, and cyanosis in TNF-treated animals. Administration of the cyclo-oxygenase inhibitors indomethacin or ibuprofen 2h prior to the TNF injection prevented the initiation of all of these toxic effects of TNF, as well as providing complete protection against the very rapid killing seen with high-dose TNF. Other investigators have demonstrated similar protective actions of cyclo-oxygenase inhibitors against the toxic effects of TNF (Marquet et al, 1987, and Talmadge et al, 1987).

Production of PgE_2 has been demonstrated to be enhanced after stimulation of mouse osteoblast-like cells (Sato et al, 1987), human synovial cells and dermal fibroblasts (Dayer et al, 1985) and murine peritoneal macrophages (Bachwich et al, 1986) with TNF. Both TNF and IL-1 have been shown to stimulate the production of PgE_2 by isolated extensor digitorum longus muscles (Moldawer et al, 1987). Although these two cytokines have been demonstrated to be synergistic in enhancing PgE_2 production by human lung fibroblasts (Elias et al, 1987), they have been reported to act in an additive fashion to activate synovial cell phospholipase A_2 (PLA_2) and to induce the generation of PgE_2 (Godfrey et al, 1988).

In the present investigation an enhanced production of PgE_2 was observed in isolated spleen cells taken from TNF-treated mice, which was significantly greater than saline-injected controls within 1h of treatment. In addition, as previously described (section 5.1), PgE_2 has been implicated in the *in vitro* proteolytic activity of TNF. These results suggest that PgE_2 may serve as an intermediate for TNF effects, although PgE_2 has also been shown to inhibit

TNF production by macrophages (Kunkel et al, 1988), suggesting a fine control for the regulation of TNF production.

The preliminary results obtained in this investigation indicated that prostaglandins may be involved in the weight loss induced by TNF; the stable PgE_2 analogue, 16,16-dimethyl PgE_2, produced weight loss in female NMRI mice, which was similar to that induced by TNF in that it was accompanied by hypophagia, a decrease in water intake and a decrease in the total body water content of animals. In addition, indomethacin administration prevented both the weight loss and the dehydration normally caused by TNF. However, Marquet et al. (1987) showed that, although indomethacin administered to rats prior to murine TNF alleviated the toxic side effects, it had no effect on the excessive wasting produced by high doses of TNF. These investigators reported up to a 25% weight loss in rats within 1 week following administration of 10mg/Kg indomethacin 1h prior to TNF (10μg) on alternate days. The absence of an inhibitory effect of indomethacin on the weight loss produced by TNF could have been due to the timing of the indomethacin administration in the investigation of Marquet et al. (1987). These workers administered indomethacin 1h prior to TNF, whereas in the present study a period of 2h was required between the indomethacin and TNF before any significant reversal of the weight loss occured. The lack of tolerance to the TNF-induced weight loss observed by Marquet et al. (1987) may have been a consequence of the dosage regimen, the gap of 1 day between injections possibly preventing tolerance from developing.

Despite the stringent time requirements for reversal of weight loss, indomethacin was equally effective in inhibiting PgE_2 production after TNF administration at all times from 0.5 to 2h prior to TNF. This result suggests that prostaglandins were not, after all, involved in the weight loss induced by TNF.

Further evidence for the lack of involvement of prostaglandins in the TNF-induced weight loss came from the observation that TNF was equally effective in stimulating spleen PgE_2 production up to 5 days of treatment, even though tolerance to the TNF-induced weight loss occured after the first

day of treatment, and by the fifth day mice were actually gaining in weight. Thus, prostaglandins do not appear to have been involved in the anorectic effect of TNF observed in female NMRI mice.

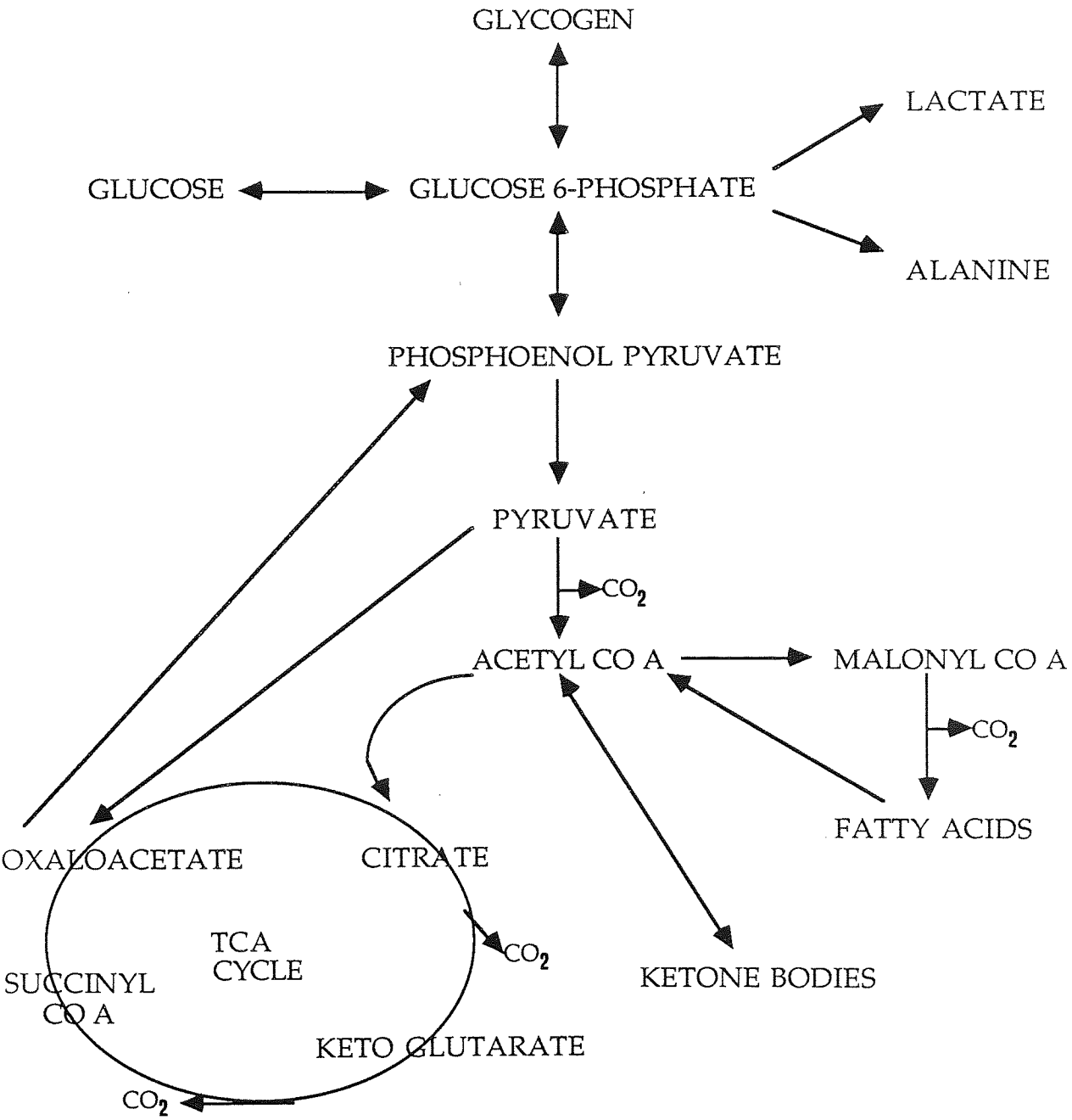
Cyclo-oxygenase inhibitors have been demonstrated to decrease sodium and water excretion in both man (Haylor, 1980) and rat (Haylor and Lote, 1980), resulting in retention of body fluid. In the present study indomethacin was shown to have no effect on urine or faeces production, either alone or in the presence of TNF, and not to cause appreciable fluid retention above saline-infused controls. However, the difficulties in accurately measuring the small urine volumes from mice have previously been described (section 5.6) and very small changes may not have been detected. It is possible that the slight increase in fluid intake induced by indomethacin, accompanied by a small, and in mice undetectable, fluid retention, resulted in the reversal by indomethacin of the TNF-induced decrease in body water content and weight loss of mice. The weight loss of mice observed with PgE_2 may, in accordance with mitozolamide and possibly TNF, have been merely due to a generalised malaise produced by high doses of this agent.

5.8 The effect of TNF on the metabolism of glucose

As described previously (section 5.1) TNF administration to female NMRI mice resulted in a marked, and possibly life-threatening hypoglycaemia, which was directly proportional to both the decrease in food and water consumption and to the decrease in weight of animals. Although oral administration of a glucose solution to mice had no greater effect on reversing the TNF-induced weight loss than water alone (section 5.6), it was of interest to discover the cause of the decrease in blood glucose to determine whether it played a role in the weight loss induced by TNF. In order to achieve this objective, glucose metabolism in TNF-injected female NMRI mice was compared with that in pair-fed controls, in order to eliminate the effects of restricted nutrient intake on the observed changes in the metabolism of glucose. A simplified schematic diagram of the possible pathways via which glucose can be metabolised is shown in figure 5.1.

Figure 5.1

A simplified schematic diagram of the metabolism of glucose



5.8.1 The effect of TNF on the concentration of glucose, glycogen, L-(+)-lactate, L-alanine pyruvate and ketone bodies, and on the rectal body temperature of mice

When administered as a single intravenous injection, TNF caused biphasic alterations in blood glucose levels similar to those reported by other workers (Tracey et al, 1986, Bauss et al, 1987, and Kettlehut et al, 1987), with an initial hyperglycaemia followed by a sharp decrease in blood glucose levels. Endotoxin has been reported to induce similar changes in the blood glucose concentrations of animals (Wolfe et al, 1977, and Raymond et al, 1981). The initial hyperglycaemia after endotoxin administration has been attributed to increased hepatic glycogen mobilisation and enhanced gluconeogenesis (Kun, 1948, Filkins, 1978, Kelleher et al, 1982, and Lang et al, 1985), whereas impaired liver gluconeogenesis was reported to be largely responsible for the endotoxin-induced hypoglycaemia (Shands and Senterfitt, 1972). The mechanisms involved in the TNF-induced alterations in glucose metabolism have not previously been elucidated.

The marked decrease in liver glycogen levels which accompanied the TNF-induced hypoglycaemia, may have been mediated via increased circulatory levels of glucagon or catecholamines, which have been shown to be elevated, together with corticosterone levels, after TNF administration (Warren et al, 1987a, and Bagby et al, 1988), correlating with an increased glucose rate of appearance (Bagby et al, 1988).

Although studies have shown no effect of TNF on glycogenolysis, gluconeogenesis or ketogenesis in isolated rat hepatocytes in short term incubations (Rofe et al, 1987), Lee et al. (1987) demonstrated that TNF increased glycogenolysis, glucose uptake and lactate production in fully differentiated L-6 myotubes derived from neonatal rat myoblasts. The increased lactate production could not have been accounted for by the rate of glycogenolysis, and did not occur as a consequence of elevated glucose transport, since TNF-induced increases in glucose transport were delayed several hours, requiring the synthesis of glucose transporters. This implies that TNF must have increased lactate production, in part, by increasing the

proportion of glucose taken up from the medium that was directed to lactate. Tracey et al. (1986) also reported elevated levels of lactate *in vivo* following TNF administration. However, no increase in blood lactate, pyruvate or alanine levels were observed over the short time interval employed in the present study. An increase in blood alanine may have been expected if TNF is the mediator of the catabolic effects of cachexia. Thus, this corroborated our findings that TNF administration to female NMRI mice did not induce muscle catabolism, although, as previously described (section 5.1), discrepancies exist in the literature regarding the possible proteolytic properties of TNF.

The dose-related decrease in body temperature observed in TNF-injected mice indicates that the blood glucose was not being utilized to provide energy for heat generation or that there was a defect in the thermoregulatory system in TNF-injected mice. Although several workers have demonstrated pyrogenic effects of TNF (Dinarello et al, 1986, and Rothwell, 1988), others have reported a decrease in body temperature similar to that observed in the present investigation (Kettlehut et al, 1987). It appears that this effect of TNF is highly dependent on the dose administered; at low doses TNF has been reported to cause fever, as may occur in mild infections, and at high levels it has been demonstrated to cause hypothermia, such as that associated with septic shock (Kettlehut et al, 1987).

The elevation of acetoacetate and 3-hydroxybutyrate observed after TNF administration was similar to that which normally occurs during prolonged starvation (Stryer, 1988, and section 1.9.1). However, this effect was not due to the reduction in food intake in TNF-treated mice since it was not observed in pair-fed animals. This suggests an elevated production of acetyl Co A. Since ketosis does not occur in clinical cancer cachexia, this is another example of a difference between the effects of TNF and those of cancer cachexia. In fact, it has been reported (Bibby et al, 1987) that the animal model of cachexia used in this investigation, the MAC 16 adenocarcinoma, does not cause ketosis in recipient animals despite massive losses in carcass fat.

5.8.2 The effect of TNF on the oxidative metabolism and resting oxygen consumption of mice

The observation that the conversion of glucose into CO_2 was reduced following TNF administration, when compared with pair-fed controls, suggests that the reduction in blood glucose was not due to an increased oxidative metabolism via the tricarboxylic acid (TCA) cycle in TNF-treated animals. In addition, since the production of $^{14}CO_2$ from [U-^{14}C] palmitate was not affected, TNF administration did not result in a malfunction of the TCA cycle. Instead, there appears to have been a shift in the metabolism of glucose from the TCA cycle towards anabolic reactions, possibly to ketone bodies and fatty acids. A similar diversion of the metabolism of acetyl Co A is observed in anorexia and in diabetes due to a decrease in carbohydrate levels (Stryer, 1988). In these circumstances oxaloacetate is used to form glucose and is thus unavailable for condensation with acetyl Co A for entry into the TCA cycle.

The decreased metabolic rate of animals, as determined by a decrease in the resting oxygen consumption of mice, observed in the present investigation correlates with the findings that TNF induced hypothermia and a decline in the oxidative metabolism of glucose. However, Rothwell (1988) reported an increase in the body temperature of rats following intracerebroventricular administration of TNF, accompanied by an increased metabolic rate and stimulation of brown adipose fat activity. The discrepancy in these two studies may lie in differences between the routes of administration of TNF, and also in the dose of TNF employed, much smaller doses being used in the study by Rothwell (1988).

5.8.3 Glucose utilization by tissues

Glucose utilization of different tissues was investigated *in vivo* by the 2-deoxyglucose tracer method. This analogue of glucose undergoes the first stages of glycolysis in the same way as glucose, but, instead of glucose 6-phosphate, 2-deoxyglucose 6-phosphate is formed. However, whereas glucose

6-phosphate then undergoes isomerization to fructose 6-phosphate, the absence of an oxygen atom on the 2 position of the pyranose ring of 2-deoxyglucose 6-phosphate hinders isomerization. Thus, glycolysis of the 2-deoxy analogue is blocked at this stage and 2-deoxyglucose 6-phosphate accumulates within the tissues.

The 2-deoxyglucose tracer method was previously only applied to the study of the brain (Sokoloff et al, 1977) and muscle (Meszaros et al, 1987a) as these tissues are deficient in glucose 6-phosphatase. This enzyme is abundant in tissues such as liver, kidney and intestine, and hydrolytically cleaves the phosphorylated glucose, which cannot readily diffuse out of cells, to glucose. Thus 2-deoxyglucose 6-phosphate would not be expected to accumulate within these tissues. However, this method has recently been successfully applied to the determination of glucose utilization by various other tissues (Meszaros et al, 1987b).

The present investigation confirmed the findings of Meszaros et al. (1987b) that TNF induced an increased glucose utilization in several organs. When calculated on a whole organ basis, the major contributor to the increased glucose utilization after TNF administration was seen to be the liver, because of the greater size of this organ compared with the colon. Since glucose turnover was also maximally elevated in the liver after TNF administration, this suggests that glucose was being utilized by the liver for anabolic reactions. In addition, since the elevated glucose consumption was not due to conversion into CO_2 or lactate, this suggests an enhanced lipogenesis.

5.8.4 The effect of TNF on the plasma levels of lipids and lipogenesis from glucose

An enhanced fatty acid and sterol synthesis has been observed following administration of TNF to the rat (Feingold et al, 1987). The elevated conversion of [U-^{14}C] glucose to lipids in the liver, spleen, adipose tissue, blood and colon of mice, observed in the present study following TNF administration, confirms that the glucose served to maintain an increased lipogenesis. These results indicate that the hyperlipidaemia observed

following TNF administration to NMRI mice may not have been solely due to inhibition of adipocyte LPL (Beutler et al, 1985a, and Semb et al, 1987), but may have also been due to an increased hepatic output. The results recently obtained by Grunfeld et al. (in press, b) corroborate the findings of this investigation and place doubt on the role of TNF in cancer cachexia. These workers demonstrated that, under conditions which acutely increased serum triglycerides (90min post human TNF administration to the rat), no decrease in LPL activity was seen in epididymal fat pads. However, by 16h after TNF administration, LPL activity was decreased by 44% in these lipid stores. Thus, the TNF-induced rise in serum triglycerides preceded the decrease in LPL in epididymal fat. In addition, the TNF-induced decrease in LPL activity appeared to be limited to only a few adipose tissue sites. These authors thus concluded that the initial hypertriglyceridaemia induced by TNF in rats was not due to inhibition of LPL, but instead, was solely due to a TNF-induced increase in lipogenesis, which was observed in these experiments, and which correlated well with the increase in serum lipids (Grunfeld et al, in press, b). This would explain the accumulation of labelled lipids in adipose tissue after TNF administration in the present investigation, an effect which would not be expected if LPL were suppressed at this time.

Thus, the initial increase in the plasma level of triglycerides and FFA observed in this study 2 to 4h after TNF administration, was possibly due solely to the increased hepatic lipogenesis. However, this increase in plasma lipid levels only persisted for a maximum of 8h, whereas Feingold et al. (1987) demonstrated an increase in serum triglyceride levels for up to 17h following TNF administration in the rat. The discrepancy in these results may reflect the different doses of TNF and animal species utilized in these experiments.

The decrease in plasma FFA levels observed 24h following TNF administration to mice in the present investigation may have been due to a TNF-induced suppression of adipocyte LPL activity at this later time point, although no change in plasma triglyceride levels was observed. In contrast, since a similar decline in FFA levels was seen 24h after pair-feeding mice (section 5.5), this effect may have been due to fat utilization as an energy source in these nutrient-restricted animals. The decrease in plasma FFA

levels, accompanied by marked hypertriglyceridaemia, seen 90min after the second of 2 injections of TNF (section 5.1), indicates that LPL suppression may have been important at this time, although measurements of LPL have not been undertaken in this investigation. Thus, it would appear that the dosage regimen and time of measurements were important in these effects of TNF, and that both the suppression of LPL and the increased lipogenesis played roles in the lipid alterations observed following TNF administration to mice.

5.9 **Conclusion**

The results of this study indicate that the hypoglycaemia induced by TNF administration to female NMRI mice did not contribute to the weight loss observed. Instead, the glucose was utilized to serve an increased lipogenesis in these animals. Since the yield from the complete oxidation of fatty acids is about 9Kcal/g, in contrast to about 4Kcal/g for carbohydrates and proteins (Stryer, 1988), the conversion of glucose to fats served to provide the animal with a very high source of energy. A possible explanation for this effect of TNF is that the mobilisation of energy stores was preparing the host for trauma, such as that involved in infection. In fact, similar alterations in lipid metabolism have been observed in several infections, including those of bacterial, viral and parasitic origins (Fiser et al, 1972, Lees et al, 1972, Kaufmann et al, 1976, and Rouzer and Cerami, 1980). However, further investigations will need to be carried out before a full explanation of these effects of TNF can be determined.

The effects of TNF on blood glucose and plasma FFA and triglyceride levels, and on prostaglandin production, have been shown to be distinct from the weight loss observed following TNF administration to female NMRI mice. In fact, the weight loss induced by TNF appears to have been solely due to an anorectic effect of this agent and was largely a result of dehydration.

Previous reports have demonstrated that a number of cytokines, including TNF, IL-1, IFN-α and IFN-γ inhibit adipose tissue LPL activity (Kawakami et al, 1982, Beutler and Cerami, 1985, Patton et al, 1986, Price et al, 1986a, and

Price et al, 1986b). Additionally these cytokines and IFN-β have been shown to inhibit tissue lipogenesis (Keay and Grossberg, 1980, Pekala et al, 1983, Kawakami et al, 1982, and Patton et al, 1986). More recently, Feingold et al. (in press, b) have demonstrated that IL-1 and IFN-α can also rapidly stimulate hepatic fatty acid synthesis, and that TNF-β, IL-1 and IFN-γ increase hepatic cholesterol synthesis. Thus, there are a large number of potential interactions between the immune system and lipid metabolism, and it is likely that these interactions account for the hyperlipidaemia that has been noted to accompany infectious diseases.

Taking into account the large number of similarities between the multiple cytokines from different cell types which act through different receptors, it is surprising that TNF alone has been implicated as the mediator of cachexia. The observation that many of these cytokines inhibit adipocyte LPL activity, and also cause anorexia and weight loss in animals (McCarthy et al, 1986, and Plata-Salaman et al, 1988), makes this distinction even more incomprehensible. The results presented in this thesis indicate that the weight loss induced by TNF in female NMRI mice was solely due to an anorectic and dehydrating effect of this cytokine. No catabolic properties of TNF have been observed, but rather distinct anabolic effects. Although it seems likely that a cascade of multiple cytokines is involved in many biological functions, whether cachexia is one of these has yet to be determined. However, it seems evident that TNF alone does not cause the complex metabolic alterations induced by cancer cachexia. Indeed, work in our laboratory is at present being undertaken to isolate a factor which seems far more likely to be involved in mediating the effects of cancer-associated cachexia.

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APPENDICES

A1. The *in vivo* proteolytic activity of TNF and the MAC 16 adenocarcinoma

It has been demonstrated in the main text of this thesis that TNF possesses *in vitro* proteolytic activity at high doses (section 4.1.6). However, no protein breakdown, as determined by muscle weight, has been observed following administration of TNF to female NMRI mice with the dosage schedules used in these investigations (table 4.9). This study was undertaken to determine whether *in vivo* proteolysis could be detected following prolonged administration of TNF to mice if a more sensitive assay method were employed, and if increasing doses of TNF were used to maintain a constant weight loss. Protein breakdown was studied *in vivo* in the gastrocnemius plus thigh muscles and in the tumours of TNF-treated and MAC 16 tumour-bearing female NMRI mice after labelling with [^{14}C] bicarbonate.

A1.1 Materials

$NaH^{14}CO_3$ (sp. act. 56mCi/mmol) was purchased from Amersham International, Buckinghamshire.
Bio-Rad Protein Assay was purchased from Bio-Rad Laboratories Ltd, Watford, Hertfordshire.

All other materials were as in the main text of this thesis.

A1.2 Methods

A1.2.1 Treatment of animals

Female NMRI mice (19±0.7g) were administered $NaH^{14}CO_3$ (400μCi/Kg body weight, i.v.) in 0.9% NaCl, and they were then injected i.p. with TNF (day 1). Increasing doses of TNF were administered i.p. twice daily, at the same time each day, over a 5 day period, to maintain a constant weight loss as shown overleaf:

Day	TNF (U/Kg X 10^7, i.p.) 9am	9pm	Weight change over 24h
1	1.5	2.1	---
2	4.5	6.0	-0.7±0.3
3	9.0	12.0	-0.7±0.2
4	15.0	22.5	-0.3±0.2
5	27.0	30.0	+0.1±0.3
6	---	---	-0.6±0.2

Controls were injected with 200µl of 0.9% NaCl at the same time each day over a 5 day period. Pair-fed controls were injected with 0.9% NaCl and were given the same amount of food and water each day as that consumed by the TNF-treated animals. On day 6, mice were killed by cervical dislocation, the gastrocnemius and thigh muscles were removed and pooled and were used for the determination of protein and radioactivity contents.

MAC 16 tumour-bearing female NMRI mice (19±1g) were injected with $NaH^{14}CO_3$ (400µCi/Kg body weight, i.p.) in 0.9% NaCl on day 15 after transplantation. Mice were killed 5 days later (day 21) by cervical dislocation, the gastrocnemius and thigh muscles were removed and pooled, and the tumours were also removed. These tissues were then used for the determination of protein and radioactivity contents.

A1.2.2 Protein and radioactivity determination

Protein was determined with the use of the Bio-Rad Protein Assay, which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595nm when binding to protein occurs.

Trichloroacetic acid-insoluble proteins were processed for lipid extraction, extensively hydrolysed (12h at 90°C) in 0.3M NaOH, and counted for radioactivity in 10ml Optiphase scintillation fluid using a Packard TRI-CARB 2000CA liquid scintillation analyser (for further details see Baccino et al, 1982,

and Tessitore et al, 1987). The specific protein activity (radioactivity per mg of protein) was then calculated.

A1.3 Results

TNF administration to female NMRI mice, with the dosage regimen used in this assay, resulted in a weight loss representing 12% of the original weight of the animals (table A1.1). Pair-fed controls exhibited a weight loss of similar magnitude to the TNF-injected mice. Both TNF-injected animals and pair-fed controls showed slight decreases in muscle weight when compared with saline-injected controls. However, no significant alteration in the muscle protein content and specific muscle protein radioactivity of mice was observed when compared with saline-injected animals.

No significant decrease in muscle weight or muscle protein content was observed in MAC 16 tumour-bearing animals, up to a weight loss of 4.3±0.4g, representing a 23% decrease in the weight of the animals (table A1.2). However, a significant decrease in muscle weight was observed when mice had lost 6.3±0.2g, representing a weight reduction of 33%. The specific muscle protein radioactivity of these animals was also significantly decreased, although no change in muscle protein content was observed. The decline in muscle weight and incorporation of radioactivity in the muscles of MAC 16 tumour-bearing mice was accompanied by a significant increase in both of these parameters in the tumours of these animals.

A1.4 Discussion

Although a decline in the muscle wet weight was observed in TNF-treated mice, this was not significantly different from pair-fed controls. Since no alteration in muscle protein content was observed in both TNF-injected and pair-fed animals, the decline in muscle wet weight may have been due to loss of water. In addition, no alteration in the incorporation of radioactivity into muscle protein was seen. Thus TNF did not alter the synthesis of muscle proteins. These results suggest that TNF does not possess *in vivo* proteolytic activity, the observed effects on weight and body composition being solely due

to the TNF-induced anorexia in female NMRI mice.

In contrast, the MAC 16 adenocarcinoma was demonstrated to possess potent *in vivo* proteolytic activity and to decrease the synthesis of muscle protein. Concomitant with this depletion of muscle, an increase in tumour weight and tumour protein content was observed, accompanied by an increase in the incorporation of radioactivity into tumour protein. This indicates that the MAC 16 tumour possesses direct proteolytic activity in order to serve the needs of the tumour.

These results were not incorporated into the main text of this thesis as more extensive work needs to be undertaken in this area. MAC 16 tumour-bearing mice exhibiting similar weight losses to the TNF-injected animals did not demonstrate protein breakdown in this assay. Thus, in order to compare TNF-treated and MAC 16 tumour-bearing mice, the dosage schedule of TNF must be adjusted to maximise the TNF-induced weight loss. It would be of interest to perform this experiment in animals continually administered TNF, eg, in mice bearing CHO cells transfected with the human TNF gene (Oliff et al, 1987). However, since very high doses of TNF were administered to mice, with no significant alteration in muscle mass and protein content when compared with pair-fed controls, it would appear that TNF does not possess *in vivo* proteolytic activity in female NMRI mice under the conditions of this assay.

A1.5 References

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Table A1.1

The *in vivo* proteolytic activity of TNF

Female NMRI mice were administered $NaH^{14}CO_3$ (400μCi/Kg, i.v.) in 0.9% NaCl followed by an i.p. injection of TNF. Increasing doses of TNF were injected i.p. twice daily over a 5 day period (section A1.2.1). Controls were pair-fed and/or were injected i.p. with 200μl of 0.9% NaCl. On day 6, mice were killed by cervical dislocation, the gastrocnemius and thigh muscles were removed and pooled, and were used for the determination of protein and radioactivity content. The values represent the means ± S.E.M. of 6 animals in each group. $^{a}p\leq0.05$, $^{b}p\leq0.005$ and $^{c}p\leq0.001$ from saline-injected controls by analysis of variance.

	Control (saline)	Control (pair-fed)	TNF
Weight change (g)	+0.7±0.5	-2.4±0.2^{c}	-2.3±0.3^{c}
Muscle wet weight (g)	0.54±0.02	0.47±0.02^{a}	0.43±0.02^{b}
Muscle protein content (mg)	19.85±1.16	16.96±1.37	15.34±0.86
Specific muscle protein radioactivity (dpm/mg protein)	16.55±2.30	16.14±2.68	17.95±1.74

Table A1.2

The *in vivo* proteolytic activity of the MAC 16 adenocarcinoma

MAC 16 tumour-bearing female NMRI mice (19±1g) were injected with $NaH^{14}CO_3$ (400μCi/Kg, i.p.) in 0.9% NaCl on day 15 after transplantation. Mice were killed 5 days later (day 21) by cervical dislocation, the gastrocnemius and thigh muscles were removed and pooled, and the tumours were also removed. These tissues were then used for the determination of protein and radioactivity content. Mice were separated into 4 groups according to the extent of weight loss for comparison between groups. The values represent the means ± S.E.M. of 4 to 6 animals in each group. $^{a}p\leq0.01$, $^{b}p\leq0.05$, $^{c}p\leq0.005$ and $^{d}p\leq0.001$ from A (no weight loss), $^{e}p\leq0.05$ and $^{f}p\leq0.01$ from B, and $^{g}p\leq0.05$ from C by analysis of variance.

	A	B	C	D
Weight change (g)	-0.3±0.2	-1.8±0.2^{d}	-4.3±0.4^{d}	-6.3±0.2^{d}
Muscle wet weight (g)	0.52±0.02	0.49±0.02	0.46±0.01	0.38±0.03^{c,f}
Muscle protein content (mg)	22.0±2.6	22.4±2.7	22.1±0.8	16.7±2.6
Specific muscle protein radioactivity (dpm/mg protein)	17.08±2.6	15.88±1.9	11.67±2.6	10.71±1.1^{b}
Tumour wet weight (g)	0.11±0.01	0.21±0.05	0.26±0.03	0.43±0.09^{b}
Tumour protein content (mg)	0.99±0.13	1.54±0.42	1.81±0.18	1.87±0.47^{b}
Specific tumour protein radioactivity (dpm/mg protein)	209±34	228±39	262±24	515±101^{e,g,a}

A2. The effect of megestrol acetate (Megace) on the weight change, food and water consumption, and body compositions of TNF-treated female NMRI mice

Megestrol acetate, a synthetic orally active progesterone which is widely used for the therapy of advanced breast cancer, has been reported to produce weight gain as a, frequently undesirable, side effect of this agent (Ross et al, 1982). Tchekmedyian et al. (1987) demonstrated a stimulation of appetite and weight gain in patients following megestrol acetate administration and, as such, have implicated megestrol acetate as a possible treatment for the anorexia which is often associated with cancer cachexia.

The aim of this investigation was to determine whether megestrol acetate (Megace) was capable of stimulating the appetite, and preventing the weight loss, in TNF-treated female NMRI mice.

A2.1 Materials

Megestrol acetate (Megace$^{(R)}$) was synthesised and donated by Bristol Myers, Evensville, Indiana, USA.
Corn oil was obtained from Asda Superstore, Birmingham.

All other materials were as in the main text of this thesis.

A2.2 Methods

Female NMRI mice (19±0.5g) were administered 7.5 X 10^7U/Kg TNF intravenously (i.v.), and 150mg/Kg Megace in corn oil (17mg/ml) subcutaneously (s.c.), either alone or in combination. Controls were injected with 200µl of 0.9% NaCl, i.v., followed by 150mg/Kg Megace in corn oil s.c. After 24h, mice were weighed and the food and water consumption was determined (section 3.1.2). Body composition analysis was performed as described previously (section 3.1.4).

A2.3 Results

Megace prevented the TNF-induced weight loss in female NMRI mice and resulted in a slight, but not significant, increase in the consumption of both food and water by these animals (figure A2.1). The reversal of weight loss was accompanied by a return to control values of the carcass lipid and water contents of mice, although no significant difference was observed between these values in TNF/Megace-injected animals when compared to Megace alone (table A2.1).

A2.4 Discussion

These results have not been incorporated into the main text of this thesis as they are preliminary, and further extensive work in this area is being undertaken at present in our laboratory. However, it was felt necessary to introduce these results as they corroborate the findings reported in this thesis.

The results of this investigation have demonstrated that Megace is capable of preventing the weight loss induced by TNF in female NMRI mice. It would appear that this effect is due to the slight, though not significant, increase in appetite of these animals and to a slight, but again not significant, rehydrating effect of Megace on the TNF-treated mice. When compared to the values previously obtained for saline-injected controls (figure 4.2 and table 4.2), it seems evident that Megace has a similar appetite-stimulating and weight-gaining effect on these control mice. However, more extensive experiments will need to be performed, with larger n values and with the inclusion of saline-injected controls, before any conclusion as to the mechanisms involved in the Megace-induced weight gain can be made. Nevertheless, the same dose of Megace did not reverse the weight loss induced by the MAC 16 adenocarcinoma (S.A. Beck, personal communication). Since the MAC 16 tumour-induced weight loss was not accompanied by anorexia (Beck and Tisdale, 1987), these results suggest that a Megace-induced reversal of anorexia was responsible for the weight gain observed in NMRI mice. This is further evidence that the weight loss induced by TNF in female NMRI mice is solely

due to an anorectic and dehydrating effect of this cytokine.

A2.5 References

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Figure A2.1

The effect of Megace on the weight change and food and water consumption of TNF-treated female NMRI mice

Female NMRI mice (19±0.5g) were administered 7.5 X 10^7U/Kg TNF, i.v., and 150mg/Kg Megace, s.c., either alone or in combination. Controls were injected with 200µl of 0.9% NaCl, i.v., followed by 150mg/Kg Megace, s.c. The body weight change and food and water consumption of mice were then measured over a 24h period. The values represent the means ± S.E.M. of 4 to 5 animals in each group. $^{*}p\leq0.05$, $^{**}p\leq0.005$ and $^{***}p\leq0.001$ from Megace alone, and $^{****}p\leq0.001$ from TNF alone by analysis of variance.

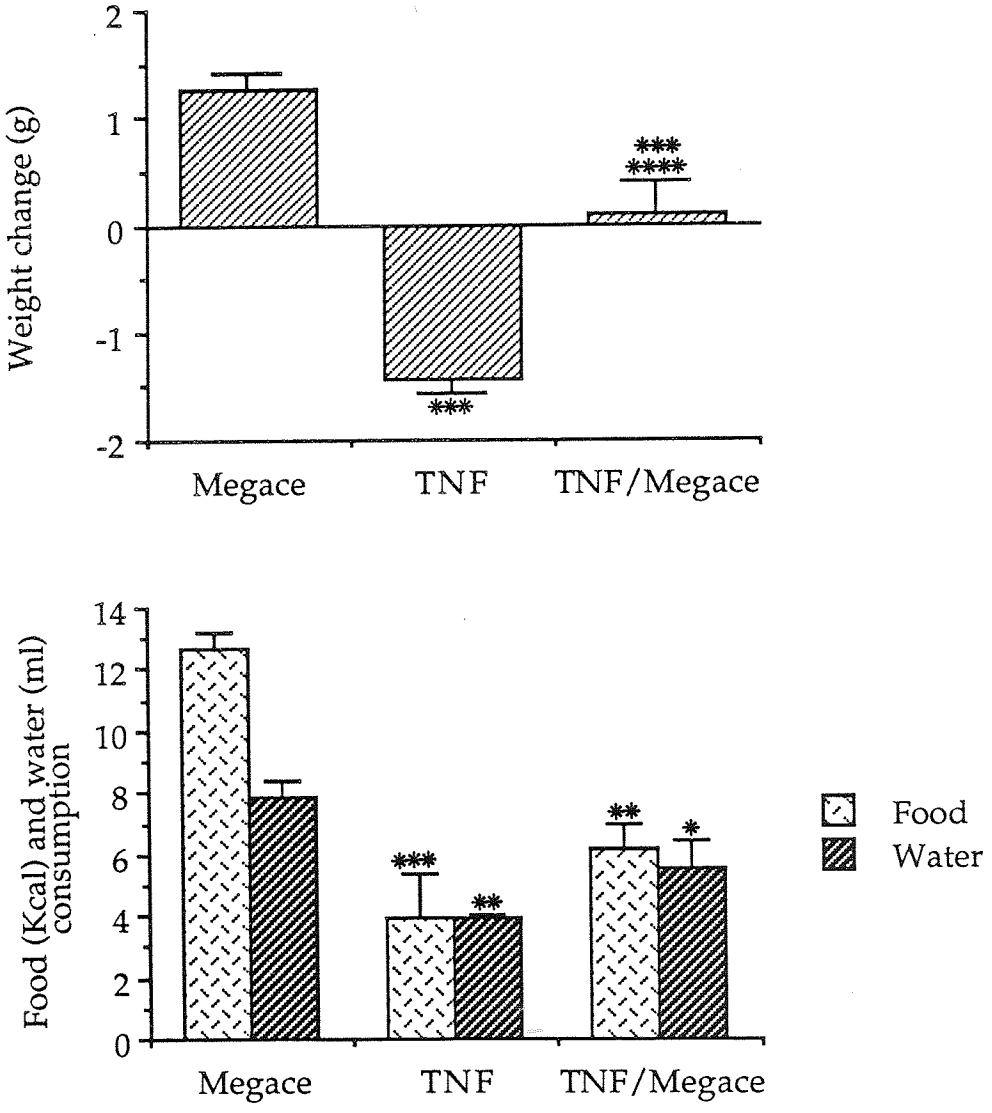

Table A2.1

The effect of Megace on the body compositions of TNF-treated female NMRI mice

Female NMRI mice (19±0.5g) were administered 7.5 X 10^7U/Kg TNF. i.v., and 150mg/Kg Megace, s.c., either alone or in combination. Controls were injected with 200µl of 0.9% NaCl, i.v., followed by 150mg/Kg Megace, s.c. Body composition analysis was performed 24h later. The values represent the means ± S.E.M. of 4 to 5 animals in each group. $^a p \leq 0.05$ and $^b p \leq 0.005$ from Megace alone by analysis of variance.

	Water content (g)	Fat content (g)	Left thigh and gastrocnemius muscle content (g)
Megace	13.6±0.3	1.8±0.1	0.06±0.002
TNF	12.2±0.2^b	1.3±0.1^a	0.06±0.002
TNF/Megace	12.9±0.2	1.6±0.1	0.06±0.003

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Br. J. Cancer (1988), 57, 183–189

Comparison of weight loss induced by recombinant tumour necrosis factor with that produced by a cachexia-inducing tumour

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Summary A comparison has been made of the cachectic effects produced by the transplantable murine adenocarcinoma of the mouse colon (MAC16) with tumour necrosis factor-α (cachectin). Tumour necrosis factor-α (TNF α) produced a dose-related weight reduction that was accompanied by a decrease in both food and water intake. The degree of weight loss was directly proportional to the decreased food and water intake. In contrast weight loss produced by the MAC16 tumour occurred without a reduction in fluid or nutrient intake. Both the MAC16 tumour and TNF-α produced hypoglycaemia and a reduction in the circulatory level of free fatty acids (FFA), but had opposite effects on the level of plasma triglycerides with the MAC16 tumour-induced cachexia causing a decrease and TNF-α producing an increase. The MAC16 tumour elaborated a lipolytic factor which caused an immediate release of FFA from adipose tissue. In contrast TNF-α had no effect on mobilization of adipose triglycerides over a short time period. Both TNF-α and extracts from the MAC16 tumour caused an enhanced release of amino acids from mouse diaphragm, which was suppressible with indomethacin and heat labile. No TNF was detected in the MAC16 tumour or in the serum of tumour-bearing animals. Both tumour and non-tumour-bearing animals responded with a similar elevation of their serum TNF levels 90 min after a single injection of endotoxin. It is concluded that weight loss produced by TNF-α arises from an anorexic effect and that this differs from the complex metabolic changes associated with cancer cachexia.

We have been investigating a chemically induced, transplantable adenocarcinoma of the colon (MAC16), passaged in inbred NMRI mice as an experimental model of cachexia (Bibby *et al.*, 1987). This tumour produces weight loss at small tumour burdens (< 1% of the host weight) and without a reduction in the intake of either food or water. The weight loss, which is directly proportional to the tumour weight, is associated with a decrease in both carcass fat and muscle dry weight (Beck & Tisdale, 1987). The cachectic effect of the tumour has been attributed to the production of both lipolytic and proteolytic factors, which are present in the circulation of tumour-bearing animals.

Endotoxin-induced cells of the reticuloendothelial system have been shown to elaborate a mediator called cachectin (tumour necrosis factor, TNF), which induces a state of cachexia in recipient animals (Cerami *et al.*, 1985). When chronically secreted by host macrophages cachectin has been suggested to contribute to a catabolic state, which ultimately leads to cachexia (Beutler & Cerami, 1986). Torti *et al.* (1985) have shown that cachectin acts to suppress the biosynthesis of several adipocyte-specific mRNA molecules and prevents morphological differentiation of pre-adipocytes. Lipoprotein lipase is one of the many enzymes whose transcription is suppressed by the action of this hormone (Price *et al.*, 1986*b*). Inhibition of lipoprotein lipase would prevent adipocytes from extracting fatty acids from plasma lipoproteins for storage. This would result in a net flux of lipid into the circulation, where the host defence could use it as an energy source. With chronic infectious challenge, however, wasting could persist and death would ensue (Beutler & Cerami, 1986).

In order to evaluate the role of TNF in cachexia we have compared the parameters contributing to weight loss in animals bearing the MAC16 tumour with that produced by human recombinant TNF-α, and sought to determine the presence of TNF either in tumour extracts or in the serum of tumour-bearing mice.

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Materials and methods

Animals

Pure strain NMRI mice (age 6–8 weeks) were purchased from Banting and Kingman, Hull and fed *ad libitum* a rat and mouse breeding diet (Pilsbury's, Birmingham, UK). All animals were given free access to water and both food and water intake were monitored daily. Fragments of the MAC16 or MAC13 tumours (1 × 2 mm in size) were implanted into the flank by means of a trocar as described (Bibby *et al.*, 1987). Positive takes can only be identified 14 days after transplantation.

TNF

Human recombinant TNF-α (6×10^7 U mg^{-1}) was kindly donated by Boehringer Ingelheim Ltd., Bracknell, Berks, and was stored at 4°C. The endotoxin content was < 0.125 EU ml^{-1} and there was no proteolytic contamination. Fresh solutions of TNF-α were made up daily in 0.9% NaCl and 200 μl of the appropriate concentration of TNF-α was injected into the tail veins of female NMRI mice. Controls were injected with 200 μl 0.9% NaCl. Body weights and food and water intake were monitored daily. A second injection of TNF-α was given 24 h after the first injection. Blood was removed by cardiac punture from animals under anaesthesia 1 h after the final injection of TNF-α.

Metabolite determinations

Blood glucose was determined on whole blood with the use of the *o*-toluidine reagent kit (Sigma Chemical Co., Dorset, UK). Free fatty acid (FFA) levels were measured in plasma with a Wako NEFA C kit (Alpha laboratories). Plasma triglycerides were determined with a triglyceride diagnostic kit (Sigma Diagnostic, Dorset, UK).

Primed TNF production

Non-tumour-bearing and MAC16 and MAC13 tumour-bearing male NMRI mice were administered 1.25 mg kg^{-1} *E. coli* lipopolysaccharide (Sigma Chemical Co., Dorset, UK) into the tail veins and blood was removed 1.5 h later by cardiac punture from animals under anaesthesia. Blood was allowed to clot, centrifuged and the resulting serum was used for TNF determinations.

TNF assay

TNF was determined by an *in vitro* method similar to that previously described by Ruff and Gifford (1981). L929 cells were seeded at a concentration of 3×10^4 per well into 96-well flat-bottom microtitre trays (Nunc, Denmark) in 100 µl RPMI 1640 medium (Gibco Europe, Paisley, Scotland) containing 10% foetal calf serum, and incubated at 37 C overnight under an atmosphere of 5% CO_2 in air. The medium was then removed and was replaced with varying dilutions of TNF-containing medium and actinomycin D (1 µg ml^{-1}) to a final volume of 100 µl. Controls contained only medium and actinomycin D. Internal standards contained medium with 1 unit of recombinant human TNF and actinomycin D. The plates were re-incubated for 16 to 18 h and the cells were stained with crystal violet. Rinsed and dried plates were enumerated spectrophotometrically at 570 nm on a Titerteck Multiscanner (Flow Laboratories) and the percentage of cell cytotoxicity was calculated as described by Flick and Gifford (1984, 1986).

Determination of lipolytic activity

The epididymal adipose tissue was removed from male BALB/c mice and minced in Krebs-Ringer bicarbonate buffer, pH 7.6. Approximately 50–100 mg of the adipose tissue was incubated with either the MAC16 tumour supernatant or TNF in a total volume of 0.25 ml of the Krebs-Ringer buffer. Controls containing adipose tissue and buffer alone were included in each experiment and the spontaneous release of free fatty acids (FFA) was subtracted from the values obtained with tumour present. The release of FFA by MAC16 tumour extracts was linear up to 2 h (Beck & Tisdale, 1987), and incubations were normally conducted for a 2 h period at 37 C. The concentration of FFA in the cell-free supernatants was determined immediately using a Wako NEFA C kit.

Determination of proteolytic activity

Male BALB/c mice were killed by cervical dislocation and diaphragms were carefully dissected out, blotted, cut in half, weighed and each half placed in a stoppered vial containing 0.75 ml Krebs-Ringer bicarbonate buffer and gassed for 20 sec with 5% CO_2 in air. Preincubations were carried out for 30 min at 37°C, and the diaphragms were then blotted and transferred to clean vials containing either tumour extract or TNF and the Krebs-Ringer buffer, in a total volume of 0.75 ml. The vials were gassed and incubated for a further 2 h at 37°C. Incubations were terminated by mixing 0.5 ml assay mixture with 0.125 ml of cold 50% TCA, mixing and centrifuging for 10 min at 3000 rpm. The supernatants were neutralised with 1 N NaOH and 0.2 ml of the neutralised sample was mixed with 1 ml of ninhydrin reagent, held in a boiling water bath for 20 min, and after dilution to 5 ml with *n*-propanol:water (1:1), the concentration of amino acids was determined spectrophotometrically at 570 nm. The spontaneous release of amino acids from the diaphragms in the absence of any additions was subtracted from the final readings.

Results

The characteristics of weight loss produced by the MAC16 adenocarcinoma passaged in NMRI mice has previously been reported (Bibby *et al.*, 1987, Beck and Tisdale, 1987). Briefly weight loss starts to occur when the tumour mass exceeds 0.1 g and reaches 10 g in a 30 g male mouse when the tumour mass is 0.7 g, representing just 2% of the weight of the animal. Both muscle and adipose mass decrease in direct proportion to the weight of the tumour (Beck & Tisdale, 1987). The average food intake in MAC16 tumour-bearing animals (15.1 ± 0.6 kcal day^{-1}) is not significantly different from that in non-tumour-bearing animals (14.9 ± 0.9 kcal day^{-1}). Also the water intake in tumour-bearing animals (4.6 ± 0.27 ml day^{-1}) does not differ from that of controls (4.8 ± 0.16 ml day^{-1}).

We have used female NMRI mice to study weight loss induced by TNF-α since they display a less aggressive behaviour than males, which may result in selective individuals being deprived food and water. Human recombinant TNF-α administered i.v. causes a dose-related weight loss after two separate injections over a 24 h period (Figure 1), which is significantly greater than saline injected controls at all concentrations of TNF-α employed. Qualitatively similar results were obtained with murine recombinant TNF-α, obtained from Dr W. Fiers, Biogent, Belgium (Marmenout *et al.*, 1985). No mortality was observed with any of the concentrations of TNF-α. This weight loss differs from that observed in MAC16 tumour-bearing animals in that it is associated with a dose-dependent decrease in both food (Figure 2) and water (Figure 3) consumption. The decrease in food and water intake is directly proportional to the decrease in body weight (Figure 4).

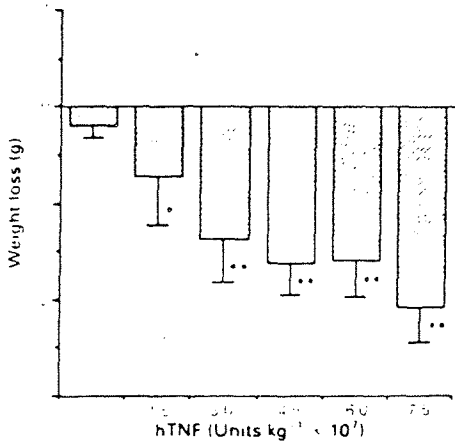

Figure 1 Effect of acute administration of TNF-α on the weight of female NMRI mice. Human recombinant TNF-α was administered i.v. as two separate injections over a 24 h period and the animals were killed 1 h after the last injection. The values represent the means ± s.e.m. for 4 to 11 animals for each concentration of TNF. $^*P<0.01$, $^{**}P<0.001$ from control by Students *t* test.

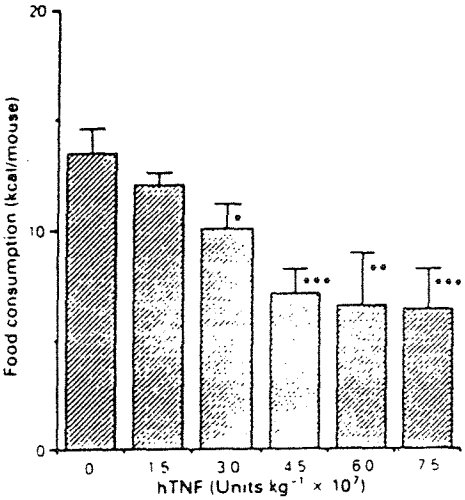

Figure 2 Effect of acute administration of TNF-α on food consumption of female NMRI mice during a 24 h period. The values represent the means ± s.e.m. for 4 to 11 animals for each concentration of TNF-α. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ from control by Students *t* test.

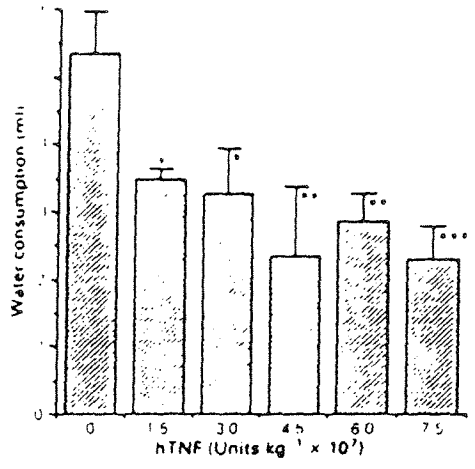

Figure 3 Effect of acute administration of TNF-α on water consumption of female NMRI mice during a 24 h period. The values represent the means ± s.e.m. for 4 to 11 animals for each concentration of TNF-α. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ from control by Students *t* test.

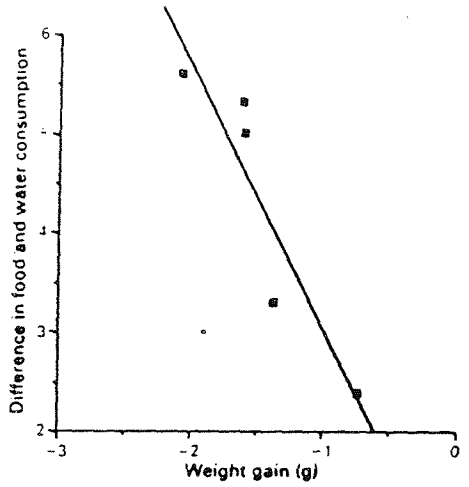

Figure 4 Variation of weight loss during a 24 h period after administration of TNF-α with the difference in food (kcal/mouse) and water (ml) consumption between a saline infused group and the TNF-α treated groups. The results were fitted to a linear model by means of a least squares analysis ($r=-0.99$).

Animals bearing the MAC16 tumour display a reduced blood glucose level. TNF-α treated mice also show a highly significant dose-related hypoglycaemia, which is much more pronounced than observed in weight-losing tumour-bearing animals (Table I). Plasma triglyceride levels are also reduced in tumour-bearing animals, whereas TNF-α causes an increase in circulatory triglycerides, presumably due to an inhibition of adipocyte lipoprotein lipase activity (Table I). Plasma levels of FFA are reduced after TNF-α administration, as might be expected from an inhibition of lipoprotein lipase and also in tumour-bearing animals, possibly due to increased tumour utilization.

The loss of body fat in MAC16 tumour-bearing animals has been correlated with the presence of a lipolytic substance produced by the tumour (Beck and Tisdale, 1987). This material is quantitated by the extent of release of FFA from mouse epididymal adipocytes. The results in Table II show that while extracts of the MAC16 tumour cause an enhanced release of FFA, TNF-α has no effect on the release of FFA under the conditions of the assay up to a concentration of 4×10^{5} units ml^{-1}. The MAC16 tumour also has high levels of proteolytic activity, which may be responsible for the muscle wasting (Beck & Tisdale, 1987) (Figure 5). Using the mouse diaphragm as a model of skeletal muscle, TNF-α at high concentrations also causes an enhanced release of amino acids (Figure 5). This effect is not due to contamination by endotoxin, since when the TNF-α is heated to 70°C for 15 min, which should destroy the TNF, but does not affect endotoxin, the proteolytic activity is completely destroyed. The proteolytic effect of TNF-α is almost completely suppressed by indomethacin and human α-1 antitrypsin. The proteolytic activity of the MAC16 tumour extract is also partially suppressed by indomethacin and there is a synergistic inhibition by a combination of indomethacin and antitrypsin (Figure 5). Proteolysis by trypsin is also inhibited by indomethacin. An enhanced amino acid release is also observed when diaphragms are incubated in the presence of PGE_2 or PGE_1, but not in the presence of $PGF_{1\alpha}$ or $PGF_{2\alpha}$ (Table III).

No TNF was detected either in the MAC16 tumour or in the serum of tumour-bearing mice using the L929 cytotoxicity assay. TNF was detected in the serum of non-tumour-bearing animals and in the serum of animals bearing the MAC16 and the non-cachexia inducing colon adenocarcinoma, MAC13, 90 min after a single i.v. injection of 25 μg endotoxin (Figure 6). However, there was no difference in the extent of response between non-tumour-bearing animals and animals bearing either type of tumour or in the levels of TNF in the two tumour types.

Discussion

The MAC16 tumour can be considered as an appropriate model for human cancer where weight loss occurs due to the biochemical effect of the tumour in patients with adequate

Table I Effect of recombinant TNF-α and the MAC16 tumour on the plasma level of glucose, FFA and triglycerides[a]

Treatment	*Glucose* (*mg 100 ml⁻¹*)	*FFA* (*mg 100 ml⁻¹*)	*Triglyceride* (*mM*)
Non-tumour-bearing	136 ± 5	29 ± 2	1.15 ± 0.11
Non-tumour-bearing (saline)	124 ± 5	32 ± 5	0.93 ± 0.31
MAC16 tumour-bearing	108 ± 11[b]	10 ± 1[c]	0.50 ± 0.07[d]
TNF-α 0.25 mg kg⁻¹	82 ± 8[b]	17 ± 2[b]	2.72 ± 0.15[e]
TNF-α 0.5 mg kg⁻¹	74 ± 7[d]	15 ± 3[b]	2.52 ± 0.14[e]
TNF-α 0.75 mg kg⁻¹	59 ± 4[d]	19 ± 3[b]	2.24 ± 0.32[e]

[a]Results are given as means ± s.e.m.; [b]$P<0.05$ from non-tumour-bearing animals; [c]$P<0.01$ from non-tumour-bearing saline infused animals; [d]$P<0.001$ from non-tumour-bearing saline infused animals; [e]$P<0.005$ from non-tumour-bearing saline infused animals.

Table II Effect of recombinant TNF-α and the MAC16 tumour on the release of FFA from adipocytes

Addition	nmol FFA mg protein $^{-1}$ h $^{-1}$ ± s.e.m.^{a}
MAC16 tumour extract	148.1 ± 8.3^a
4×10^1 units TNF-α^b	0
4×10^2 units TNF-α^b	0
4×10^3 units TNF-α^b	0

^{a}Results are expressed as means ± s.e.m., ^{b}TNF-α in units ml^{-1} of the assay mixture; ^{a}Mean of 11 determinations.

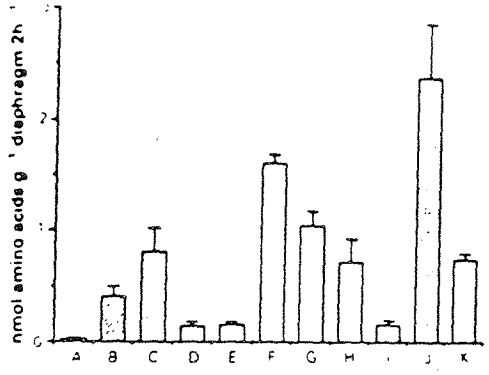

Figure 5 Rates of release of amino acids from mouse diaphragms by MAC16 tumour homogenate and TNF-α. (a) 10^2U TNF-α per assay. (b) 10^4U TNF-α per assay. (c) 10^3U TNF-α per assay. (d) 10^3U TNF-α + 1.0 mM indomethacin. (e) 10^3U TNF-α + 1 mg ml^{-1} α − 1 antitrypsin. (f) MAC16 tumour extract; 2.9 mg protein ml^{-1}. (g) MAC16 tumour extract + 1.0 mM indomethacin. (h) MAC16 tumour extract + 1 mg ml^{-1} antitrypsin. (i) MAC16 tumour extract + 1.0 mM indomethacin + 1 mg ml^{-1} antitrypsin. (j) Trypsin: 0.1 mg ml^{-1}. (k) Trypsin 0.1 mg ml^{-1} + 1.0 mM indomethacin. (b) and (c) $P<0.05$ from Krebs Ringer buffer alone. (d) and (e) $P<0.05$ from (c). (c) $P<0.05$ from (f). (h) and (i) $P<0.001$ from (f), by Students t test.

Table III Effect of prostaglandins on the release of amino acids from mouse diaphragm

	Concentration (μg ml^{-1})	nmoles amino acid released g.diaphragm^{-1} 2h^{-1} ± s.e.m.
PGE_1	5	0.028 ± 0.024
	10	0.085 ± 0.006^a
	20	0.234 ± 0.066^b
PGE_2	5	0.069 ± 0.022^b
	10	0.242 ± 0.079^b
	20	0.369 ± 0.036^a
PGF_1	5	0.000
	10	0.000
	20	0.040
PGF_2	5	0.000
	10	0.000
	20	0.000

$^a P<0.005$ from spontaneous release; $^b P<0.05$ from spontaneous release.

nutrient intake and without intestinal malfunction. In contrast TNF induces a state of anorexia and the ensuing weight loss is directly proportional to the decrease in food and water intake. A similar effect has been observed in mice injected with dialyzed conditioned medium obtained from lipopolysaccharide-induced peritoneal macrophages (Cerami *et al.*, 1985). Although all the experiments have been performed with human TNF-α similar results were obtained

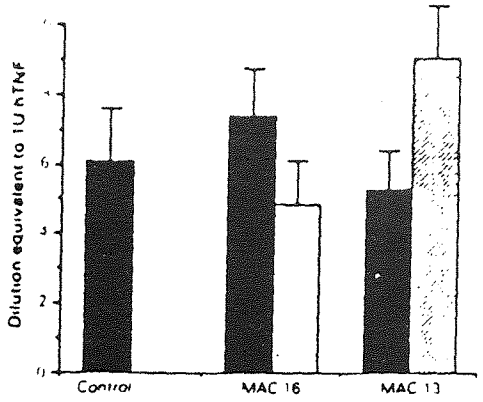

Figure 6 Production of TNF by endotoxin in unprimed mice. The TNF concentration in serum (■) and tumour (▨) was determined by means of the L929 cytotoxicity assay as described in Materials and methods.

with murine TNF-α. Marmenout *et al.* (1985) have shown that in spite of the apparent species specificity of TNF, human TNF is about 80% homologous to mouse TNF, and its hydrophilicity plot is also very similar.

The weight loss produced by both TNF-α and the MAC16 tumour is associated with hypoglycaemia, although TNF produces a more marked and possibly life-threatening decline in blood glucose levels. While administration of lipopolysaccharide has been shown to induce hypoglycaemia, Satomi *et al.* (1985) reported no hypoglycaemia in mice administered highly purified TNF. However, Kettlehut *et al.* (1987) have recently demonstrated large biphasic changes in blood glucose levels after TNF injection, with an initial hyperglycaemia followed by a sharp decrease in blood glucose. It has been suggested (Kettlehut *et al.*, 1987) that TNF may stimulate glucose uptake and oxidation contributing to the severe hypoglycaemia. In contrast the hypoglycaemia observed in animals bearing the MAC16 tumour probably arises from an increased consumption of glucose by the tumour (Tisdale & Brennan, 1986).

The MAC16 tumour and TNF-α differ as regards their effect on lipid metabolism in weight-losing animals. Thus, whereas animals bearing the MAC16 tumour have a reduced circulatory level of both FFA and triglycerides, TNF-α causes an increase in plasma triglyceride levels probably due to an inhibition of lipoprotein lipase activity. While lipoprotein lipase activity has been shown to be decreased in mice with the development of Sarcoma 180 (Masuno & Okuda, 1986) we have no evidence for an effect on lipoprotein lipase activity in animals bearing the MAC16 tumour, despite a massive loss of adipose tissue. This catabolism of adipose tissue has been attributed to the production by the tumour of a lipolytic factor (Beck & Tisdale, 1987). However, we have observed no increased breakdown of stored triglycerides in adipose tissue in the presence of TNF-α. While Kawakami *et al.* (1987) have reported that TNF-α increased the lipolysis of stored fat in 3T3-L1 adipocytes, even in the presence of 50 ng ml^{-1} of insulin, Price *et al.* (1986*b*) have shown that while crude preparations of TNF were able to suppress the activity of key lipogenic enzymes and stimulate lipolysis, recombinant TNF-α had no effect on either the ability of the adipocytes to synthesize and store or to mobilize triacylglycerols. The lipolytic activity of stimulated macrophages was attributed to interleukin 1, which both suppressed lipoprotein lipase activity and stimulated lipolysis (Price *et al.*, 1986*a*). Another possible reason for the absence of lipolysis we observed with our TNF-α preparation was the relatively short incubation time that we employed (2 h). Kawakami *et al.* (1987) did not observe an increase in glycerol production

in 3T3-L1 cells until 12 h after the addition of TNF-α, after which there was a linear increase in production up to 24 h. Kettlehut *et al.* (1987) have shown that the toxic and metabolic effects of TNF probably arise from an increased prostaglandin E_2 production since the cyclooxygenase inhibitors indomethacin or ibuprofen administered before TNF reduced the lethality and changes in blood glucose. We have shown (Beck & Tisdale, 1988) that the lipolytic substance elaborated by the MAC16 tumour is not a prostaglandin since indomethacin had no effect on FFA release at concentrations up to 1 mM.

The MAC16 tumour also elaborates a serine-protease when measured by an accelerate rate of release of amino acids from mouse diaphragm as a model of skeletal muscle (Beck & Tisdale, 1987). Using a similar assay we have detected a proteolytic activity associated with high level of TNF-α. This activity was not due to the small amount of endotoxin contamination since it was destroyed by heating, and not due to the presence of endogenous proteases in the TNF-α preparation (Boehringer Ingelheim, pers. comm.). Proteolysis induced by both TNF-α and the MAC16 tumour extract is suppressible by indomethacin suggesting the possibility of a prostaglandin intermediate. We have shown that prostaglandins of the E series, but not of the F, are also effective in inducing amino acid release from mouse diaphgram. PGE_2 is believed to be an important stimulus for the production of intracellular proteases (Rodemann & Goldberg, 1982). Moreover, TNF-α has been reported to stimulate collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts (Dayer *et al.*, 1985). This suggests that the enhanced release of amino acids from mouse diaphragm in the presence of TNF is due to an elevation of PGE_2 levels.

We have been unable to detect TNF either in the MAC16 tumour, or in the serum of tumour-bearing animals. Animals bearing either the MAC16 or the non-cachexing-inducing MAC13 colon adenocarcinomas do not respond to endotoxin with an increased TNF production compared with non-tumour bearing controls. This negates against a synergistic influence of the presence of a tumour on TNF production in response to endotoxin.

The results suggest that TNF has no role in the induction of cachexia seen in animals bearing the MAC16 tumour. Although we have compared the chronic secretion of factors produced by the MAC16 tumour with the acute effects of TNF we have shown (Mahony and Tisdale, unpublished results) that chronic exposure to TNF does not differ appreciably from the acute effects. Furthermore the weight loss produced by TNF appears to arise from an anorexic effect of this agent and this differs from the changes associated with cancer cachexia.

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Induction of weight loss and metabolic alterations by human recombinant tumour necrosis factor

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Summary A comparison has been made of the weight loss produced by tumour necrosis factor (TNF) (cachectin) with that produced by a restricted food and water intake (pair-fed controls), and by mitozolomide, a drug which in toxic doses induces weight loss with a similar decrease in nutrient and water intake. When administered as two separate injections over a 24 h period (acute administration) TNF produced a dose-related weight reduction that was accompanied by and directly proportional to a decrease in both food and water intake. When administered daily by i.v. injection over a 5-day period (chronic administration) the major weight loss was found to occur during the first 24 h after injection and thereafter the weight of treated mice increased toward that of controls. Acute administration of TNF produced hypoglycaemia that was more severe than observed with either mitozolomide or in pair-fed controls, a reduction in the circulatory level of free fatty acids (FFA) and an increase in plasma triglycerides, while mitozolomide and pair-feeding had no effect on the level of blood glucose or plasma triglycerides. Body composition analysis showed a loss of adipose tissue in TNF-injected and pair-fed animals after both acute and chronic treatment. Acute administration of TNF also induced a decrease in the total body water content of treated animals which was similar to pair-fed controls. It is concluded that the weight loss produced by TNF arises from a combination of semi-starvation and a reduced water intake, and that the effect only occurred with the first administration of TNF.

Cachexia is often reported as the most frequent cause of death in cancer patients (Robbins, 1962) and is characterized by the development of progressive weakness, weight loss and wasting. The frequency of cancer cachexia varies with tumour type, with gastrointestinal cancers and lung cancer having the greatest incidence (Strain, 1979). Cachexia may occur with a small primary tumour and may precede the clinical diagnosis. The weight loss associated with cachexia may be accompanied by marked anorexia (Garrattini *et al.*, 1980). However, cancer cachexia is more like the condition produced by a major injury or sepsis, rather than that due to simple starvation (Brennan, 1977). Anorexia seems to be only a partial cause of the wasting process, since in both rat and man loss of both muscle and adipose tissue frequently precedes a fall in food intake (Costa, 1963). This suggests that metabolic disturbances within the tumour or the host tissues also contribute.

Several tumour-associated factors with a possible significance in the aetiology of the cachectic syndrome have been reported. Most recently a macrophage product, cachectin, has been suggested to orchestrate the complex metabolic changes that lead to cachexia. Cachectin has been shown to inhibit lipoprotein lipase activity in adipose tissue resulting in a marked elevation of plasma very low density lipoprotein (Beutler *et al.*, 1985*a*). Cachectin is an acidic protein which has been shown to be homologous to tumour necrosis factor (TNF) (Beutler *et al.*, 1985*b*), a macrophage product of molecular weight 17,000 that can be induced by endotoxin and other microbial products. When mice were passively immunized with a highly specific polyclonal rabbit antiserum directed against murine TNF they were protected against the lethal effect of the endotoxin lypopolysaccharide produced by *E. coli* (Beutler *et al.*, 1985*c*). This suggests that cachectin/TNF is one of the principal mediators of the lethal effect of endotoxin. In addition a considerable amount of evidence has implicated cachectin as a central mediator of the wasting that accompanies chronic invasive disease states (Beutler & Cerami, 1986). Animals inoculated intramuscularly with a rodent tumour cell line which continuously secretes human TNF were recently shown to develop severe cachexia and weight loss (Oliff *et al.*, 1987).

In order to evaluate the role of TNF in cachexia we have compared the parameters contributing to weight loss produced by human recombinant TNF with that in pair-fed animals and in animals injected with mitozolomide, a drug which in toxic doses also induces weight loss with a decrease in nutrient and water intake. The effects of acute and of chronic administration of TNF on NMRI mice are compared.

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Materials and methods

Animals

Pure strain NMRI mice (age 6–8 weeks) were purchased from Banting and Kingman, Hull, UK, and were fed *ad lib.* a rat and mouse breeding diet (Pilsbury's, Birmingham, West Midlands, UK). All animals were given free access to food and water and both food and water intake were monitored daily.

TNF

Human recombinant TNF (6×10^7 U mg^{-1}) was kindly donated by Boehringer Ingelheim Ltd., Bracknell, Berks and stored at 4°C. The endotoxin content was <0.125 EU ml^{-1}. Fresh solutions of TNF were made up daily in 0.9% NaCl and 200 µl of the appropriate concentration of TNF was injected into the tail veins of female NMRI mice (19–22 g). Controls were injected with 200 µl 0.9% NaCl. Injections were administered at the same time each day for 5 days (chronic dosage) or as two separate injections over a 24 h period (acute dosage). Body weights and food and water intake were monitored daily. Food intake was measured by weighing the pellets remaining. Food wastage was minimal using pelleted food. Water consumption was determined by volume. Water bottles contained a ball valve to prevent dripping. Blood was removed by cardiac puncture from animals under anaesthesia 1 h after the final injection of TNF.

Mitozolomide

Fresh solutions of mitozolomide (May and Baker Ltd., Dagenham, UK) in arachis oil containing 10% DMSO were made up daily and 20 mg kg^{-1} were injected i.p. into female NMRI mice (19.3 ± 0.15 g). Controls were injected with

Table I Effect of recombinant TNF, mitozolomide and pair-feeding on body weight, blood glucose and the plasma level of triglycerides[a]

Treatment	Weight change[b] (g)	Glucose (mg 100ml^{-1})	Triglyceride (mM)
Controls no treatment	+0.08 ± 0.3	122 ± 5	1.23 ± 0.2
Controls i.v. saline	−0.19 ± 0.1	120 ± 4	1.01 ± 0.2
Controls i.p. arachis oil + 10% DMSO	−0.46 ± 0.2	117 ± 5	1.32 ± 0.2
Controls pair-fed	−2.34 ± 0.3[c]	128 ± 11[c]	1.49 ± 0.4
Mitozolomide 20 mg kg^{-1}	−1.22 ± 0.2[d]	105 ± 7	1.47 ± 0.1
TNF 1.5 × 10^7 U kg^{-1}	−0.73 ± 0.5	89 ± 3[d]	2.71 ± 0.2[d]
TNF 3.0 × 10^7 U kg^{-1}	−1.38 ± 0.4[d]	71 ± 8[d]	2.52 ± 0.1[d]
TNF 4.5 × 10^7 U kg^{-1}	−1.62 ± 0.3[d]	58 ± 3[d]	2.43 ± 0.4[d]
TNF 6.0 × 10^7 U kg^{-1}	−1.60 ± 0.4[d]	56 ± 9[d]	2.34 ± 0.1[d]
TNF 7.5 × 10^7 U kg^{-1}	−2.08 ± 0.4[d]	44 ± 4[d]	2.37 ± 0.2[d]

[a]Results are given as means ± s.e.m. for 6 to 13 animals per group. [b]The weight change over 24 h for TNF-treated, pair-fed and mitozolomide-treated mice; [c]$P < 0.001$ from controls (no treatment); [d]$P < 0.001$ from arachis oil + 10% DMSO infused controls; [d]$P < 0.001$ from saline infused on pair-fed controls.

arachis oil containing 10% DMSO. Body weights and food and water intake were monitored and blood was removed by cardiac puncture from animals under anaesthesia 24 h after the injection.

Pair-feeding

Female NMRI mice (19.2 ± 0.46 g) were given the same amount of food and water (given every 6 h) both over a 24 h period (acute) and over a 5 day period (chronic) as that consumed by mice following injection of 7.5 × 10^7 U kg^{-1} TNF. Body weights were then monitored and blood was removed by cardiac puncture from animals under anaesthesia.

Metabolite determinations

Blood glucose was determined on whole blood with the use of the *o*-toluidine reagent kit (Sigma Chemical Co., Dorset, UK). FFA levels were measured in plasma with a WAKO NEFA C kit (Alpha Laboratories, Hampshire, UK). Plasma triglycerides were determined with a triglyceride diagnostic kit (Sigma Diagnostics, Dorset, UK).

Body composition analysis

The gastrocnemius and thigh muscles from the left hind leg of mouse carcasses were carefully dissected out and weighed, together with the whole carcass. Each carcass plus muscles were heated at 80°C until a constant weight was achieved. Carcasses were then reweighed and the water content was determined from the difference between the wet and dry weights. Total carcass fat was determined by the method of Lundholm *et al.* (1980).

Statistical analysis

All results were analysed statistically using the analysis of variance or F-ratio.

Results

Human recombinant TNF administered i.v. causes a dose-related weight loss after two separate injections over a 24 h period (Table I), which is significantly greater than the saline injected controls at all concentrations of TNF employed. Mice receiving daily injections of TNF exhibit a biphasic decrease in the relative body mass, which is dose-related (Figure 1). No morbidity or mortality was observed with any of the concentrations of TNF employed. When the actual daily weight loss for the three concentrations of TNF are plotted (Figure 2), it can be seen that all of the weight loss occurs during the first 24 h after injection and thereafter the body weight increases towards that of saline injected controls, despite further daily injections of TNF.

Both the food and water consumption of mice receiving daily injections of TNF closely follows the pattern of weight loss (Figures 3 and 4) with an initial dose-dependent sharp decrease, followed after a period of 1 to 2 days in an

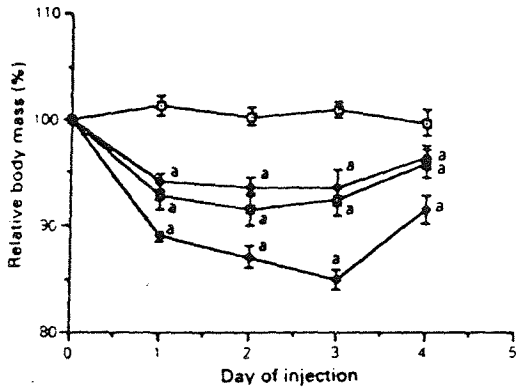

Figure 1 Effect of daily administration of TNF on the weight of female NMRI mice expressed as percentage relative body mass. Human recombinant TNF was administered daily by i.v. injection over a 5 day period and the animals were killed 1 h after the final injection. The average of the body mass of the animals in each group at day 0 was taken as 100%. Animals were infused with saline (□) or with 4.5 × 10^7 U kg^{-1} (◆), 6.0 × 10^7 U kg^{-1} (■) or 7.5 × 10^7 U kg^{-1} (◇) of TNF. (a) $P < 0.001$ from control by analysis of variance.

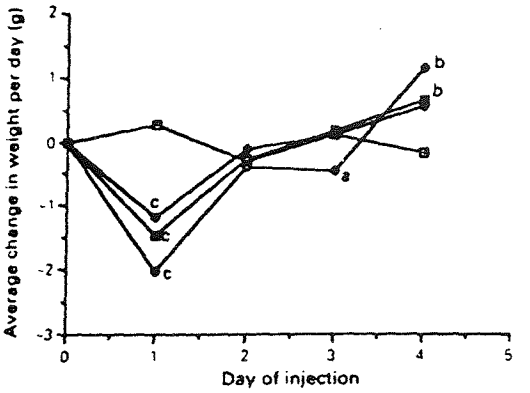

Figure 2 Effect of daily administration of TNF on the weight female NMRI mice. Animals were infused with saline (□) or with 4.5 × 10^7 U kg^{-1} (◆), 6.0 × 10^7 U kg^{-1} (■) or 7.5 × 10^7 U kg^{-1} (◇) of TNF. The values represent means ± s.e.m. for 6 to 13 animals for each concentration of TNF. Error bars were omitted in order to simplify the diagram. (a) $P < 0.01$, (b) $P < 0.005$, (c) $P < 0.001$ from control by analysis of variance.

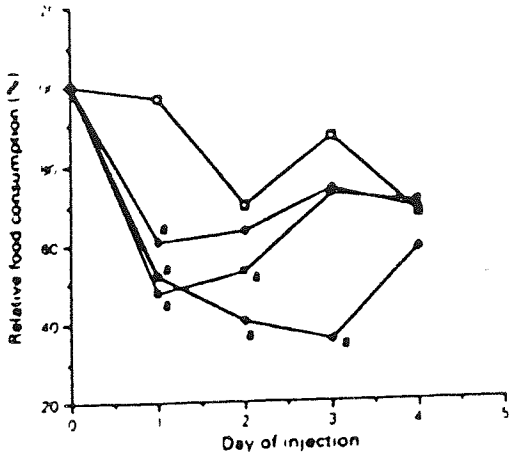

Figure 3 Effect of daily administration of TNF on the food consumption of female NMRI mice as measured at the same time each day. The values represent the means ± s.e.m. for 4 to 13 animals for each concentration of TNF. Error bars were omitted in order to simplify the diagram.. The symbols represent the same concentration of TNF as in Figures 1 and 2. (a) $P<0.005$ from control by analysis of variance.

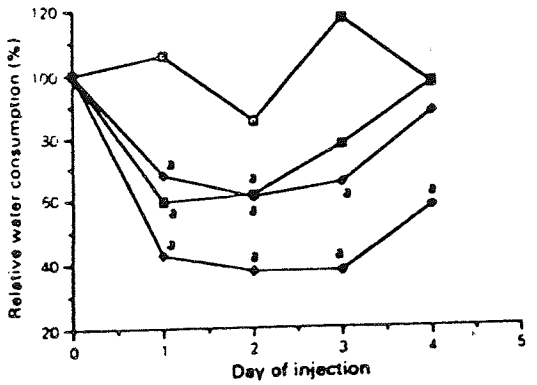

Figure 4 Effect of daily administration of TNF on the water consumption of female NMRI mice as measured at the same time each day. The values represent the means ± s.e.m. for 4 to 13 animals for each concentration of TNF. Error bars were omitted in order to simplify the diagram. The symbols represent the same concentrations of TNF as in Figures 1 and 2. (a) $P<0.005$ from control by analysis of variance.

increased consumption towards the levels found in the controls. The initial decrease in food and water intake is directly proportional to the decrease in body weight of the animals over the first 24 h (Figure 5). Animals given the same amount of food and water as that consumed by those injected with the highest concentration of TNF (7.5×10^7 U kg^{-1}) lost the same amount of weight over a 24 h period (Table I). Mitozolomide is a cytotoxic drug, which at a concentration of 20 mg kg^{-1} causes general malaise and a decrease in food and water consumption equal to that obtained with 7.5×10^7 U kg^{-1} of TNF. The results in Table I show that there is no significant difference between the weight loss produced by mitozolomide and the equivalent concentration of TNF, suggesting that weight loss produced by TNF may be due to a generalized cytotoxicity.

TNF treated mice show a highly significant hypoglycaemia 60 to 90 min after the second of 2 injections over a 24 h period, but not after 5 daily injections of TNF (Figure 6, Table I). The TNF-induced hypoglycaemia is directly proportional to both the decrease in body weight (Figure 7) and

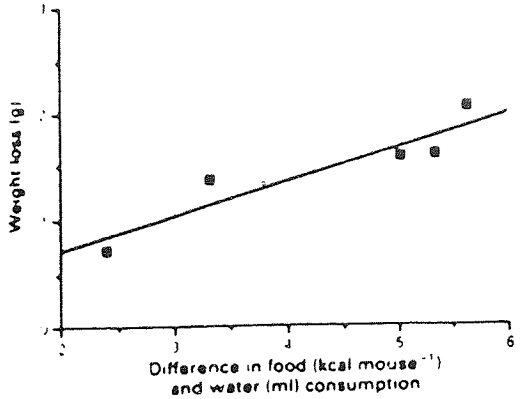

Figure 5 Variation of weight loss (g) during a 24 h period after administration of TNF with the difference in food (Kcal mouse^{-1}) and water (ml) consumption between a saline infused group and TNF treated groups. The results were fitted to a linear model by means of a least squares analysis ($r=-0.92$).

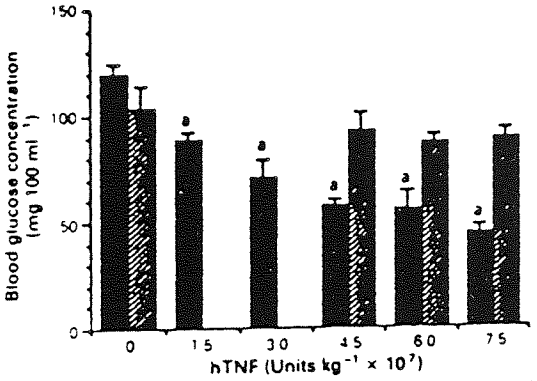

Figure 6 Comparison of the effects of acute and chronic administration of TNF on the blood glucose concentration of female NMRI mice. The values represent the means ± s.e.m. for 4 to 11 (acute dosage) (■) or 6 to 13 (chronic dosage) (▨) animals for each concentration of TNF. (a) $P<0.001$ from saline infused controls by analysis of variance.

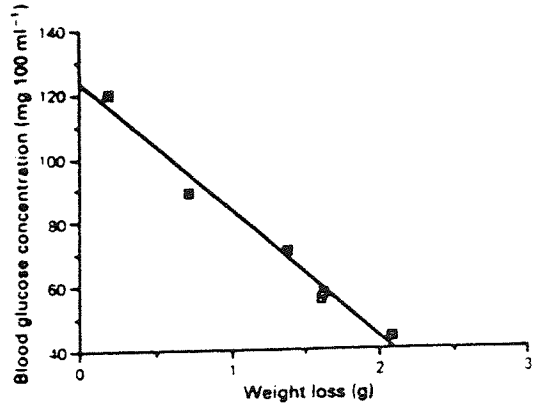

Figure 7 Variation of blood glucose concentration (mg 100 ml^{-1}) with weight loss (g) during a 24 h period after administration of TNF. The results were fitted to a linear model by means of a least square analysis ($r=-0.99$).

to the decrease in food and water consumption over the first 24 h following injection (Figure 8), and is much more

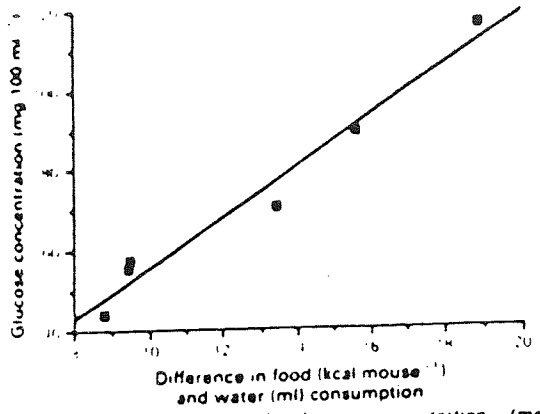

Figure 8 Variation of blood glucose concentration (mg 100 ml^{-1}) with the difference of food (Kcal mouse^{-1}) and water (ml) consumption during a 24 h period between a saline infused group and the TNF treated groups. The results were fitted to a linear model by means of a least squares analysis ($r = 0.98$).

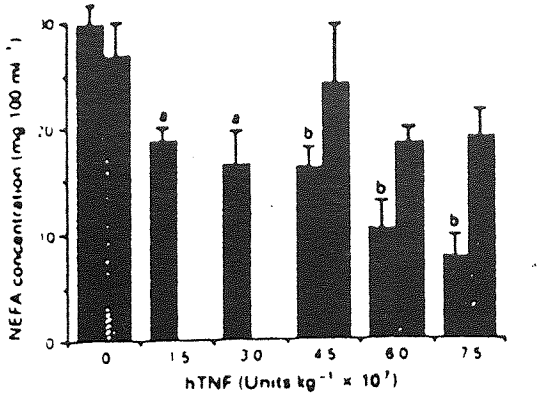

Figure 10 Comparison of the effects of acute (■) and chronic (▨) administration of TNF on the plasma NEFA concentration of female NMRI mice. The values represent the means ± s.e.m. for 4 to 7 (acute dosage) or 6 to 13 (chronic dosage) animals for each concentration of TNF. (a) $P < 0.005$, (b) $P < 0.001$ from saline infused controls by analysis of variance.

pronounced than observed in weight-losing, pair-fed or mitozolomide-treated animals (Table I).

Marked hypertriglyceridemia is observed after acute administration of TNF (Figure 9, Table I) and may be either due to inhibition of lipoprotein lipase activity, or to increased hepatic triglyceride synthesis (Feingold *et al.*, 1987). This increase in triglyceride levels is also directly proportional to the decrease in food and water consumption of treated animals as compared to control ($r = -0.93$). In contrast, pair-feeding and mitozolomide induced no significant changes in plasma triglyceride levels of treated animals. Plasma levels of FFA were reduced after acute TNF administration, but not after chronic administration (Figure 10).

The decrease in body weight was accompanied by a dose-related decrease in total body fat after both acute and chronic administration of TNF when compared with saline infused controls (Table II). There was no difference in body fat content between the TNF treated and pair-fed controls. There was a decrease in total body water content after acute administration, but an increase was observed after 5 daily injections of TNF, when compared with saline infused controls. There was no difference in body water between TNF treated, pair-fed or mitozolomide treated animals. No change in thigh and gastrocnemius muscle content was observed after either acute or chronic administration of TNF. No change was observed in the body composition of mitozolomide-treated mice as compared to arachis oil injected controls except for a decrease in carcass water content.

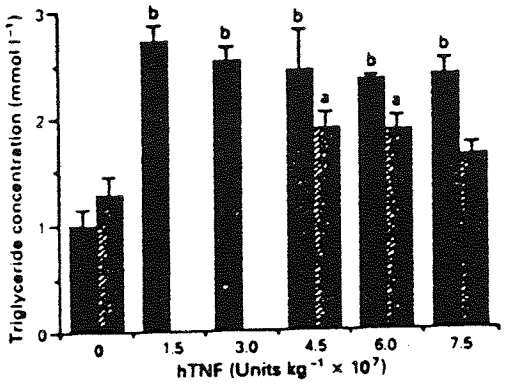

Figure 9 Comparison of the effects of acute (■) and chronic (▨) administration of TNF on the plasma triglyceride concentration of female NMRI mice. The values represent the means ± s.e.m. for 4 to 11 (acute dosage) or 6 to 13 (chronic dosage) animals for each concentration of TNF. (a) $P < 0.05$, (b) $P < 0.001$ from saline infused controls by analysis of variance.

Discussion

Daily administration of TNF to female NMRI mice has been shown to induce a transient state of anorexia with the ensuing weight loss being directly proportional to the decrease in food and water intake. A similar effect has been observed by Cerami *et al.* (1985) in mice injected with dialysed conditioned medium obtained from lipopolysaccharide-induced peritoneal macrophages. However, whereas these mice were reported to continue to lose weight, the weight loss in human recombinant TNF-treated mice only occurs over the first 24 h; thereafter the weight of treated mice returns towards that of controls, as does the food and water consumption. Thus the anorexic effects of TNF are confined to the initial exposure, and thereafter the animals become resistant to subsequent dosing. This probably explains the lack of weight loss in cancer patients administered TNF in phase I studies (Blick *et al.*, 1987). However, progressive weight loss has been observed in mice bearing CHO cells transfected with the human TNF/cachectin gene (Oliff *et al.*, 1987). This weight loss appeared to be due, at least in part, to reduced food consumption.

The weight loss induced by TNF appears to be directly related to the decrease in food and water consumption since animals fed the same amount of food and water lost the same amount of weight as the TNF treated group. In addition the body composition of the pair-fed group did not differ significantly from the TNF treated animals. No muscle breakdown occurred either after semi-starvation or after chronic administration of TNF at any of the concentrations employed.

The initial weight loss produced by TNF is associated with a marked and possibly life-threatening hypoglycaemia. While administration of lipopolysaccharide has been shown to induce hypoglycaemia, Satomia *et al.* (1985) reported no hypoglycaemia in mice administered highly purified TNF. However, Kettlehut *et al.* (1987) have recently demonstrated large biphasic changes in blood glucose levels after TNF induce hypoglycaemia, Satomi *et al.* (1985) reported no decrease in blood glucose. Since the TNF-induced hypoglycaemia which we observed is directly proportional to both the decrease in food and water intake and to the decrease in body weight of mice and, since no hypoglycaemia is observed after 5 daily injections of TNF when the mice are regaining weight, this may be an important feature in the

Table II Effect of recombinant TNF, pair feeding and mitozolomide on the body composition of female NMRI mice*

Treatment	Water content[1] (g)		Fat content[2] (g)		Muscle content[3] (g)	
	Acute	Chronic	Acute	Chronic	Acute	Chronic
Controls – no treatment	13.07 ± 0.362		2.30 ± 0.19		0.0695 ± 0.005	-
Controls – i.v. saline	13.11 ± 0.24	12.56 ± 0.20	2.20 ± 0.11	1.79 ± 0.12	0.065 ± 0.0015	0.0613 ± 0.002
Controls – i.p. arachis oil ± 10% DMSO	13.25 ± 0.52	-	1.79 ± 0.10	-	0.0685 ± 0.007	-
Control pair-fed	11.80[a] ± 0.24	11.54 ± 0.40	1.62[a] ± 0.13	0.916 ± 0.107	0.062 ± 0.003	0.053 ± 0.004
Mitozolomide 20 mg kg^{-1}	11.70[a] ± 0.168	-	1.84 ± 0.22	-	0.062 ± 0.0005	-
TNF 7.5×10^{4} U kg^{-1}	11.99[a] ± 0.09	13.35[b] ± 0.18	1.54[d] ± 0.23	0.61[d] ± 0.08	0.067 ± 0.023	0.063 ± 0.003

*Results are given as means ± s.e.m. for 6 to 13 animals per group. Starting weight for animals in all groups 19.2 ± 0.46 g ($n = 57$). [1]Body water content in g. [2]Body fat content in g. [3]Left thigh and left gastrocnemius muscles in g. [a]$P < 0.01$ from controls – no treatment; [b]$P < 0.05$ from controls – i.v. saline; [c]$P < 0.01$ from controls – arachis oil; [d]$P < 0.01$ from controls – i.v. saline.

metabolic perturbations induced by TNF. The observed hypoglycaemia is probably due to a direct action of TNF, and not merely due to a decrease in food and water consumption since the decrease in blood glucose is much more pronounced in TNF injected animals (7.5×10^{4} U kg^{-1}) than in pair-fed mice, even though the latter consumed the same amount of food and water, or in mitozolomide-treated mice despite a decrease in body weight with a decrease in nutrient intake. This severe hypoglycaemia could possibly be due to TNF stimulating glucose uptake and oxidation.

Semb *et al.* (1987) has recently shown that the suppression of lipoprotein lipase activity by TNF is confined to adipose tissue and that increased enzyme activity is observed in several other tissues, most notably the liver and also in plasma. Although fasting also leads to a decrease in lipoprotein lipase activity in epididymal adipose tissue the effect of TNF on adipocyte gene expression differs from that in the fasted state.. We have observed a marked hypertriglyceridemia and a decreased level of FFA in mice 60 to 90 min after the second of two injections of human TNF over a 24 h period. This hypertriglyceridemia probably arises from an inhibition of adipocyte lipoprotein lipase, since the pool of plasma triglyceride is comparatively small and a minor impairment of the triglyceride removal mechanism would probably increase the size of this plasma pool (Nilsson-Ehle, 1980). Although there was no correlation with the increase in triglyceride levels and the weight loss of mice, the decrease in plasma FFA observed was directly proportional to the weight loss in TNF-treated mice. Lipoprotein lipase inhibition and hence increased plasma triglyceride levels is due to a direct action of TNF and not merely due to a decrease in nutrient intake as mitozolomide-treated and pair-fed mice showed no changes in the level of circulating triglycerides.

The results suggest that the weight loss produced by TNF appears to arise from an anorexic or toxic effect of this agent, and that animals become refractory to subsequent administration of this cytokine.

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A.13 Weight loss and metabolic alterations induced by recombinant human tumour necrosis factor

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We have investigated the ability of rhTNF to elicit cachectic changes in female NMRI mice. When administered daily by i.v. injection over a 4 day period, the major weight loss was found to occur during the first 24 hrs after injection and, thereafter, the weight of the treated mice increased towards that of controls. When given as two separate injections over a 24 h period, there as a decrease in both food and water intake and a dose-dependent decrease in body weight. Hypoglycaemia, hypertriglyceridaemia and a decreased level of circulating FFA were observed 60 to 90 min after the final injection. Body composition analysis showed a loss of adipose tissue in TNF-treated animals without an effect on the lean body mass or on the water content. Thus it appears that acute administration of rhTNF has a temporary anorexic effect on mice.

Characterisation and reversal of weight loss induced by recombinant human tumour necrosis factor (TNF)

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When administered as a single i.v. injection human recombinant TNF produced a dose-related weight reduction that was accompanied by and directly proportional to a decrease in both food and water intake. When given as two separate injections over a 24 h period, hypoglycaemia, hypertriglyceridaemia and a decreased level of circulatory FFA were observed 60 to 90 min after the final injection. The degree of hypoglycaemia is proportional to both the weight loss and the decrease in food and water intake.

In order to investigate the mechanism of the weight loss produced by TNF, animals were dosed orally with either water alone or a concentrated glucose solution. Weight loss was reversed by oral administration of water; the increase in weight being accompanied by an increase in total body water. Administration of a concentrated glucose solution had no greater effect than water alone in reversing the weight loss, indicating that the observed hypoglycaemia is not important in this action of TNF. Weight loss produced by TNF was also reversed by the i.p. administration of indomethacin (10 mg kg^{-1}) 2 h prior to the TNF injection. These results suggest that weight loss produced by TNF appear to be the result of prostaglandin production and dehydration.

WA 326 THE EFFECT OF RECOMBINANT HUMAN TUMOUR NECROSIS FACTOR ON GLUCOSE METABOLISM AND UTILIZATION IN FEMALE NMRI MICE.

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Intravenous administration of rhTNF to female NMRI mice resulted in a marked decrease in blood glucose when compared with saline-injected pair-fed controls. The hypoglycaemia was significant 2h after injection and was still evident 24h later. This reduction in blood glucose was accompanied by a marked decrease in liver glycogen, a decrease in rectal body temperature and an increase in blood acetoacetate, 3-hydroxybutyrate, free fatty acid and triglyceride levels. No alteration in the blood concentrations of lactate, L-alanine or pyruvate was observed.

rhTNF-treated mice exhibited a decrease in $^{14}CO_2$ production after administration of U^{14}C glucose when compared with saline-injected controls, this being significant up to 8h after injection of rhTNF. No change in $^{14}CO_2$ production after injection of ^{14}C palmitate was observed.

Glucose utilization, as determined by the 2-deoxyglucose tracer technique, was increased in the liver, spleen, kidneys, colon and diaphragm 2h after injection of rhTNF. No change was observed in glucose utilization by the heart, lungs and pancreas and by thigh and gastrocnemius muscles.

A single injection of rhTNF to female NMRI mice thus appears to result in rapid utilization of glucose by several tissues, most markedly the liver. The decrease in CO_2 production from glucose, the lack of change in blood lactate and pyruvate and the increase in concentration of acetoacetate and 3-hydroxybutyrate suggest that the glucose is being used for synthetic purposes via acetyl Co A. The most likely end product is the plasma triglycerides and free fatty acids which we have shown to be increased. Further investigations into this theory are at present being undertaken.