Purification and characterisation of two cancer cachexia factors

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Summary.

Cachexia is a wasting phenomenon that often accompanies malignant disease. Its manifestation is associated with shortened survival and reduced responsiveness to anti-tumour therapy and as yet there is no established, effective amelioratory treatment.

The MAC 16 model of cancer cachexia has been shown by many studies to closely mirror the human condition. Thus, cachexia is mediated by the presence of a small, slow-growing solid tumour that is mainly resistant to chemotherapy. In addition, the condition is largely attributable to aberrations in metabolic processes, while weight loss due to anorexia is negligible. Cachexia induced by the MAC 16 tumour, has been shown to be mediated by the production of tumour-derived circulatory catabolic factors, and the further elucidation of the structure of these molecules contributes towards the main content of this report. Thus, a factor with *in vitro* lipid-mobilising activity has been purified from the MAC 16 tumour, and has been found to have similarities to tumour-derived lipolytic factors published to date. Further work demonstrated that this factor was also purifiable from the urine of a patient with pancreatic cancer, and that it was capable of inducing weight loss in non tumour-bearing mice. Sequence analysis of the homogeneous material revealed an identity to Zn-α-2-glycoprotein, the significance of which is discussed. An additional factor, first detected as a result of its specific reactivity with a monoclonal antibody produced by fusion of splenocytes from MAC 16 tumour-bearing mice with mouse BALB/c myeloma cells, was identified as a co-purificant during studies to isolate the lipolytic factor. Subsequent purification of this material to homogeneity resulted in the determination of 18 of the N-terminal amino acids and revealed the highly glycosylated nature of its structure. Thus, this material (P24) was found to have an apparent molecular mass of 24kD of which 2kD was due to protein, while the remainder (92%) was due to the presence of carbohydrate groups. Sequence analysis of the protein core of P24 revealed an identity with *Streptococcal* pre-absorbing antigen (PA-Ag) in 11 of the amino acids, and the significance of this is discussed. P24 was shown to induce muscle protein breakdown *in vitro* and to induce cachexia *in vivo*, as measured by the depletion of fat (29%) and muscle (14%) tissue in the absence of a reduction of food and water intake. Further studies revealed that the same material was purifiable from the urine of patients with pancreatic cancer and was found to be detectable in the urine of cancer patients with weight loss greater than 1.5kg/month. Thus, cachexia induced by the MAC 16 tumour in mice and by malignant disease in humans may be induced by similar mediators.

Attempts to isolate the gene for P24 using information provided by the N-terminal protein sequence were unsuccessful. This was probably due to the low abundance of the material, as determined by protein purification studies; and the nature of the amino acids of the N-terminal sequence, which conferred a high degree of degeneracy to the oligonucleotides designed for the polymerase chain reaction.

For Mum and Dad

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Abbreviations.

ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cm	Centimetre (s)
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
DEPC	Diethylpyrocarbonate
dGTP	Deoxyguanosine 5'-triphosphate
DHA	Docosahexaenoic acid
(c)DNA	(complementary) deoxyribonucleic acid
dTTP	Deoxythymidine 5'triphosphate
DTT	Dithiothreitol
ECL	Emission chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether)
ELISA	Enzyme-linked immunosorbent assay
E. coli	*Escherichia coli*
EPA	Eicosapentaenoic acid
FPLC	Fast protein liquid chromatography
GAG	Glycosaminoglycan
GLA	Gamma linolenic acid
HPLC	High performance liquid chromatography
IFN-γ	Interferon-γ
IL-1	Interleukin-1
IL-6	Interleukin-6
IPTG	Isopropyl β-D-thiogalactopyranoside
kD	Kilodaltons
l	Litre (s)
LB	Luria broth
LPL	Lipoprotein lipase
LIF	Leukaemia inhibitory factor
M	Mole (s) per litre
MAC	Murine adenocarcinoma of the colon
μ	Micro
mg	Milligram (s)

MHC	Major histocompatibility complex
ml	Millilitre (s)
mm	Millimetre (s)
MOPS	3 [N Morpholino]propanesulphonic acid
MW	Molecular weight
NADH	α-Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
nm	Nanometre (s)
NSAID	Non-steroidal anti-inflammatory drug
OD	Optical density
OPD	o-Phenylenediamine
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming units
$PGE_{2/3}$	Prostaglandin $E_{2/3}$
pI	Isoelectric point
PMSF	Phenylmethylsulfonylflouride
PUFA	Polyunsaturated fatty acid
(m)RNA	(Messenger) Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SLS	Sodium N-laurylsarcosine
TBE	Tris borate EDTA
TBS (T)	Tris buffered saline (+ Tween 20)
TFA	Triflouroacetic acid
TGF	Transforming growth factor-β
TNF	Tumour necrosis factor-α
Tween 20	Polyoxyethyle-sorbitan
TEMED	N,N,N',N'-tetramethylethylenediamine
TMAC	Tetramethylammonium chloride
Tris	Tris (hydroxymethyl) aminomethane
v/v	Volume by volume
w/v	Weight by volume
X-gal	5-Bromo-4-chloro-3-indoyl β-D-galactopyranoside
YAH	Yoshida ascites hepatoma

CHAPTER 1: GENERAL INTRODUCTION

Cachexia is one of the most frequent adverse systemic effects of malignancy (De Wys *et al*, 1980). The syndrome, reported to be present in 16-73% of cancer patients (Nixon *et al*, 1980) commonly accompanies advanced disease but it may also be one of the first signs of the presence of a cancer - a reflector of a 'hidden cancer' (Brennan, 1977), such that an otherwise healthy individual presenting sudden unexplained weight loss should be investigated for the presence of a possible tumour (Costa, 1977). It has been shown that cachexia may often be a major cause of death in cancer patients (Warren, 1932; Harnet, 1952), the syndrome often results in a worse prognosis with respect to long term survival and response to chemotherapy (De Wys *et al*, 1980) and a significantly higher postoperative morbidity and mortality (Landel *et al*, 1985). Progressive weight loss may be the major symptom experienced by cancer patients (De Wys, 1986), they may appear chronically ill and emaciated with pale and atrophied skin (Costa, 1977) and with the symptoms of anorexia and nausea that frequently accompany cachexia (Theologides, 1972), they often have a reduced feeling of well-being and in the end, 'the progressive fading of physical wholeness is eventually associated with a flatness of the affects and a longing for annihilation' (Costa, 1977).

1.1. Cancer cachexia versus cancer anorexia

The occurrence of cancer cachexia has been associated with many different malignancies and does not appear to be correlated to tumour burden, tumour cell type, or anatomical site of involvement (Costa, 1977). As a general though not absolute observation, it has been reported to be present in 30-35% of patients with favourable non-Hodgkins lymphoma, breast cancer, acute non-lympholytic leukaemia and sarcomas; 48-61% of patients with non-favourable non-Hodgkins lymphomas, colon, prostate and lung

cancer; and 83-87% of patients with pancreatic or gastric cancer (De Wys *et al*, 1980). The cause of the syndrome is unknown, though it is generally believed that cachexia in cancer patients results from the combination of metabolic abnormalities and reduced food intake. Anorexia, characterised by loss of appetite and early satiety may be the underlying cause in some cases of weight loss associated with cancer. Anorexia is an extremely distressing syndrome as appetite and the ability to eat have been reported to be the most important factors in the physical aspects of a patients quality of life (Padilla, 1986).

Contributing factors to the development of anorexia include change in perception of the taste and smell of food resulting in increased sweet and decreased bitter thresholds which may correlate with weight loss and extent of disease (De Wys and Walters, 1975, Carson and Cormican, 1977). Appetite may also be affected by chemotherapy, radiotherapy and surgery as well as depression and feelings of malaise and distress (Theologides, 1972).

Early satiety is often reported by anorexic cancer patients (Theologides, 1979) and they frequently report of a feeling of fullness after ingestion of a small amount of food. This phenomenon may result from encroachment of the tumour on the gastrointestinal tract which could hinder the passage of food, or cause abnormalities in the mucosa thus resulting in malabsorption (Knox, 1983). It has been postulated that reduced appetite and early satiety may be caused by the production of factors by the tumour which exert their effects by their action on the hypothalamic sensory cells. In an experimental model of tumour-induced anorexia, a lesion introduced into the lateral, appetite-controlling region of the hypothalamus caused a fall in food intake before that induced by the tumour (Baillie *et al*, 1965). A possible candidate for such a factor are the satietins (Knoll, 1988); these have been purified from human plasma and been found to consist of two proteins that co-purify until separation by affinity chromatography. The larger protein has been characterised as an extensively glycosylated α1-acid-glycoprotein of molecular weight 64kD and is probably a vehicle for satietin D, a 41kD glycoprotein. Satietin D has been

shown to produce a long-lasting anorectic affect when injected into rats and is thought to work by activation of the ventromedial region of the hypothalamus however, its role in the development of anorexia in humans is unknown. More recently, it has been suggested that anorexia in tumour-bearing animals may be caused by a dysfunction in the hypothalamic membrane cyclic AMP second messenger system, as determined by a reduced responsiveness of adenylate cyclase to the inhibitory effect of neuropeptide Y and stimulatory effect of isoprenaline (Chance *et al*, 1995). Delayed gastric emptying times due to autonomic dysfunction may also result in early satiety (Vigano *et al*, 1994) thus causing an increase in the time interval between meals.

Increased serotoninergic activity within the central nervous system secondary to enhanced availability to the brain of tryptophan has been proposed as a possible cause of anorexia. Thus, a close relationship between elevated plasma free tryptophan and anorexia was observed in patients with cancer and reduced food intake (Cangiano *et al*, 1994). Anti-tumour surgery that was associated with alleviation of anorexia also resulted in the reduction of plasma tryptophan levels. In contrast, there was no change in patients who remained anorexic.

Anorexia due to loss of appetite and early satiety may develop during cachexia as a result of tumour-induced aberrant metabolic processes resulting in abnormal blood metabolite concentrations (De Wys, 1979). This association of anorexia and cachexia and use of both terms together to describe an "anorexia-cachexia" syndrome (Body and Borkowski, 1987, Nelson *et al*, 1994) has led to difficulties in interpreting the results of some clinical studies where cancer patients were recruited on the basis of weight-loss without clarification of the contribution of reduced food intake. Indeed in one study, patients presenting weight loss and reporting no change in food intake were in fact found to have reduced their caloric intake by as much as 40% (Costa, 1977). Clarification of the contribution of reduced food intake to the observed weight loss is important as prognosis may differ markedly for anorexia and cachexia, and treatment of the two conditions may

require radically different strategies (section 1.3.). Thus, weight loss had a prognostic effect in patients with limited tumour extent indicative of true cachexia, while no affect on survival was observed in patients with more advanced cancer in which the weight loss was more likely to be due to reduced caloric intake (De Wys *et al*, 1980). In addition, anorexia may be treatable by protein-caloric supplementation while this may be detrimental to patients with cancer cachexia.

True cancer cachexia is now recognised to be manifested by abnormalities in carbohydrate, lipid and protein metabolism due to distant catabolic effects of the tumour and/or due to host-mediated response to the presence of the tumour. This may result in loss of body fat and protein, symptoms of insulin resistance, hypoglycaemia, anaemia, muscle weakness, impaired visceral function and immune-defence mechanisms, and elevation of basal metabolic rate (Lindsey, 1986). In these cases, initial food intake is usually unchanged, though anorexia may develop as the disease progresses

1.2. Pathophysiology of cancer cachexia

1.2.1. Elevation of basal metabolic rate

Elevation of basal metabolic rate is believed to be an important factor in the development of cancer cachexia. In the absence of increased, or modified food intake, host tissues may be utilised in an attempt to cope with the increased daily energy expenditure caused by systemic catabolic effects of the tumour. Basal metabolic rate measured by indirect methods involving the monitoring of respiratory gases or direct whole body calorimetry after a 12h fast, have been shown to be elevated in cancer patients by many studies. The opposite phenomenon is observed in chronic starvation during which the basal metabolic rate is reduced as the body adapts to conserve tissues and energy in a low protein-calorie environment (Brennan, 1977).

Increased daily energy expenditure has been found to be elevated by approximately 12% in weight-losing cancer patients compared to non-cancer controls matched for age, sex, physical activity and food intake (Warnold *et al*, 1978, Lindmark *et al*, 1984); and increased by up to 20% in 60% of a mixed cancer patient population and correlated to the degree of weight loss (Bozzeti *et al*, 1980, Knox *et al*, 1983). A correlation between increasing basal metabolic rate and disease progression has been observed by some workers (Warnold *et al*, 1978, Knox *et al*, 1983, Fredrix *et al*, 1991), as well as a decrease with response to therapy (Warnold *et al*, 1978, Bozzeti *et al* 1980, Fredrix *et al*, 1991). However the importance of selecting an adequate control population is demonstrated by some of these studies. Thus, although elevation of basal metabolic rate above that for healthy non weight-losing controls has been observed in one study (Fredrix *et al*, 1991), another study could find no difference between weight-losing cancer patients compared to normal healthy subjects, while an increase was observed after comparison of the same patients to hospitalised, malnourished non-cancer patients (Lindmark *et al*, 1984). In addition, Nixon *et al* (1988) observed no change in the basal metabolic rate of weight-losing lung and colon cancer patients when they were compared to healthy controls, patients with weight-loss due to gastrointestinal disease, chronic lung disease and other miscellaneous diseases. However, in a subset of their cancer patients namely, female lung cancer patients, daily energy expenditure was found to be elevated above that for female patients with anorexia nervosa, thus suggesting that raised metabolic rate may not be unique to the cancer situation.

Other workers however have failed to show any increase hence, Heber *et al* (1982) found no elevation of basal metabolic rate in a group of pre-cachectic lung cancer patients, no difference was found between patients with benign and malignant gastrointestinal disease by Burke and co-workers (1980), and in another study, an increase was observed only in those cancer patients with metastastic disease (Macfie *et al*, 1982).

The tumour type studied may also be important in determining whether or not a rise in basal metabolic rate is detected hence, an increase of 12% above that for healthy controls was measured in patients with non small-cell lung carcinoma but not in patients with gastric or colorectal cancer (Fredrix *et al*, 1991). Indeed, in a mixed cancer patient group with gastrointestinal, gynaecological and genitourinary cancers, 33% were found to be hypometabolic, 41% had average metabolic rates and 26% were hypermetabolic (Knox *et al*, 1983).

It is generally accepted that basal metabolic rate is raised in cachectic cancer patients indeed, even before the onset of weight-loss (Hyltander *et al*, 1991a) suggesting that it may be a contributing factor rather than a consequence of the condition. It has been calculated that an elevation of 12% in the metabolic rate could account for the loss of 1-2kg of body weight per month (Lindmark *et al*, 1984). The phenomenon is more clearly demonstrated by some tumour types than others and is most apparent when the controls used are patients who have lost weight due to chronic starvation. The reasons why the basal metabolic rate is increased in patients with cancer cachexia are unknown, although the various aberrations in metabolic processes observed in this group of cancer patients are believed to be important.

1.2.2. Carbohydrate metabolism

Solid tumours, due to their poor vascularisation and hence hypoxic nature rely almost exclusively on the anaerobic metabolism of glucose as their main energy source, with most being converted to lactate (Nolop *et al*, 1987)). Glucose uptake and lactate release by human colonic carcinomas has been found to exceed the peripheral tissue exchange rate by thirty and forty three-fold respectively (Holm *et al*, 1995). Indeed, selective transcriptional regulation of hexokinase isoforms by a tumour may enable it to have a growth advantage over normal cells. Thus, the type 11 isoform has been shown to be the dominant form expressed in AS-30D hepatoma cells in contrast to the type 1V

isoform in hepatocytes (Mathupala *et al*, 1995). These authors also demonstrated that promoter activity of type 11 hexokinase was resistant to normal hormonal control thus enabling tumour cells to maintain glycolysis at an optimal rate regardless of the metabolic state of neighbouring healthy cells. Glycolysis represents a relatively inefficient system for the production of energy from glucose, resulting in only two molecules of ATP per molecule compared to the release of thirty ATP molecules from oxidative metabolism via the tricarboxylic acid cycle. The tumour therefore, has a high requirement for glucose and it has been suggested that this could cause a drain on available glucose to the detriment of the host (Shapot and Blinov, 1974). In order to maintain blood glucose concentrations, glucose production from lactate, alanine and glycerol via gluconeogenesis would be increased. This process is normally responsible for approximately 15% of the glucose utilised. An increase in the rate of gluconeogenesis, a high energy-requiring process especially for the conversion of lactate to glucose could contribute towards an elevated basal metabolic rate.

Cori-cycle activity representing gluconeogenesis from lactate has been found to be three times higher in patients with cancer and progressive weight loss compared to non-weight-losing cancer patients and healthy controls, and appeared to be correlated to an elevated basal metabolic rate (Holroyde and Reichard, 1981). However, it has been suggested that increased gluconegenesis from lactate alone would not result in an overall rise in daily energy expenditure (Young, 1979). Increased glucose production from alanine has also been reported in weight-losing cancer patients (Waterhouse *et al,* 1979; Heber *et al,* 1985). The former authors observed that alanine disappearance from the blood was comparable to that of controls, but glucose production from alanine was increased two-fold. In this study, gluconeogenesis was suppressed by approximately 20% in the presence of glucose with a concomitant rise in plasma alanine concentrations. This indicated that the process was still sensitive to metabolic control in contrast to findings by other authors which suggested that there may be impaired feedback control in

patients with cancer since gluconeogenesis did not slow down when the glucose pool expanded (Lundholm *et al.* 1981).

Alterations in glucose oxidative metabolism have been observed. Thus, Waterhouse and Kemperman (1971) observed that in the postabsorptive state cachectic cancer patients and controls both showed similar rates of oxidative metabolism from glucose and fatty acids. However, after a glucose load, oxidative metabolism from glucose in the control subjects increased by 62% and that from fatty acids fell by 70%, but there was little change in oxidative metabolism pattern in the cachectic patients. This suggested that weight-losing cancer patients may maintain a fasting oxidative metabolism profile even in the presence of a glucose load. These authors also observed slower glucose disappearance from the blood after a glucose load compared to control subjects. This may be indicative of reduced uptake of glucose by the peripheral tissues, a phenomenon also observed by other workers (Schein *et al,* 1979), thus suggesting a degree of glucose intolerance in cancer patients with cancer cachexia. Reduced utilisation of glucose by peripheral tissues was also suggested following observations of a decrease in activity of glycolytic and glucose oxidative enzymes in biopsies from the rectus abdominal muscle of patients with heterogenous malignancies (Lundholm *et al*, 1976).

Decreased insulin sensitivity in weight-losing cancer patients has been reported (Lundholm *et al,* 1978, Schein *et al* , 1979). In both studies, response of blood glucose to insulin challenge was found to be reduced in cancer patients though plasma insulin concentrations were similar to those in controls and insulin receptor binding was unaltered. Thus, uptake of glucose by the forearm muscle was found to be similar to that of controls, though conversion of glucose to glycogen was less in response to insulin (Lundholm *et al*, 1978). More recently, Byerley *et al* (1991) found no difference in glucose disposal rates in response to insulin infusion in patients with head and neck cancer and no evidence of insulin resistance, but glucose production and insulin clearance was enhanced in comparison to control subjects. The authors suggest that this illustrated

an attempt by the host to maintain blood glucose levels in an environment of high glucose flux. Three types of insulin response following a glucose load were observed in weight-losing cancer patients in another study (Rofe *et al*, 1994). Those patients with the greatest degree of weight loss demonstrated abnormal insulin:glucose ratios represented by either an elevated or reduced value with equal occurrence.

In conclusion, patients with cancer cachexia may have increased glucose production and turnover and relative insulin resistance. They differ from patients with Diabetes Mellitus, by a lack of increase in protein-calorie intake and minimum increase in plasma ketone body concentration (Legaspi *et al*, 1987). The cachectic situation also differs from that of chronic starvation during which glucose production, turnover and basal metabolic rate are reduced (Brennan, 1977).

1.2.2.1. Animal studies

Increased gluconeogenesis has been observed in several experimental models of cancer cachexia. Transplantation of Fischer F344 rats with a methylcholanthrene-induced tumour (MCG 101) resulted in a 27% increase in the rate of glucose production from alanine and lactate in isolated hepatocytes compared to non-tumour-bearing controls (Roh *et al*, 1984). Alanine transport across the hepatocyte membrane was also increased by 55%. In agreement with human studies (Waterhouse *et al*, 1979), enhanced gluconeogenesis was suppressed by the infusion of glucose, though suppression in hepatocytes from tumour-bearing rats required a three-fold increase in the concentration of glucose compared to non tumour-bearing controls.

In most studies measurement of gluconeogenesis occurs during the postabsorptive phase after a 12h fast however, a more accurate measurement may be obtained after a meal. Emery and co-workers (1993) demonstrated elevated glucose production in Fischer 344 rats transplanted with the Leydig mammary tumour, as measured by liver glycogen

synthesis, to be most apparent 2-3h postpranially. This increase was completely inhibited by the administration of 3-mercaptopicolinic acid, a potent inhibitor of gluconeogenesis. This group suggested that the difficulty sometimes encountered in detecting elevated glucose production and basal metabolic rate could be due to the lower rates observed in the postabsorptive phase; in this study, the rate was 20-fold less during this phase. Enhanced gluconeogenesis was further emphasised in this study by the observation of almost complete inhibition of postpranial glycogen synthesis in pair-fed controls, and gives further support to the image of the hypermetabolic cachectic state in contrast to the hypometabolic starved state. It has been suggested by these authors that enhanced postpranial glycogen synthesis may contribute towards the development of anorexia due to reduced appetite and a subsequent delay in re-feeding.

Reduced glucose utilisation by peripheral tissues observed in human studies (Lundholm *et al*, 1976) has also been observed in an experimental model. Tumours induced by exposure to 1,2-dimethylhydrazine hydrochloride and transplanted into NMR I mice (MAC 16 tumour-bearing mice) were observed to have a higher glucose utilisation rate when compared to all peripheral tissues with the exception of the brain (Mulligan and Tisdale, 1991a). Elevated tumour and reduced peripheral glucose utilisation were also observed in a non-cachectic tumour model (MAC 13 tumour-bearing mice) suggesting that the effects were due to the presence of a tumour alone. These authors suggested that weight loss may occur in the MAC 16 tumour model due to a failure to meet increased energy demands of alternative glucose metabolic pathways such as lipogenesis by increasing protein-calorie intake.

1.2.3. Protein metabolism

Lean body mass and visceral protein depletion is characteristic of patients with cancer cachexia, and the degree of depletion may be associated with reduced survival (Nixon *et al*, 1980). The major site of this protein loss has been observed to be the skeletal musculature (McMillan *et al*, 1994). It is the alteration in protein metabolism in these patients that demonstrates most clearly the difference between weight loss due to starvation and that due to cachexia. In the latter case, there is early rapid proteolysis resulting in amino acid mobilisation from muscle and an increase in gluconeogenesis, urinary nitrogen may rise to approximately 12g/day thus, representing the loss of 75g of muscle protein per day. To prevent further loss of lean body mass, the body switches to fat oxidation and production of ketone bodies which become the main energy source for the brain. Urinary nitrogen then drops to 3g/day and in this way, man can survive for long periods with minimum loss of muscle mass (Brennan, 1977). This adaptive response for protein conservation has been shown to be impaired or absent in patients with cancer cachexia. Thus, though absolute body cell mass, a measure of the total muscle mass, was observed to be significantly depleted in patients with anorexia nervosa and a mean weight loss of 30%; relative sparing of this compartment was observed as indicated by a rise as a percentage of the total body weight (Moley *et al*, 1987). In contrast, cancer patients with less depletion of total body weight demonstrated a proportional decline in body cell mass with no change in body cell mass as a percentage of body weight. Alterations in lean body mass in human studies have been determined by measurement of total body potassium, amino acid isotope tracer studies, urinary creatinine, urea and 3-methylhistidine excretion, plasma proteins and anthropomorphic measurements such as mid-arm and mid-calf circumference of the non-dominant limb.

An increase in whole body protein turnover of 32-35% and a whole body protein synthesis rate of 35-54% above that for controls was observed for weight-losing cancer patients by Jeevanandam and co-workers (1984). A reduced rate of protein synthesis and

increased rate of degradation was observed in muscle biopsies from 43 newly diagnosed cancer patients with weight loss (Lundholm *et al*, 1976). These authors also observed an increase in cathepsin D activity in biopsies from the rectus abdominal muscle an increase that was correlated with the rate of protein degradation. Indeed in one patient with non-responsive, progressive disease cathepsin D activity was found to have increased three-fold and leucine incorporation reduced by one third on subsequent re-examination. In another study, a decrease in muscle protein synthesis was also observed in weight-losing cancer patients (Emery *et al*, 1984) though these authors detected no change in total body synthesis or degradation. This group calculated that muscle protein synthesis only accounted for approximately 8% of the total body synthesis in these patients compared to 53% for healthy controls. The observed maintenance of the total protein synthetic rate in these patients may therefore be due to a two-fold increase in non-skeletal muscle protein synthesis. This may be caused by increased hepatic protein synthesis due to the enhanced production of acute phase proteins (section 1.4.2.), as other studies have observed that slow-growing solid tumours had a protein synthetic rate that was comparable to the tissue of origin and were therefore unlikely to contribute to whole body protein synthesis (Jeevanandam *et al*, 1984). In addition, elevated whole body protein turnover may also be apparent in patients with a small tumour burden (Fearon *et al*, 1988). Shaw *et al* (1990) compared patients with cancer cachexia to two groups of controls, namely patients with weight loss and benign disease and those with cancer but no weight loss. They observed that whole body protein catabolism was elevated in the first group as was the rate of appearance and oxidation of leucine. In contrast to a previous study (Lundholm *et al*, 1976), significantly higher fractional synthetic rates were measured for muscle, liver and albumin though these showed less of an increase than that observed for catabolism. The authors suggest that these synthetic rates may be increased in response to muscle breakdown but are insufficient to prevent overall whole body protein catabolism. In one study, increased total body protein turnover was observed in patients with pre-cachectic lung cancer (Heber *et al*, 1982). Elevated protein catabolism was also already apparent in

these patients and was found to be inversely proportional to the small degree of weight loss that had occurred.

Other observations that may result in alteration of the normal protein homeostasis include increased uptake of amino acids by tumours when compared to the corresponding normal tissue (Stein *et al*, 1978; Heber *et al*, 1982), though tumour tissue may be less efficient at utilising these amino acids, as indicated by a lower utilisation rate (Stein *et al*, 1978). Human colonic tumours were observed to have a specific requirement for serine and for the branched-chain amino acids valine, leucine and isoleucine when compared to normal colon tissue (Holm *et al*, 1995). However, retention of total amino acids between the two tissues was similar. A significant correlation between histidine induced, enhanced formiminoglutamic acid (FIGLU) excretion, elevated basal metabolic rate and reduced serum albumin was observed in seven patients with small cell carcinoma of the lung. This suggested the presence of disturbances in protein synthesis associated with a deficit of one- carbon units (Sengelov *et al*, 1994). FIGLU excretion was reduced in patients who showed a positive response to chemotherapy while there was no change in a patient with progressive disease. Requirement of host one-carbon unit donors by the tumour was demonstrated by a reduction of FIGLU excretion after a histidine load by exogenous DL-methionine administration.

In conclusion, patients with cancer cachexia have been shown to have increased protein turnover rates represented by increased catabolism and increased synthesis. Initially, the ratio of these processes may be maintained (Lazo, 1984) resulting in a balance of total body nitrogen however, the protein synthetic rate may be unable to match the high rate of muscle breakdown associated with disease progression and hence a negative nitrogen balance may develop.

Protein turnover accounts for a significant fraction of the basal metabolic rate (Young, 1977), hence the increase in protein cycling observed in cancer cachexia could

conceivably contribute to an elevated daily energy expenditure. Indeed, a positive correlation between protein turnover and extent of disease has been observed by some studies (Carmichael *et al*, 1980; O'Keefe *et al*, 1990). However the association of increased protein turnover with elevated basal metabolic rate may be an over-simplification as other workers have failed to demonstrate a correlation between these parameters (Fearon *et al*, 1988).

1.2.3.1. Animal studies

Depletion of lean body mass observed in patients with cancer cachexia is also a characteristic of experimental models of cachexia. Protein loss is usually measured from the gastrocnemius muscle which is thought to represent metabolism in the musculature of the whole body, and protein loss from this muscle has been shown to involve mainly white fast oxidative type IIa and white fast glycolytic type IIb fibres (Lundholm *et al*, 1981). Decreased protein synthesis was thought to be the most important process behind protein loss from the gastrocnemius muscle of rats transplanted with the Walker 256 sarcoma, as measured by incorporation of labelled amino acid into isolated polyribosomes (Goodlad and Clark, 1972), and it has been suggested that there may be a defect in the post initiation stage of the translation processes (Costa, 1977). The gastrocnemius muscle was observed to be reduced by 33% in mice implanted with the MAC 16 tumour (Plumb *et al*, 1991) and as protein synthesis rates were unchanged, this was thought to be due to an increased rate of protein degradative processes. More recently however, both decreased protein synthesis and increased degradation were observed in the same model of cancer cachexia (Smith and Tisdale, 1993a). Protein synthesis was reduced by 70% while degradation was increased five-fold, and a correlation was observed between loss of protein from the liver and skeletal muscle and increase in protein content in the tumour. Protein loss due to increased degradation has also been observed in rats bearing the Yoshida ascites hepatoma AH-130 (Tessitore *et al*, 1993a). The major sites of protein loss were the heart and gastrocnemius muscle while the liver and other viscera were

relatively preserved; this was in contrast to pair-fed controls in which protein loss occurred from all tissues. In addition, plasma amino acid concentrations were shown to be maintained in tumour-bearing rats, while in pair-fed controls the concentration gradually decreased as starvation-induced adaptations to conserve protein became effective.

Increased mobilisation of amino acids may be required by host tissues for glucose production via gluconeogenesis and by tumours as an additional energy source to glucose (Rivera *et al*, 1988). Tumours may require certain amino acids as precursors for nucleotide synthesis demanded by their high proliferative rates. In agreement with observations in human colonic carcinomas (Holm *et al*, 1995), the MCG 101 sarcoma was observed to have a specific requirement for serine, and by selective alteration of enzyme activities associated with the various metabolic pathways for this amino acid, serine was almost exclusively utilised for purine and pyrimidine synthesis (Snell *et al*, 1988). Indeed this model and a human colon carcinoma were found to have elevated activities of 3-phosphoglycerate dehydrogenase and serine hydroxymethyltransferase compared to the respective normal tissues, suggesting increased synthesis and utilisation of serine.

1.2.4. Lipid metabolism

Loss of whole body fat is a feature of cancer cachexia and alterations in fat metabolism include increased fatty acid turnover as a result of increased lipolysis and mobilisation, and increased whole body fatty acid oxidation. Methods used to measure changes in body fat include isotope tracer methods using triglyceride and free fatty acids, glycerol concentration and clearance determinations, and anthropomorphic measurement of triceps skin fold.

Increased plasma concentrations of glycerol, free fatty acids and triglycerides were observed in a heterogeneous group of cancer patients with an average weight loss of 13% of original body weight (Legaspi *et al*, 1987). Basal fatty acid turnover was elevated by 25% above that for controls and was found to be similar to the rate observed for patients with severe burns. Lipolysis was increased by 40% in these patients in whom complete triglyceride hydrolysis without re-esterification was observed and a 20% increase in fatty acid oxidation. Increased utilisation of fatty acids as a preferred energy source has been observed by workers even in the presence of high plasma glucose concentrations (Waterhouse and Kemperman, 1971), thus suggesting that in the presence of certain tumours, host tissues may increase their utilisation of fatty acids as an energy source. Eden *et al* (1985) also observed an increase in lipolysis however, others have reported no increase above that caused by a reduction in food intake (Klein and Wolfe, 1990). Jeevanandam *et al* (1986) did not observe an increase in whole body lipolysis but instead attributed the loss of body fat contributing to a total weight loss of 11% in the patients in their study, to reduced lipogenesis. This difference perhaps could be attributed to the apparently greater degree of cachexia in patients in the former study (Legaspi *et al*, 1987) as indicated by the lower mean plasma albumin concentration (3.3g/100ml as compared to 4.1g/100ml) and the observed elevation of basal metabolic rate. Other anomalies of fat metabolism observed include elevated clearance of triglycerides, even after 8 days of constant infusion in patients with cancer cachexia, thus resulting in a lack of plasma accumulation compared to controls with weight loss (Waterhouse and Nye, 1961) and further illustrating an increased utilisation of fat in the cachectic state.

The relationship between cachexia and lipid metabolism in patients is therefore unclear. Whole body lipolytic rates and fatty acid oxidation have been shown to be elevated by some workers and normal by others. The situation differs from that of chronic starvation though, during which mobilised fatty acids undergo β-oxidation in the liver and conversion to ketone bodies. Increased fatty acid oxidation in the absence of increased dietary fat intake would result in a depletion of fat stores, while increased

triglyceride-fatty acid cycling and gluconeogenesis from glycerol could result in an increase in metabolic rate. All of these processes therefore, have the potential to contribute towards a net loss of body weight.

1.2.4.1. Animal studies

Depletion of fat stores as a result of enhanced lipolytic activity and fatty acid mobilisation observed in patients has been reproduced in several experimental models (Costa and Holland, 1962; Kravolic *et al*, 1977; Lundholm *et al*, 1980; Bibby *et al*, 1987; Tanaka *et al*, 1990 and Balint, 1991). An elevated rate of lipolysis was observed in Wistar rats bearing the Walker 256 carcinoma before a loss of body lipids was apparent (Kravolic *et al* 1977) while the tumour was just palpable and mobilisation of fatty acids paralleled an increase in weight loss. A triphasic pattern of fat loss appeared to result during the five week growth period of the Krebs 2 carcinoma transplanted into mice (Costa and Holland, 1962). Thus, approximately 50% of total body fat was lost during the first seven day period while the tumour was just palpable, and food intake was unchanged. Further fat loss was then minimum until the third stage, when a drop in food intake was also observed. Hence, an adaptive response to the initial rapid fat loss was apparent in this model, perhaps involving an increase in the rate of lipogenesis as has been reported by other workers (Mulligan and Tisdale 1991b).

Lipogenesis from glucose was elevated in the liver, kidney and epididymal adipose tissue of mice bearing the MAC 16 adenocarcinoma, and an increase in the activity of fatty acid synthase in the liver was also detected (Tisdale and Leung, 1988). An increased rate of lipogenesis was also apparent in peripheral tissues of mice bearing the similar but non cachexia-inducing MAC 13 tumour. However, an increased food intake was also observed in this model which may compensate for the increased energy requirements of the lipogenic process, and thus may prevent the development of cachexia. In contrast to the MAC 16 model of cachexia, a postpranial suppression of lipid synthesis

was observed by other workers (Emery *et al*, 1993). Rats transplanted with the Leydig cell tumour appeared to utilise a greater proportion of ingested carbohydrate for gluconeogenesis than pair-fed controls in which chronic dietary restriction resulted in increased postpranial fatty acid synthesis. Detection of an alteration in the rate of peripheral tissue lipogenesis during cachexia may depend on experimental conditions, especially with respect to the timing of measurements. Thus, the rate of lipogenesis may not be elevated during the entire process of weight loss but may become more apparent once fat stores have reached a certain level of depletion due to increased lipolysis. The experimental model of cachexia chosen may also determine whether or not an alteration in lipogenesis is detected. Thus, a tumour with a high growth requirement for fatty acids but a low *de novo* synthetic ability may induce an increased rate of lipogenesis.

Requirement for fatty acids was observed in mice transplanted with the MAC 16 tumour (Mulligan *et al*, 1992). Lipid oxidation by peripheral tissues was elevated as measured by the production of CO_2 from triolein and palmitate, and incorporation of mobilised fatty acids from adipose tissue into tumour lipids was also increased seventeen-fold above that observed for mice bearing the MAC 13 tumour. In addition, heart and adipose tissues of MAC 16 tumour-bearing mice accumulated less dietary lipid even though the activity of lipoprotein lipase was elevated in these tissues (Briddon *et al*, 1991), further illustrating increased utilisation of fatty acids in this model. The requirement of this tumour model for fatty acids was also demonstrated by the tumour growth-stimulatory effects of a hypolipidaemic drug (Mulligan and Tisdale, 1991c). Bezofibrate increased the growth rate of the MAC 16 tumour *in vivo*, but not *in vitro* by two-fold, but had no effect on growth of the MAC 13 tumour. An elevated incorporation of lipid into tumour tissue was observed, and it is thought that the drug may cause weakening of the bonds involved in circulatory fatty acid binding to albumin thus, increasing the pool of incorporable fatty acids. Incorporation of fatty acids by the MAC 16 tumour appeared to be non-specific (Hudson and Tisdale, 1994), though a decrease in

the plasma linoleic:arachidonic acid ratio was observed and tumour tissue stearic:oleic acid ratio was increased in correlation with weight loss.

The MAC 16 tumour therefore, appeared to have an greater requirement for fatty acids than the MAC 13 tumour, a requirement probably in excess of its biosynthetic capability which appeared to be similar to that of tissues with low lipid synthetic rates such as the brain (Mulligan and Tisdale, 1991b). The elevated lipolytic and lipogenic rates associated with this tumour may therefore serve to ensure the availability of fatty acids as a high calorific energy source for host tissues and for the growth requirements of the tumour tissue.

Increased mobilisation of fatty acids is associated with cancer cachexia but although hyperlipidaemia has been observed in some experimental models (Kravolic *et al,* 1977), it may be absent in others (Bibby *et al*, 1987), and in patients (Waterhouse and Nye, 1961). Administration of a diet in which 50% of the calories were in the form of fat resulted in hepatic lipid accumulation in non-tumour-bearing rats but a complete absence in tumour-bearing rats (Dagnelie *et al*, 1994). This may be a consequence of differences in tissue lipoprotein lipase activities, and hepatic fatty acid metabolism, as determined by the requirements of the host and tumour tissues.

1.3. Treatment of cancer cachexia

Attempts to treat cancer cachexia to date have often sought to correct the weight loss by targeting the anorexia that may also be present. Treatments have therefore included enteral or parenteral hyperalimentation and appetite-stimulating drugs.

1.3.1. Hyperalimentation

Aggressive nutritional support has been used immediately prior to cancer treatment in an attempt to improve the nutritional status of a patient who may otherwise have been unable to undergo rigorous anti-cancer regimes. In one study, 52 patients from a total of 56 gained sufficient weight to be able to undergo chemotherapy after three days of enteral nutrition during which they received 30-35 calories and 0.2-0.3g nitrogen/kg/24h (Copeland *et al*, 1975). Subsequent chemotherapy resulted in tumour regression in 36% of these patients. However in another study, nutritional support in combination with chemotherapy did not improve response rates drug tolerance or long term survival (Evans *et al*, 1985). Other workers reported a lack of improvement in myeloid recovery following supportive nutrition of their patients, thus rendering intensive chemotherapy unsuitable (Levine *et al*, 1982). Normalisation of metabolic aberrations associated with cancer cachexia have been observed as a result of nutritional support by some workers. Thus, eight patients receiving total parenteral nutrition in one study gained weight after five days of treatment, and blood glucose, insulin and lactate concentrations all increased while the free fatty acid concentration was reduced (Holroyde *et al*, 1977). An increase in oxidative glucose metabolism was also observed in these patients as was a decrease in Cori-cycle activity. However, in a later study in which a similar phenomenon was observed, there was no weight gain and no reversal of muscle catabolism (Holroyde *et al*, 1981). Although nutritional support has been reported to stimulate the synthesis of fat (Nixon *et al*, 1981), most of the weight gain attributed to this treatment is believed to be fluid due to the nature of its rapid appearance and disappearance (Evans *et al*, 1985).

Repletion of lean body mass following nutritional support has not been observed. Nitrogen balance showed partial improvement during one study, but remained negative even after the nitrogen content of the diet was increased by a factor of six (Levine *et al*, 1982). These authors calculated that a caloric intake of 1875 calories/m^2/day and a nitrogen intake of 10g/day would be required to obtain nitrogen balance in these patients,

an amount rendered impractical by the amount of fluid it represents. Nixon and co-workers (1981) were able to show some improvement in lean body mass parameters such as serum albumin, creatinine/height ratio and mid-arm circumference measurement in patients with cancer cachexia during venous hyperalimention. These increments were less than those observed for malnourished patients without cancer also undergoing nutritional support however, and in contrast to the control group, the lean body mass apparently being synthesised was impaired, as indicated by the abnormal nitrogen, phosphate and calcium retention ratios. Studies by other workers have shown increases in the fractional synthetic rates of protein in muscle and whole body protein synthesis in patients receiving total parenteral nutrition (Shaw *et al*, 1990). Hyltander and co-workers (1991b) observed an increase in total body protein synthesis when the caloric content of the intravenous nutritional support was increased to 100% above that corresponding to daily energy expenditure. In addition, supplementation of total parenteral nutrition with branched-chain amino acids was observed to result in a decrease in protein oxidation and an increase in protein synthesis and fractional albumin synthetic rate (Hunter *et al*, 1989). However it is not known whether these alterations would result in long term reversal of muscle mass depletion, and observations of increased retention of leucine, isoleucine and valine by human colonic tumours compared to normal tissue (Holm *et al*, 1995) indicates the possible inappropriateness of such treatment. Recently it has been suggested that supplementation of supportive nutrition with β_2-adrenergic agonists may enable result in skeletal muscle mass repletion. Thus, supplementation with cimaterol to MCG-101 sarcoma-bearing rats resulted in an increase in muscle mass of 10-16% above that for nutritional support alone with a concomitant reduction in 3-methylhistidine production (Stallion *et al*, 1991). These authors suggest that these agents may be useful in the alleviation of cachexia, perhaps in conjunction with β-blockers to counteract possible side effects of tachycardia and elevated basal metabolic rate.

Treatment of cachexia by nutritional support has thus far been unsuccessful, indeed the method may be deleterious to a patient with cancer cachexia. Parenteral

nutrition may cause problems due to fluid load, leading to congestive heart failure in a patient with impaired visceral function (De Wys, 1985), and increased susceptibility to infection when immune-defence systems are impaired. In addition, animal studies have demonstrated stimulation of tumour growth in the presence of aggressive nutritional support (Cameron, 1981). The effect of this treatment on human tumours is unclear ,with many studies reporting relatively little change in overall survival and hence, one would assume on tumour growth. However, the observations that plasma lactate concentrations were increased in patients receiving total parenteral nutrition in one study (Holroyde *et al*, 1977), and that the fractional synthetic rate was found to have increased two-fold in human colorectal cancer by other workers (Heys *et al*, 1989), suggests that stimulation of tumour growth may be a possibility.

Although hyperalimentation has had little effect in reversing cachexia, the treatment may be beneficial to patients who have lost weight entirely due to reduced protein-calorie intake or to cachectic patients with curative tumours in order that they may undergo anti-cancer treatment.

1.3.2. Appetite-stimulant drugs

Megestrol (Medroxyprogesterone) acetate is the most extensively tested of the appetite-stimulant drugs to date with respect to its role in the treatment of cancer cachexia. The drug has been used for the hormonal management of breast cancer and it was observed that it resulted in weight gain, increased appetite and an increased sense of well being in patients being treated (Aisner *et al*, 1987). It has recently been shown that megestrol acetate may result in improved appetite through its action on the satiety centre through modulation of residual calcium channel current, resulting in attenuation of the firing of ventromedial hypothalamus nucleus neurones (Costa *et al*, 1995). As a result of these observations, several clinics have tested its possible efficacy in cancer patients with cachexia. Several clinical trials have reported improvements in the cachectic condition in

terms of weight gain, reduced nausea, improved appetite and taste sensation, increased protein-caloric intake and sense of well being (Loprinzi *et al*, 1990, 1993, 1994; Bruera *et al*, 1990; Feliu *et al*, 1991; Mantovani *et al*, 1995). Increases in triceps skin fold and mid-arm and calf circumference, were also noted (Feliu *et al*, 1991; Loprinzi *et al*, 1993), as were plasma markers thyroxine binding pre-albumin and retinol binding protein (Downer *et al*, 1993), thus suggesting possible improvements in body composition in terms of increased fat and protein. Treatment of cachexia induced by an experimental model however, demonstrated that much of the increase in weight gain due to megestrol acetate was in the form of water (Beck and Tisdale, 1990). Moreover, an increase in tumour weight was observed in the treated group by these authors, possibly as a result of increased availability of nutrients due to increased food and water intake. No improvement in survival have been reported for the treatment of cancer cachexia with megestrol acetate, therefore its main role may be in the improvement of the quality of life especially in view of its very low toxicity. The expense of the drug may limit its widespread use, the optimum dose was found to be 800mg/day (Loprinzi *et al*, 1994) at a cost of $15-20/ day.

1.3.3. Experimental treatments

1.3.3.1. Anabolic steroids

Anabolic steroids have been shown to increase muscular mass in non-malignant conditions in humans (Hassager *et al*, 1989), their effectiveness against protein loss in cachexia however seem limited (Lyden *et al*, 1995). Results from this animal study suggested that the gain in weight observed was due mainly to water and the slight gain in protein mass was represented by reduced degradation of hepatic structural proteins. There was no increase in survival and no increase in total body nitrogen retention.

1.3.3.2. Insulin

Decreased responsiveness to insulin has been observed in both human and animal studies. The low insulin : glucagon ratio that presides in this situation would favour the mobilisation of stored nutrients and an increase in hepatic glucose production, thus perpetuating the metabolic abnormalities associated with cachexia. Insulin therapy might therefore be expected to reverse this catabolism and result in increased storage of nutrients.

Moley *et al* (1985) observed an improvement in host condition in F344 rats bearing the methylcholanthrene induced sarcoma treated with insulin (2u/day). Food intake and body weight were increased even when insulin was administered during late cachectic decline. However, long term treatment was associated with reduced survival, probably due to hypoglycaemic death; and insulin failed to reverse the elevation of basal metabolic rate associated with cachexia in this model.

Treatment of MAC 16 tumour-bearing mice with 15-20 units of insulin per day resulted in a reduction of weight loss due to fat and protein loss (Beck and Tisdale, 1989a). However, tumour growth was stimulated by 50% with these doses of insulin thus limiting its usefulness.

Increasing plasma insulin concentrations by exogenous administration would result in stimulation of glucagon secretion and would thus have a counteractive effect on the anabolic properties of the former hormone. In addition, an association between elevated plasma glucagon concentration, disease progression and development of cachexia has been observed in patients with breast cancer (Knapp *et al*, 1991). Hence combined hormone therapy involving insulin, somotostatin analogue (octreotide) and growth hormone has been tested in Lewis/Wistar rats transplanted with the MAC 33 mammary adenocarcinoma (Bartlett *et al*, 1993). Octreotide was included as a glucagon inhibitor

and its insulin and growth hormone-inhibitory effects were reversed by exogenous co-administration of these two agents. Results included an 18% gain in weight, reduction in the tumour:carcass ratio, increase in hamstring muscle weight and protein content and an increase in total body protein of 40%. Tumour protein content was reduced as was the proportion of tumour cells in the synthesis phase of the cell cycle, thus suggesting significant suppression of protein synthesis, cell division and tumour cell proliferation. In a more recent study by this group, combined therapy with insulin and octreotide resulted in a 100-fold increase in the insulin:glucagon ratio but was also associated with an unexpected further increase in gluconeogenesis compared to non-treated tumour-bearing controls as indicated by increased activity of fructose 1.6-diphosphatase and lactate dehydrogenase (Bartlett *et al*, 1995). The authors suggest that glucagon-independent factors must intervene to increase hepatic glucose production in an attempt to restore circulating glucose levels. Survival data was not provided by these studies but preliminary data indicate that combined hormone therapy may be more effective against cachexia than the individual hormones alone. However its long-term use may be limited due to problems associated with hypoglycaemia, while its apparent stimulatory effect on gluconeogenesis may counteract any initial weight gain and reduction in tumour growth.

1.3.3.3. Insulin-like growth factor -1

Insulin-like growth factor-1 (IGF-1) mediates many of the anabolic effects of growth hormone and was therefore thought to be a possible candidate for reversal of some of the effects of cachexia. Fischer 344 rats transplanted with the MCG 101 sarcoma were administered 400μg of IGF-1 per day and as a result, a reduction in weight loss, increase in muscle protein, DNA and RNA content and a decrease in the proportion of tumour cells in active proliferative state were observed (Eng-Hen *et al*, 1992). Therefore IGF-1 administration was observed to beneficially influence host preservation of lean tissue mass in this experimental model.

1.3.3.4. Dietary manipulation

Most tumours have a reduced capacity to utilise fat compared to normal tissue. Although tumour incorporation of dietary lipid does occur (Mulligan and Tisdale, 1992), the transport of free fatty acids from the plasma was observed in one study to be so slow compared to the rate of plasma clearance that it was estimated that less than 1% of the fatty acids leaving the circulation were incorporated into tumour lipids (Baker *et al,* 1977). More recently, Holm and co-workers (1995) demonstrated the marked contrast between the high degree of fatty acid mobilisation from peripheral tissues and low uptake by human colonic carcinomas. In the cachectic host, fatty acids are increasingly used by peripheral tissues as an energy source hence, dietary manipulation may be able to provide a nutritional environment that supports the host and deprives the tumour. Promising results have indeed been obtained by such dietary manipulation thus, Buzby and co-workers (1980) demonstrated that a diet in which the total calorie content was in the form of fat, resulted in greater weight gain without stimulation of tumour growth in Lewis/Wistar rats transplanted with an adenocarcinoma compared to other diets.

Many tumours also have an impaired ability to utilise ketone bodies as an energy source. Retention of acetoacetate by human colonic carcinomas was observed to be negligible during substrate balance studies (Holm *et al*, 1995) and *in vitro* utilisation of D-3-hydroxybutyrate by mouse lymphoma, human erythroleukaemic, human bladder and mouse bladder carcinoma cell lines over a period of seven days was found to be minimal (Tisdale and Brennan, 1983). Inability to utilise ketone bodies may be due to reduced activity of succinyl-coenzyme A : acetoacetate CoA-transferase (3-oxo acid-CoA transferase) in tumour tissue, as observed by these authors. In addition, there was no induction of the activity of this enzyme during long-term exposure to D-3-hydroxybutyrate (Tisdale, 1984). However, increased activity of 3-oxo acid-CoA transferase has been observed, together with increased tricarboxylate carrier in freshly isolated Morris Hepatoma 7777 cells (Hildebrandt *et al*, 1995), in agreement with the

observation that these cells utilise ketone bodies as an energy source and for lipogenesis.

Dietary induction of ketosis therefore, may result in caloric restriction in tumours with low activity of the rate-limiting enzyme for ketone body metabolism, while providing adequate source of energy for host tissues. Elevated plasma ketone body concentration may also alleviate the weight loss associated with cachexia by reduction of lipolysis through substrate-product inhibition or by direct inhibition of hormone-sensitive lipase activity (Bjorntorp, 1966). In addition, lean body mass may be conserved by the inhibitory effect of ketone bodies on the process of protein degradation (Tisdale and Brennan, 1983).

Ketosis was achieved in MAC 16 tumour-bearing mice by the administration of an isonitrogenous, isocaloric diet supplemented with D-3-hydroxybutyrate in which 68-80% of the caloric intake was in the form of medium chain triglycerides (Tisdale *et al*, 1987). Host weight loss was reduced in proportion to the fat content of the diet, and was attributable to both an increase in carcass fat and reduction of plasma free fatty acid concentration. An increase in non-fat tissue was also observed as a result of this diet while total body water content was unchanged. Final tumour weight was reduced in proportion to the fat content of the diet hence tumours from mice receiving 80% of their calories in the form of fat were approximately 50% smaller than those from tumour-bearing mice fed a standard diet. In contrast, a diet in which long-chain triglycerides were the caloric source did not induce ketosis and was less effective in reducing weight loss and tumour growth (Tisdale and Brennan, 1988).

A ketogenic diet comprising 80% medium chain triglycerides was effective against loss of lean body tissue (Beck and Tisdale, 1989b). Nitrogen balance and plasma amino acid levels were restored, creatinine excretion was increased and urea excretion reduced in MAC 16 tumour-bearing mice on this diet, thus indicating a reduction of gluconeogenesis from alanine and an increase in muscle mass.

Induction of ketosis in five cancer patients was achieved by enteral administration for seven days of a diet consisting of calories in the form of 70% medium chain triglycerides (Fearon *et al*, 1988). A mean weight gain of 2kg was observed in these patients in addition to an increased performance score. Rates of total body protein turnover, protein synthesis and degradation were decreased in three of the patients, though there was no significant change for the group as a whole. Improvements observed in such grossly cachectic patients (mean weight loss 32%) further supports the efficacy of a ketogenic diet and the suggestion that it may be a valid anti-cachectic therapy.

High fat diets in which the lipid content was in the form of n-3 series fatty acids were observed to result in an even greater improvement in cachectic parameters. Thus, an isocaloric, isonitrogenous diet in which 50% of the calories were in the form of fish oil was observed to result in almost complete cessation of weight loss in mice transplanted with the MAC 16 tumour (Tisdale and Dhesi, 1990). Tumour growth was also inhibited by approximately 50% indeed, a similar reduction of tumour volume was achieved by administration of 5-fluorouracil (120mg/kg) only with considerable toxicity, as indicated by exacerbation of host weight loss. Body composition data indicated that most of this weight gain was due to fat and muscle mass, and no alteration in water content was observed.

Other studies have also demonstrated the effectiveness of fish oil in the treatment of cancer cachexia, hence approximately 85% inhibition of weight loss was observed in Fisher F1 hybrid rats transplanted with the MAT-LyLu rat prostrate carcinoma fed a diet containing 50% fish oil (Dagnelie *et al*, 1994). Tumour growth was not inhibited in this case however, and may be due to differences in tumour kinetics. Thus, fish oil may be more effective against slow-growing tumours such as the MAC 16 colon adenocarcinoma.

1.3.3.5. Eicosapentaenoic acid (EPA)

The effectiveness of a fish oil diet in the treatment of cachexia in two experimental models led to a search for the identity of the active component. Fish oil is rich in the n-3 series of polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Administration of EPA (5g/kg) to MAC 16 tumour-bearing mice resulted in complete inhibition of weight loss in contrast, DHA and the n-6 fatty acid linoleic acid (LA) at similar dose levels had no effect (Tisdale and Beck, 1991). EPA also inhibited tumour growth in this model while DHA was growth-stimulatory and LA had no effect. As EPA and DHA were equally cytotoxic *in vitro*, and as LA was able to reverse the anti-tumour, but not anti-cachectic effect of EPA *in vivo* (Hudson *et al*, 1993), it has been suggested that EPA may be primarily anti-cachectic, with secondary effects on tumour growth. EPA has been shown to exert its tumour cytostatic effect by increasing the cell loss rate (Hudson *et al*, 1993), perhaps by reducing the supply of circulating available substrates required for tumour growth. EPA inhibition of cachexia in the MAC 16 tumour model was dose-dependent, the optimum dose for maintenance of body weight was determined to be 1.25-2.5g/kg (Beck *et al*, 1991), and this resulted in a doubling of the survival time. EPA administration also resulted in a significant reduction in muscle protein degradation, though protein synthesis was unaffected suggesting that the degradative process may primarily be involved in muscle protein catabolism in this model. These effects appeared to be specific to EPA as the n-6 polyunsaturated fatty acid gamma linolenic acid (GLA) had markedly less effect. Thus, partial reductions in weight loss and tumour growth were observed with 5g/kg of this latter fatty acid, though toxicity was associated with this dose and lower doses had no effect. It has been proposed that the anti-cachectic and anti-tumour effects of EPA may be apparent only in certain animal models (Costelli *et al*, 1995). Thus, these authors observed no attenuating effects with the fatty acid (1.5g/kg) against cachexia induced by the Yoshida ascites hepatoma in rats. It is possible therefore that EPA has greater effectiveness against tumours that are more slow-growing and differentiated. This would explain the lack of effect of EPA observed

in this model and observed when administered as a component of fish oil to mice transplanted with MAT-LyLu rat prostrate tumour (Dagnelie *et al,* 1994). However, the limitations of these models with respect to their relevance to the human cachectic condition, become apparent in the light of recent human studies.

Administration of EPA in the form of MaxEPA capsules which contain EPA (18%) and DHA (12%) to eighteen patients with unresectable adenocarcinoma of the pancreas with a mean weight loss of 2.9kg/month, has produced promising initial results (Wigmore *et al*, 1996). Patients were able to tolerate the equivalent of 2g EPA per day for three months in this form of fish oil without toxic effects, and in eleven of the patients a net weight gain of 0.3kg/month was observed, while three others became weight stable and the rate of weight loss in the remaining four was reduced by 50%. In this short study however, there was no change in mid-arm circumference, triceps-skinfold thickness or basal metabolic rate. Plasma fatty acid composition was altered as a result of this therapy so that EPA increased in proportion to a mean of 5% of the total fatty acids and arachidonate decreased from 14.6% to 9.0%.

A similar trial undertaken by this group with GLA resulted in continuation of weight loss in the majority of patients taking part, thus illustrating further the specific anti-cachectic effects of EPA.

Patients with pancreatic cancer have a very high incidence of cachexia (De Wys, 1980) and at present there is no effective standard anti-tumour therapy (Lionetto *et al*, 1995), therefore the effectiveness of MaxEPA in reversing weight loss in the majority of patients in this clinical trial provides support for future studies to try administration of pure EPA, which has been shown by animal studies to be the active anti-cachectic component of fish oil.

1.4. Mediators of cancer cachexia

It is generally accepted that the variations in host metabolism associated with cancer cachexia cannot be caused simply by tumour growth alone, as the syndrome is commonly seen before a tumour is clinically apparent and has been reported to occur with tumours as small as 0.01% of body mass (Nathanson *et al*, 1974). It is more likely that the tumour releases a substance into the circulation which actively inhibits host energy utilisation, or causes it to be used inefficiently. Evidence that this phenomenon does indeed occur has been provided by observations from experiments involving parabiotic pairs of animals. Thus weight loss was induced in the parabiotic pair of rats transplanted with the Leydig cell mammary carcinoma and the MCG 101 sarcoma (Mordes and Rossini, 1981; Norton *et al*, 1985). In another study involving infusion of plasma from a MCG 101 sarcoma-bearing rat to a non tumour-bearing animal over a period of four days, cachectic parameters of anorexia, weight loss, negative nitrogen balance, and reduction of gastrocnemius and heart muscle were induced (Illig *et al*, 1992).

The mediators of the cachectic process are unknown but the three main theories to date suggest that the resulting metabolic aberrations may be due to a tumour-mediated host neuroendocrine response, a tumour-mediated host inflammatory response involving the cytokine network, or may be due to the production and secretion of cytokines, lipolytic factors and/or proteolytic factors by the tumour.

1.4.1. Neuroendocrine mediators

Cancer cachexia has been associated with elevated urine and serum concentrations of catecholamines and glucocorticoids in patients (Drott *et al*, 1988; Knapp *et al*, 1991; Hyltander *et al*, 1993) and in experimental models (Balint, 1991; Tessitore *et al*, 1993a, 1993b). Therefore it was thought that the metabolic alterations associated with cachexia may be caused by up-regulation of the host adrenergic state. In one study, propranolol

was observed to reduce the basal metabolic rate in patients with progressive disease by 10% (Hyltander *et al*, 1993). This was associated with a decrease in plasma free fatty acid concentration due to a decrease in the rate of lipolysis, as no change in the fatty acid oxidation rate was observed. Adipose tissue from mice transplanted with the Ehrlich ascites carcinoma had a higher basal lipolytic rate than non tumour-bearing controls (Balint, 1991) and lipolysis was more sensitive to stimulation by both catecholamines and theophylline; in turn this stimulation was also repressed to a greater extent by insulin and propranolol. A similar effect was observed in patients in whom increased sensitivity to infused adrenaline was observed (Drott *et al*, 1989). It has been shown that elevated plasma cortisol levels within 300-500μg/l can increase whole body proteolysis in healthy volunteers (Simmons *et al*, 1984). This may be in part due to hepatic stimulation of gluconeogenesis as exogenous administration of glucocorticoid was observed to increase total body glucose production by 400% in mice with the Ehrlich acites carcinoma (Shapot and Blinov, 1974). However, the importance of the contribution of glucocorticoid hormones to the development of cachexia in general may be limited, as other workers have demonstrated a complete lack of attenuating effect of the glucocorticoid receptor antagonist RU3 8486 (Llovera *et al*, 1996). Hence in the tumour-bearing state, prevalence of lipolytic and proteolytic hormones may in part explain the ongoing tissue breakdown of progressive cancer, together with other contributory factors.

1.4.2. Tumour-mediated host inflammatory response

Inflammation involves the production of acute phase proteins by the liver such as fibrinogen, immunosuppressive acidic protein and sialic acid-containing proteins. Thus a hyperplasic liver sometimes observed in experimental models of cachexia (Tessitore *et al*, 1993) and patients (Heymsfield and McManus, 1985) may be indicative of an on-going inflammatory response. C-reactive protein is an acute phase protein commonly cited as a marker of the magnitude of the response, and it has been reported that in a proportion of patients with malignancy, circulating C-reactive protein levels were raised gradually with

disease progression (Milano *et al*, 1978). An on-going inflammatory response has also been associated with altered protein kinetics associated with cancer cachexia thus, a reduction in total body potassium was observed in patients with plasma C-reactive protein levels greater than 5mg/ml when compared to normal controls (McMillan *et al*, 1994). Patients with advanced, metastatic disease and a mean weight loss of 10% were observed to have elevated total body protein synthesis and acute phase response (Fearon *et al*, 1991). In addition, treatment of patients with colonic tumours with the non-steroidal anti-inflammatory agent, ibuprofen resulted in a 50% reduction in all the acute phase proteins measured and a normalisation of whole body protein kinetics (Preston *et al*, 1995). Indomethacin administration (100mg/day) to patients with solid tumours resulted in a doubling of the survival time compared to patients on placebo. Prostaglandin synthesis intervention by indomethacin treatment was also observed to dramatically and reproducibly reduce the parameters of the cachectic process and prolong survival in tumour-bearing rodents irrespective of administration during the early or late stage of the process (Sandstrom *et al*, 1990; Gelin *et al*, 1991). Several cytokines have been proposed as important mediators in the manifestation of the inflammatory response through the action of prostaglandins. This property and their inhibitory action on lipoprotein lipase has resulted in interest in their role in the cachectic process

1.4.2.1. Tumour necrosis factor α (TNF)

TNF was proposed as a possible causative candidate in the process of cancer cachexia as a result of observations of weight loss observed in rabbits chronically infected with Trypanosoma brucei brucei. The elevated plasma triglyceride concentration observed in infected animals was shown to be due to systemic suppression of lipoprotein lipase. Activated mouse macrophages were shown to inhibit gene expression in TA 1 adipocytes resulting in the prevention of accumulation of lipid within differentiating pre-adipocytes, and loss of stored trigylcerides from mature cells (Torti *et al*, 1985). In the latter case which more closely represents physiological conditions, a reduction of adipose-

specific mRNA resulted in a 90% depletion of stored triglycerides after six days. Using its LPL-inhibitory property for detection by bioassay, TNF was subsequently purified from an endotoxin-stimulated mouse macrophage cell line (Beutler *et al*, 1985). The factor was initially assigned the name cachectin until amino acid sequence information confirmed its identity to TNF, a protein with a subunit molecular mass of 17 kD.

Several observations of the effects of purified TNF served to further confirm its suitability as a candidate for the mediation of cancer cachexia thus, recombinant TNF was observed to reduce LPL activity by 95% and mRNA levels by 75% in mouse 3T3-L1 adipocytes (Zechner *et al*, 1988). More recently, exposure of human adipocytes to TNF at a concentration of 5nmol/l for 24h was shown to cause marked reduction of LPL mRNA content and a dose-dependent increase in lipolysis to 400% above the endogenous rate (Hauner *et al*, 1995). This may suggest stimulation of the hormone-sensitive lipase through elevation of cAMP and activation of protein kinase (Zhang *et al*, 1988). Furthermore, a complete loss of the stimulatory effect of insulin on glucose transport was observed, suggesting that elevated TNF levels could be involved in the insulin resistance commonly associated with cancer cachexia perhaps by an inhibitory effect on GLUT 4 gene expression (Hauner *et al*, 1995).

Despite promising *in vitro* results supporting the role of TNF in the cachectic process, data obtained from human and animal studies is inconclusive. In one study, spontaneous TNF production from freshly isolated peripheral blood mononuclear cells was measurable by cytotoxicity assay in 70% of the patients with solid tumours and in only 15% of healthy controls (Aderka *et al*, 1985). Furthermore, in five of the patients in which malignancy and cachexia were more advanced, an enhanced sensitivity to endotoxin-stimulated TNF production was observed. These authors suggested therefore, that cachexia may develop as a result of sustained stimulation of TNF production from macrophage cells by the tumour. TNF was detected in the serum of 50% of cancer patients examined in another study (Balkwill *et al*, 1985) compared to detection in 3% of

controls and 18% of cancer patients with no clinical disease; in addition, TNF mRNA was measurable in peripheral blood mononuclear cells in 73% of patients as compared to 12.5% of controls. In one study, detection of TNF in the serum of patients with breast cancer was associated with disease progression and development of cachexia (Knapp et al, 1991). In contrast, in a group of cancer patients with severe weight loss TNF was not detected using a more sensitive ELISA method with a detection limit of 40pg/ml (Socher *et al*, 1988). TNF measured by this method was shown to be markedly elevated in 70% patients with leishmaniasis and malaria while no difference in the presence of TNF was detected between cancer patients and controls (Scuderi *et al*, 1987). The importance of the contributory role of this cytokine was further questioned by a study involving the administration of pentoxifylline to cancer patients with anorexia/cachexia. This drug has been shown to have inhibitory effects on the production of TNF but in this study, no improvement in appetite or weight gain was observed (Goldberg *et al*, 1995).

Recombinant human TNF inserted into a mammalian expression vector and transfected into Chinese hamster ovary (CHO) cells caused severe weight loss over a period of 32 days when transplanted into nude mice (Oliff *et al*, 1987). Intramuscular inoculation of the cells resulted in approximately 18% loss of body weight due to extensive muscle wasting and almost complete depletion of fat stores. Anorexia was also apparent as measured by reduced food and water intake and reduced urine and faeces output. Although clearly demonstrating the effectiveness of TNF to induce some of the characteristics of cachexia *in vivo*, this model by its design ensures that high levels of serum TNF are obtained and thus may not be comparable to the physiological situation. In contrast, transgenic mice expressing human TNF did not develop cachexia (Keffer *et al*, 1991). The Yoshida AH-130 ascites hepatoma induces weight loss in the host and elevated endogenous circulating TNF and prostaglandin E_2 levels are associated with its growth *in vivo* (Costelli *et al*, 1993). Administration of anti-TNF antibody prior to transplantation of this tumour caused abolition of detectable plasma TNF levels, while fractional rates of protein degradation in the gastrocnemius muscle, heart and liver of rats

transplanted with this tumour were reduced. Improvement was most apparent for the liver in which the protein degradation rate was maintained at levels observed for non tumour-bearing rats. Improved insulin and corticosterone homeostasis were also apparent, but no attenuation in weight loss or anorexia were observed, thus suggesting that other factors in addition to TNF must contribute to the overall condition of cachexia. This conclusion was also reached by Carbo and co-workers (1994), who demonstrated increased activity of white adipose LPL and reduced plasma concentrations of triglycerides after anti-TNF antibody administration to rats transplanted with the Yoshida AH-130 hepatoma during the early, exponential phase of tumour growth. This experimental model is associated with increased brown adipose tissue and heart LPL activity which was also reduced by administration of anti-TNF antisera. Brown adipose tissue is highly thermogenic and metabolic stimulation due to dietary modification, stress or infection is thought to contribute to elevated basal metabolic rate and hence, the cachectic process. However, antibody treatment during this study was ineffective when administered during the later stationary phase of tumour growth. This observation may explain negative results obtained by other workers with respect to anti-TNF antibody treatment (Langstein *et al*, 1991) hence, TNF may be involved in the early lipid metabolic changes that develop after transplantation and thereafter, other factors may play an increasingly prominent role.

Mice transplanted with the methylcholanthrene-induced sarcoma (MCG 101) develop anorexia and weight loss associated with depletion of total body protein and fat. Inoculation of the Lewis lung adenocarcinoma results in fat depletion in the absence of anorexia and has less of an effect on lean body mass. Anti-TNF antibody treatment in these cases resulted in a reduction of carcass lipid loss and prevention of hypertriglyceridaemia but had no effect on hypoalbuminaemia, anaemia and serum concentration of the acute phase protein amyloid P (Sherry *et al*, 1989). These authors suggest therefore that TNF may contribute to the anorexia and changes in body fat and protein metabolism in these models but may not be responsible for all the alterations

associated with cachexia. The ineffectiveness of the TNF antibody to reverse the hepatic acute phase response observed in mice transplanted with MCG 101 was also reported by Gelin *et al* (1991), who observed improvements in whole body lipid content and food intake, but no change in acute phase reactants or total body protein.

In addition to its fat-depletory effects, TNF has been observed to cause significant muscle protein catabolism *in vivo* (Oliff *et al*, 1987) and has been shown to be produced by Yoshida AH-130 hepatoma cells which induce marked muscle mass depletion in mice (Tessitore *et al*, 1993b). *In vitro*, TNF at high concentrations was observed to cause an enhanced release of amino acids from isolated mouse diaphragm (Mahony *et al*, 1988). However, no effect on protein degradation or synthesis was observed in isolated extensor digitorum longus muscle following exposure to recombinant TNF (Moldawer *et al*, 1987).

One of the mechanisms by which TNF is believed to exert its catabolic effects is via stimulation of the release of adrenocortical hormones. However, although adrenalectomy of rats transplanted with the Yoshida AH-130 ascites hepatoma corrected the associated hypertriglyceridaemia and elevated serum corticosterone levels, there was no improvement in other cachectic parameters (Tessitore *et al*, 1994). Insulin treatment however, was observed to result in a significant improvement in muscle protein and adipose tissue preservation with a reduction of plasma corticosterone level, but no change in circulating TNF levels; thus, once again opening to question the role of this cytokine in the development of the cachectic process.

Prostaglandin E_2 has been shown to be elevated in mice transplanted with the Yoshida AH-130 ascites hepatoma (Costelli *et al*, 1993) in addition, administration of recombinant TNF to non tumour-bearing mice resulted in a long-lasting elevation of this prostaglandin (Mahony and Tisdale, 1989a); hence, it was suggested that TNF may mediate its effects through prostaglandin synthesis. However, indomethacin failed to

alleviate the cachectic effects of the tumour in the former case (Tessitore *et al*, 1994) and weight loss due to exogenous administration of TNF was not prevented by prior administration of the drug despite inhibition of PGE_2 production (Mahony and Tisdale, 1989a). Indeed, in the latter case chronic dosing with recombinant TNF resulted in the development of resistance to its effects while PGE_2 levels were still elevated. This suggests therefore, that prostaglandins may not be involved in the induction of weight loss by TNF.

Hypoalbuminaemia has been observed in experimental models of cancer cachexia (Lonnroth *et al*, 1990; Langstein *et al*, 1991), and this may be attributable to a selective inhibitory effect of TNF on albumin gene expression (Brenner *et al*, 1990). Albumin mRNA was observed to be reduced by up to 90% prior to weight loss in nude mice inoculated with CHO cells transfected with the TNF gene, perhaps by modifying DNA-binding proteins that bind to the albumin gene promoter and are required for liver-specific control of gene expression.

Experiments that attempt to establish the importance of TNF in manifestation of cachexia by testing the anti-cachectic properties of neutralising antibody have in some cases provided evidence that TNF may be involved in the early changes of the process. However attempts to detect elevated levels of TNF in the serum and tissues of mice transplanted with one of these neutralisable models have failed to demonstrate levels above those observed with non tumour-bearing controls (Lonnroth *et al*, 1990). While it may be possible for TNF present in amounts below the level of detection using current bioassays and ELISA methods to induce cachexia, it seems more likely that its role as a sole inducer is minimum. In one study administration of recombinant TNF to non tumour-bearing mice was observed to cause weight loss that was in proportion to the decrease in food and water intake (Mahony *et al*, 1988). This weight loss was most apparent during the 24h post injection and in contrast to pair-fed controls, hypoglycaemia, reduction of plasma free fatty acids and marked hypertriyglyceridaemia were observed

(Mahony and Tisdale, 1988). Body composition analysis revealed similar degrees of loss of adipose tissue and total body water content in TNF treated and pair-fed mice, though there was no change in lean body mass as measured by thigh and gastrocnemius muscle content. Thus unlike true cachexia, weight loss caused by TNF may primarily be a consequence of reduced food intake. In addition, weight loss caused by TNF was reversible by force feeding with glucose and even by administering the equivalent volume of water (Mahony and Tisdale, 1989b).

Other metabolic effects observed after single injection of TNF include hypoglycaemia, liver glycogen depletion and a drop in rectal body temperature compared to pair-fed controls (Mahony and Tisdale, 1990). Plasma levels of alanine, lactate and pyruvate were not altered thus suggesting no elevation of the rate of gluconeogenesis as has also been observed by other workers (Rofe *et al*, 1987). Plasma free fatty acids and trigylcerides were transiently elevated while ketone bodies acetoacetate and 3-hydroxybutyrate were elevated over a longer period of time. Enhanced lipogenesis was observed to account for the increased glucose utilisation by the liver after a single injection of recombinant TNF. Lipogenesis from glucose was elevated two-fold and plasma and adipose tissue concentrations of newly synthesised fatty acids were also elevated by the same degree. Hence, the elevated plasma free fatty acids may result from increased hepatic output and hypertriglyceridaemia from esterification of fatty acids to very low density lipoprotein by the liver.

In contrast to some observations (Hauner *et al*, 1995) TNF did not stimulate lipolysis in isolated rat (Rofe *et al*, 1987) or mouse adipocytes (Mahony *et al*, 1988), though enhanced stimulation of adrenaline-induced lipolysis was observed after pre-incubation with TNF (Rofe *et al*, 1987). These authors suggest therefore, that TNF may make the adipocyte more sensitive to β-adrenergic stimulation so that in the stressed state, TNF may facilitate the availability of fatty acids in the circulation for host metabolism.

The role of TNF in the development of cachexia is unclear. Alleviation of some cachectic parameters is observed by the administration of anti-TNF antibody in certain experimental models, but since TNF has been shown to cause severe anorexia it may be that in these responsive models, this parameter is the major component of cachexia, as evidenced by the observed decrease in food and water intake soon after transplantation. The importance of TNF may also appear questionable in view of the observation that the chronic administration of this cytokine has been shown to render the host refractory to its effects (Mahony and Tisdale, 1988; Stovroff *et al*, 1989). Anti-TNF antibody treatment failed to have any effect on cachexia conferred by models that more closely represent the human cachectic condition namely, the colon 26 adenocarcinoma (Strassmann *et al*, 1992a) or the MAC 16 tumour (Mulligan *et al*, 1992)). Indeed, TNF mRNA was only measurable at the tumour site and in the spleen of mice bearing the non-cachexigenic variant of the former model (Yasumoto *et al*, 1995).

In conclusion, the induction of the cancer cachexia syndrome by an TNF-producing tumour has not yet been demonstrated and there is no evidence to date that cachectin is a toxohormone produced by cancer cells (Iseki *et al*, 1995). Indeed, one study has demonstrated a role for TNF in the mediation of obesity and in particular, the insulin resistance that often accompanies this condition (Gokhan *et al*, 1993). Induction of weight-loss by TNF may be attributable to anorexia (Milenkovic *et al*, 1989) or to toxicity (Mahony and Tisdale, 1988). These negative findings reported highlight the need for investigators to 'determine the specific properties of this highly purified and commercially available cytokine before it falls into disrepute clinically through studies based on the more global properties implied in its present names TNF/cachectin' (Rofe *et al*, 1987).

1.4.2.2. Interleukin-6 (IL-6)

IL-6 was believed to be a possible mediator of the cachectic process as a result of its lipoprotein lipase inhibitory properties (Greenberg *et al*, 1992). The role of IL-6 in cancer cachexia has been extensively studied due to the development of the colon 26 model of cachexia (Tanaka *et al*, 1990). Colon 26 adenocarcinoma was induced by exposure to N-nitroso-N-methylurethan; and in CDF 1 mice, was observed to induce many parameters of the cachectic process with a low tumour burden (1-2%) and no change in food and water intake. This model caused weight loss due mainly to fat depletion (85%), though muscle protein was also reduced (15%). The tumour also induced hypoglycaemia, hypercorticism, elevation of acute phase proteins, and reduction in the activities of hepatic catalase and drug metabolising enzymes. Progression of weight loss in this model was associated with increasing serum IL-6 concentrations, a phenomenon that was reversed after resection of the tumour (Strassmann *et al*, 1992a).

IL-6 was also detected in the conditioned medium of the colon 26 cell line, though at concentrations that were 60-fold less than in freshly isolated cells. This observation was later shown to be the result of stimulation of tumour IL-6 production by IL-1 released from infiltrating macrophages comprising approximately 6% of the tumour *in vivo* (Strassmann *et al*, 1992b). Hence, it was proposed that host macrophages may interact with the tumour cells and cause potentiation of the effects of tumour-produced IL-6, resulting in the induction of cachexia. *In vitro* studies further supported this hypothesis by the observation of the presence of high affinity tumour cell receptors for IL-1. IL-1 receptor antagonists injected intratumourally resulted in a reduction of serum IL-6 concentration and a reduction in fat and muscle loss (Strassmann *et al*, 1993a). The demonstration of the long-lasting stimulation of IL-6 by IL-1, in contrast to the acute stimulation caused by endotoxin also served to support the feasibility of the role of this process in a chronic progressive condition such as cachexia. (Strassmann *et al*, 1992b).

Suramin (polysulphated naphthylurea), a drug that has been observed to block the receptor binding of various growth factors, was observed to prevent the binding of IL-6 to its gp80 membrane glycoprotein receptor and administration *in vivo* inhibited the cachectic process by 60% (Strassmann *et al*, 1993b). Anti-mouse IL-6 antibody was observed to suppress the development of cachexia by 15% in terms of weight loss, as represented by an increase in fat and protein of 75% and 14% respectively, compared to untreated tumour-bearing mice.

Development of two clones from the colon 26 tumour however, have cast doubt on the importance of IL-6 alone to induce the cachectic process. Mice bearing the cachexigenic clone 20 tumour rapidly lost weight with similar reductions of fat and muscle tissue as that observed for colon 26 carcinoma. However in the isolated clone, weight loss was also associated with a decrease in food and water intake though pair-feeding experiments demonstrated that the weight loss observed was not entirely due to anorexia (Soda *et al*, 1994). IL-6 mRNA was detected at the tumour site of clone 20 but not the tumour site of the non-cachexigenic clone 5 (Yasumoto *et al*, 1995). These authors also observed the presence of mRNA for the naturally occurring IL-1 receptor antagonist only at the tumour site of clone 5. However, speculation that this could account for the lack of detection of IL-6 in this clone was dispelled by the failure to prevent cachexia following transfection of clone 20 with cDNA for the receptor antagonist despite a resulting decrease in IL-6 serum concentration. IL-6 concentration was elevated in the serum of mice bearing clone 20 by day 15 after transplantation however similar levels were also reached in the serum of mice by day 25 after inoculation with clone 5 (Fujimoto-Ouchi *et al*, 1995).

Administration of anti-mouse IL-6 antibody to clone 20 tumour bearing mice between days 9 and 16 was observed to partially prevent the development of cachexia, as determined by an slight increase in carcass weight, adipose tissue and gastrocnemius weights compared to untreated controls (Yasumoto *et al*, 1995; Fujimoto-Ouchi *et al*,

1995). However, even in antibody treated clone 20 tumour-bearing mice, extensive weight loss was observed, thus adding further confusion to the role of IL-6 in the development of cachexia in this model. In addition, continuous infusion of recombinant mouse IL-6 failed to induce the cachectic syndrome in clone 5 tumour-bearing mice (Soda *et al*, 1995). Lipolysis and proteolysis were only observed during administration of large doses during this study and in another study, recombinant human IL-6 was shown to have no effect on the rate of protein breakdown in isolated rat muscle (Garcia-Martinez *et al*, 1994).

In the MAC 16 tumour model of cancer cachexia during which cachexia is induced by a low tumour burden and without a decrease in food and water intake (Bibby *et al*, 1987), elevation of IL-6 was not detected in the serum during any stage of the cachectic process (Mulligan *et al*, 1992). In addition in another study, cachexia was not induced in transgenic mice expressing IL-6 constitutively (Suematsu *et al*, 1989)

1.4.2.3. γ-Interferon (IFN)

Although elevated serum IL-6 levels have been shown to correlate with poor prognosis in a group of patients with ovarian cancer (Scambia *et al*, 1995), other patient studies have cast doubt on the contributory role of IL-6 as a mediator of cancer cachexia. Circulatory IL-6 levels have been shown to be elevated in cancer patients with advanced metastatic disease together with elevated whole body protein turnover and acute phase response (Fearon *et al*, 1991). In addition, IL-6 has been shown to cause a dose-dependent increase in the production of positive acute phase proteins, especially C-reactive protein; and a decrease in the production of negative acute phase proteins, namely prealbumin and transferrin, by primary human hepatocyte cultures (O'Riordain *et al*, 1995). Hence, IL-6 may mediate the acute phase response and account for the observed increased rate in whole body protein synthesis. However, IL-6 levels were reported to be elevated in patients with unresectable pancreatic cancer, and in whom elevated resting energy expenditure and acute phase response was apparent, but no correlation between these parameters could be determined (Falconer *et al*, 1994a). In addition, the same

workers have shown that although ibuprofen treatment alleviated the elevated acute phase response and normalised whole body protein kinetics in patients with colonic tumours, circulating IL-6 levels remained high (Preston *et al*, 1995). These authors suggest that local IL-6 production from blood monocytes may be important for the mediation of hepatic acute phase response and this may have been inhibited by ibuprofen. In contrast, IL-6 circulatory levels were reduced by indomethacin in mice with colon 26 adenocarcinoma by 96% with a reduction in adipose tissue and protein loss (Fujimoto-Ouchi *et al*, 1995) however, a 74% increase in tumour growth was also observed.

The role of IL-6 in the development of cachexia is unclear, it seems unlikely that it is responsible alone for the cachectic parameters induced by the tumour models examined so far but it may induce cachexia through its interaction of other cytokines or as yet unidentified factors.

1.4.2.3. γ *-Interferon* (IFN)

Interest in the role of IFN in the pathogenesis of cancer cachexia developed as a result of observations confirming that it shared similar properties to TNF with respect to fat metabolism *in vitro* (Patton *et al*, 1986).

Weight loss in mice bearing the Lewis lung tumour was antagonised by treatment with anti-IFN antibody or enhanced by exogenous IFN (Matthys *et al*, 1991a). Tumour-bearing mice exhibited weight loss that was due to depletion of total body fat and protein in the absence of anorexia. Administration of anti-IFN antibody reduced the depletion of fat but had no significant effect on total body protein. A growth-inhibitory effect on the tumour was also observed with administration soon after transplantation suggesting that this cytokine may be important for tumour growth during the early stages of cell proliferation. The role of IFN in the development of cachexia in rats transplanted with the MCG 101 sarcoma has also been implicated as a result of a reduction of the severity of the

cachectic parameters of weight loss and anorexia observed after administration of anti-IFN antibody (Langstein *et al*, 1991). Survival was increased in antibody-treated rats, however there was no change in hypoalbuminaemia or hypertriglyceridaemia and the treatment was partial and short-lived suggesting that IFN may not be the sole mediator.

In another study, repetitive sub-lethal doses of recombinant IFN to non tumour-bearing mice over a period of 10 days resulted in anorexia and weight loss and unlike a similar experiment using TNF (Mahony and Tisdale 1989), the effect observed in this case was sustained during chronic administration (Langstein *et al*, 1991).

Inoculation of CHO cells transfected with the IFN gene resulted in a dose-related development of anorexia and marked weight loss due to fat and muscle depletion in mice not wholly attributable to the reduction in food intake (Matthys *et al*, 1991b). Pre-treatment with anti-IFN antibody prevented this effect but was ineffective when administered 24h after transplantation.

In addition to its inhibitory action on LPL, it has been proposed that IFN may exert its cachectic effect through the stimulation of indoleamine 2,3, dioxygenase (IDO), thus resulting in depletion of plasma tryptophan levels, possibly leading to a negative nitrogen balance and protein catabolism (Iwagaki *et al*, 1995a). The resulting increase in kynurenin could also result in mental and metabolic disorders. Neopterin is commonly used as a biochemical marker for the activation of cellular immunity as it is produced from activated macrophages as a result of production of IFN from activated T cells. Levels of this marker were observed to be elevated in patients with gastrointestinal tumours and cachexia compared to controls and a correspondingly elevated level of the macrophage-derived protein immunosuppressive acidic protein (IAP) was also observed. Hence the observed relationship between neopterin and IAP suggested that host immune activation involving IFN may play a role in the immunosuppression of patients with cancer cachexia. In contrast to patients with acute infections, a higher serum ratio for

neopterin:C-reactive protein was also observed in patients with cancer cachexia hence while response to infection involves an increase in both markers, the cancer situation is characterised by a major increase in neopterin thus leading to the suggestion that IFN may play the major role in the pathogenesis of cancer cachexia while IL-6 may modify the disease status (Iwagaki *et al*, 1995b).

1.4.2.4. Transforming growth factor β (TGF)

While being tested for anti-tumour activity, TGF was observed to have systemic cachectic effects thus, extensive weight loss was induced in mice transplanted with MDA-MB-231 mammary tumour cells and injected with TGF (Zugmaier *et al*, 1991). This effect was reversed on cessation of treatment. In addition, weight loss was observed in inhibin-deficient mice and correlated with the development of gonadal tumours (Matzuk *et al*, 1994). Elevated levels of activins which are members of the TGF family of cytokines were found to be elevated by a factor of 3 due to the presence of the tumour. In contrast, inhibin-deficient gonadectomised mice did not develop tumours or cachexia suggesting that the secretion of avidins was responsible for the development of the cachexia syndrome. However, the weight loss was largely attributable to anorexia perhaps as a result of mucosal atrophy of the glandular stomach.

Elevated TGF has also been associated with tumour growth and the development of cachexia in the colon 26 adenocarcinoma when transplanted into CDF1 mice (Tanaka *et al*, 1993), and has been shown to cause a rapid decrease in expression of adipose genes when incubated with fully differentiated adipocytes (Torti et al, 1989).

1.4.2.5. Leukaemia inhibitory factor (LIF)

The human melanoma cell line, SEK1 was observed to induce severe cachexia in tumour-bearing mice, and addition of conditioned medium from this cell line caused a

decrease in the activity of LPL in cultured adipocytes with no effect on the viability of the cells (Kawakami *et al*, 1991). This inhibition was similar to that caused by TNF in terms of dose and time course however, no TNF was detected in the medium. A protein with LPL-inhibitory effects has been purified from conditioned medium of these cells and found to be identical to LIF (Mori *et al*, 1989). Several studies have produced results which suggest that LIF may be important in the development of cachexia thus, LIF mRNA was observed to be present in two melanoma xenografts that induce weight loss while none was detected in non-inducing xenografts (Mori *et al*, 1991).

In another study, five human tumour cell lines with the ability to cause cachexia were screened for the presence of cytokines. Conditioned media from all the cell lines exhibited LPL-inhibitory activity but in two this activity was greater by 70% namely, SEK1 and neurepithelioma (NAGAI) (Iseki *et al*, 1995). LIF mRNA was detected in these cell lines and when transplanted into nude mice, plasma LIF was elevated to approximately 1700pg/ml and 1400pg/ml respectively. The resulting cachectic parameters included weight loss, skin dryness, loss of appetite, low body temperature, reduced physical activity, hypoglycaemia and elevated IAP. The mechanism involved in the development of cachexia in the other three models was unknown as TNF and IL-6, also proposed as potential mediators were not detected.

LIF may exert its cachectic effect through the inhibition of lipoprotein lipase as recombinant LIF was observed to produce a dose and time-dependent inhibition of the enzyme activity in 3T3-L1 adipocytes (Berg *et al*, 1994). The inhibitory effect was approximately 50% less than that caused by TNF and appeared to be transcriptional. In contrast to TNF, LIF was also observed to increase lipogenesis in these cells as measured by uptake of [^{3}H]-acetate (Marshall *et al*, 1994).

In conclusion, LIF was thought to fulfil many of the criteria for a toxohormone because it was produced by tumour cells, secreted into the circulation, and was associated

with the development of some of the parameters of cachexia. However, other symptoms accompanying the weight loss induced by high levels of LIF suggested that some of the effects may be due to toxicity. Thus, mice inoculated with cells expressing recombinant LIF were observed to develop marked abnormalities in calcium metabolism, and in the adrenal cortex and ovarial corpora lutea (Metcalf and Gearing, 1989). In addition, thymus atrophy and pancreatitis were apparent.

1.4.2.6. Interleukin-1 (IL-1)

IL-1 has been shown to have many similar effects to TNF in various tissues and cells including suppression of LPL activity and enhancement of intracellular lipolysis and they are believed to be secreted in combination by macrophages. When co-administered, they were observed to have a synergistic effect on the suppression of LPL activity in cultured mouse adipocytes but appeared to act by different mechanisms to cause the inhibition of hormone-sensitive lipase (Ogawa *et al*, 1989).

Administration of recombinant IL-1 was observed to induce anorexia, weight loss, hypoalbuminaemia and elevated amyloid P levels in the mouse (Moldawer *et al*, 1988). Indeed this cytokine was observed to have a greater anorexigenic effect than TNF when administered in isomolar quantities. However in a tumour model of cachexia, IL-1 mRNA was detectable in the spleen, liver, intestine and brain of mice transplanted with the MCG 101 tumour, but only in the spleen was up-regulation of expression detected (Lonnroth *et al*, 1990). IL-1 and TNF were both detected in the tumour tissue of this model though at levels that were similar to those in normal tissues. Hence, IL-1 was not believed to be a major promoter of cachexia in this model.

Neutralising antibodies against the IL-1 receptor was observed to cause significant but minor inhibitory effects on cachexia and anorexia in mice transplanted with the MCG 101 sarcoma (Gelin *et al*, 1991). However, there was no improvement in the levels of

acute phase proteins and hypoalbuminaemia, thus suggesting that IL-1 may have a partial role in the aetiology of cancer cachexia. More recently, administration of the IL-1 receptor antagonist was observed to be completely ineffective in either reversing tumour growth or in preventing tissue depletion and protein hypercatabolism in rats transplanted with the Yoshida ascites hepatoma, with the conclusion that IL-1 may not be important for the development of cachexia in this tumour model (Costelli *et al*, 1995). This is in agreement with other workers who observed that transfection of a cachectic tumour cell line with the gene for the IL-1 receptor antagonist failed to abolish the capacity of the tumour to cause cachexia (Yasumoto *et al*, 1995). In addition, recombinant IL-1 stimulated prostaglandin E_2 production by extensor digitorum longus muscle but had no effect on protein synthesis or degradation rates (Moldawer *et al*, 1987), thus casting doubt on the role of this cytokine and prostaglandins with respect to depletion of lean body mass.

1.4.2.7 Conclusion

It has been proposed that three lines of evidence need to be satisfied in order to support the role of an agent as an important cachectic factor in the development of cancer cachexia. These include measurable circulatory levels, correlation of the serum level with the degree of cachexia and finally, abolition of the key parameters of cachexia including fat and muscle wasting, hypoglycaemia and hepatic acute phase response by inhibition of the agent (Strassmann *et al*, 1992a). Studies of IL-6, TGF and LIF to date most completely fulfil these criteria in the models examined, though their suitability as candidates in all experimental models and in human cancer cachexia await further elucidation. The lack of evidence of circulating IL-1 and TNF has led to the suggestion that these cytokines may act as paracrine or autocrine mediators rather than as circulating messengers in experimental cancer (Gelin *et al*, 1991). The studies to date strongly suggest that rather than one *bona fide* cytokine being responsible for all the cachectic parameters observed, many cytokines or endogenous factors that augment each others

effects may be involved in the development of cachexia. This has been most clearly demonstrated in the case of IL-6 whose production has been shown to be stimulated by TNF, IL-1 and IFN release from activated macrophages. IL-1, TNF and IFN are believed to stimulate or serve as a co-signal for the production of IL-6 (Strassmann *et al*, 1992a). Thus, TNF and IL-1 were observed to cause a rapid and transient increase in intracellular cAMP, an increase in protein kinase activity and induction of IL-6 mRNA in human fibroblast cells (Zhang *et al*, 1988). Thus elevated circulatory levels of IL-6 may be a reflection of the presence of these cytokines (Strassmann *et al*, 1992a; Lonnroth *et al*, 1990). More recently, Mantovani *et al* (1995) demonstrated increased concentrations of TNF, IL-1, IL-2, IL-6 and the soluble IL-2 receptor in the serum of cachectic patients with advanced head and neck carcinoma. Treatment with megestrol acetate which resulted in improved appetite and weight gain, was also associated with a reduction in serum concentration of these cytokines thus further suggesting the possibility of interplay between them in the mediation of cancer cachexia. It has also been suggested that the hypermetabolic cachectic condition may be the result of the interaction of cytokines and classical endocrine hormones. Thus, elevated levels of circulatory TNF, cortisol and glucagon were detected in cachectic patients with breast cancer (Knapp et al, 1991) and in Yoshida AH-130 hepatoma-bearing rats (Tessitore et al, 1993b). The former authors suggest that TNF may indirectly stimulate cortisol and glucagon release through increased production of IL-1.

1.4.3. Tumour-derived lipolytic and proteolytic factors

As has been discussed, weight loss in cancer cachexia is due to a depletion of whole body fat and protein represented mainly by a reduction of adipose tissue and skeletal muscle mass. The last decade therefore has involved the search for factors released from the tumour into the circulation that would directly cause catabolism in these tissues.

1.4.3.1. Lipolytic factors

The production and secretion of lipolytic factors by a tumour was implicated in an early study which observed increased fat mobilisation in rats transplanted with the Krebs-2 carcinoma (Costa and Holland, 1962). These workers observed that in the triphasic pattern of fat depletion observed for their model, the early rapid stage of fat depletion, which represented true cachexia in the absence of anorexia and with a low tumour burden, was associated with increased uptake of acetate into total body fat. They observed that the phenomenon could also be invoked by non-viable tumour preparations but did not occur during the later stage of fat loss which was associated with reduced food intake. Thus, a factor produced by this tumour may have induced fatty acid mobilisation and lipogenesis due to the increased requirement of the host for fatty acids as a result of the development of the cachectic syndrome. The action of a lipolytic factor was also suggested as an explanation for the observations of increased stimulation of lipolysis in an *in vitro* assay by ascites serum from rats transplanted with the Walker 256 carcinoma (Kravolic *et al*, 1977).

Several workers have identified and attempted to isolate lipid mobilising factors from tumour cells. Kitada *et al* (1980 and 1981) showed that a lipid mobilising factor was present in the serum of mice transplanted with a thymic lymphoma. This factor was also detected in extracts of the tumour, tissue culture medium and in the serum of a cancer patient with adenocarcinoma of the cervix and stomach, thus suggesting that the lipolytic factor was tumour-derived and circulatory. In addition, serum from tumour-bearing mice produced massive fat loss when injected into non tumour-bearing controls. Preliminary purification suggested that the factor was approximately 5kD, though it was subsequently shown to be a heat-stable protein of Mr less than 10kD. This protein caused massive fat mobilisation when injected into non tumour-bearing mice (Kitada *et al*, 1982).

Masuno *et al* (1981) purified a lipolytic factor from the ascites fluid of mice transplanted with the sarcoma 180 tumour. This was identified as an acidic, heat-labile 75kD protein with an isoelectric point of 4.7 and was called Toxohormone-L. A lipolytic factor of between 65 and 75kD was also observed in the serum of patients with hepatoma and Grawitzs tumour and was believed to be associated with an increase in plasma free fatty acids and loss of body fat (Masuno *et al*, 1984).

In another study, the conditioned medium from three human melanoma cell lines exhibited lipid mobilising activity that was higher than that produced by a benign human fibroblast cell line and this effect was correlated with the degree of fat depletion *in vivo* (Hollander *et al*, 1986). The factor responsible was shown to be less than 50kD and sensitive to heat and pH.

A lipolytic factor has also been associated with the depletion of fat observed in mice transplanted with the MAC 16 adenocarcinoma. Preliminary tests on the tumour extract suggested that the factor was peptidic in nature because it was non-dialysable, inhibited by pronase treatment and destroyed by acid and high temperature (Beck and Tisdale, 1987). After further purification from tumour extract (Beck *et al*, 1990) and tumour-bearing mouse urine (Beck and Tisdale, 1991), the lipolytic activity was assigned to a series of low molecular weight factors with apparent molecular weights of 3,1.5 and 0.7kD. Analysis of the non-cachexigenic though histologically similar MAC 13 tumour extract revealed that these factors were present in markedly reduced concentrations (Beck *et al*, 1990). A linear relationship between weight loss and the presence of lipolytic activity was observed in MAC 16 tumour-bearing mice with less than 15-20% weight loss, the serum activity then decreased with further weight depletion (Groundwater *et al*, 1990). Lipolytic factors purified from conditioned medium of MAC 16 cells induced weight loss without a decrease in food intake when injected intraperitoneally into mice transplanted with the MAC 13 tumour (Beck *et al*, 1990). These factors were shown to be absent in the urine of both non tumour-bearing mice and healthy human subjects even

after 24h starvation thus suggesting that they may differ from hormones associated with the catabolic state in starvation (Beck and Tisdale, 1991).

Lipolytic activity was detected in the serum and urine from cachectic cancer patients (Groundwater *et al*, 1990), and subsequent purification of the factors responsible determined that they were the same as those produced by the MAC 16 tumour (Beck et al, 1990; Beck and Tisdale, 1991). Sera and urine from 24 cancer patients with weight loss ranging from 0-50% of the original body weight were assayed for lipolytic activity and compared to samples from patients with Alzheimer's disease with and without a similar degree of weight loss and healthy controls (Groundwater *et al*, 1990). No differences were observed in the lipolytic activity of serum and urine from all three control groups as determined by an *in vitro* assay; in contrast, significantly higher lipolytic activity was detected in samples from cancer patients. A linear relationship between weight loss and lipolytic activity was also observed in patients with less than 20% weight loss, thus mirroring the relationship between weight loss and lipolytic activity observed in MAC 16 tumour-bearing mice. In addition, the level of serum lipolytic activity was reduced when compared to the pre-treatment values in six of the seven patients who showed a positive response to chemotherapy, and in one non-responsive patient there was no change (Beck *et al*, 1990). Purification of factors from the serum of one the responsive patients before, during and after treatment clearly demonstrated that the levels of these factors had decreased. Measurement of these factors may therefore enable the efficacy of cancer treatment to be determined.

Further support for the role of these factors in the cachectic process was provided by inhibitory studies thus, the *in vitro* lipolytic activity of the factor produced by the MAC 16 tumour was shown to be suppressible by insulin and 3-hydroxybutyrate (Beck and Tisdale, 1987). This is in agreement with the anti-cachectic effects of insulin and a ketogenic diet observed *in vivo* (Tisdale *et al*, 1987; Beck and Tisdale, 1989; Fearon *et al*, 1988). Likewise, EPA was observed to inhibit the lipolytic activity *in vitro* while other

fatty acids of the n-3 series and those of the n-6 series had no effect (Tisdale and Beck, 1991). This was also reflected in the *in vivo* situation in which EPA alone was observed to completely inhibit the cachectic process in MAC 16 tumour-bearing mice.

Lipolytic factors purified from the MAC 16 tumour therefore, appear to satisfy all the criteria for a cachectic factor (Gelin *et al*, 1991) and more importantly, the factors are not restricted to this particular model but also appear to play an important role in the development of cancer cachexia in humans.

Recently, another lipolytic factor has been purified from the conditioned medium of the human A375 melanoma cell line (Taylor *et al*, 1992). This factor was observed to have an apparent molecular weight of approximately 6kD and was heat-stable, resistant to proteases and nucleases and periodate oxidation. Intravenous injection of purified material into nude mice resulted in 85% depletion of adipose tissue and weight loss of 3.3%. Whether all these factors are indeed different tumour products or are in fact the same product and the differences simply a reflection of the purification and analytical methods employed awaits their further characterisation.

1.4.3.2. Proteolytic factors

Transplantation of mice with the MAC 16 adenocarcinoma induces depletion of skeletal muscle tissue which has been shown to be directly proportional to the weight of the tumour (Beck and Tisdale, 1987). Proteolytic activity as determined by amino acid release from mouse diaphragm *in vitro* has been detected in MAC 16 but not MAC 13 tumour extracts, and this activity was shown to be proportional to the decrease in thigh and gastrocnemius muscle dry weight. The proteolytic activity was heat and acid-labile though not destroyed by phenylmethylsulphonyl flouride, thus suggesting that the factor was not a serine protease. This tumour-derived factor has also been detected in the plasma of tumour-bearing mice and was shown to be inhibited by insulin and 3-

hydroxybutyrate, in agreement with improvements in protein homeostasis observed *in vivo* (Fearon *et al*, 1988; Beck and Tisdale, 1989). In addition, EPA had an inhibitory effect on tumour-induced proteolytic activity *in vitro*, and the rate of protein degradation determined by tyrosine release from gastrocnemius muscle isolated from mice dosed with EPA was also reduced (Beck *et al*, 1991). The observed *in vivo* effects of decreased protein synthesis and markedly increased degradation in the gastrocnemius muscle of MAC 16 tumour-bearing mice could be induced *in vitro* by serum from tumour-bearing mice (Smith and Tisdale, 1993a). This effect was most pronounced with serum from tumour-bearing mice with weight loss of between 11 and 20%, and was not observed with serum from MAC 13 or non tumour-bearing mice (Smith and Tisdale, 1993b). This provided further evidence for the involvement of a tumour-derived circulatory proteolytic factor in the manifestation of aberrant protein metabolism associated with cancer cachexia. The role of this factor in the aetiology of the human cachectic condition awaits its further characterisation.

Similar material has been detected in the plasma of 50% of cancer patients with weight loss greater than 10% (Belizario *et al*, 1991) while no activity was detected in healthy controls. The factors responsible for the proteolytic activity have not yet been characterised though they were believed to be less than 25kD as determined by filtration. In 5/13 of these cancer patients the bioactivity was mediated in part by IL-1 as determined by neutralisation with antibodies, and it has been determined that IL-1 degrades to an active fragment with proteolytic activity which causes muscle degradation *in vitro* (Clowes *et al*, 1983). However another study failed to detect proteolytic activity in the plasma from weight-losing cancer patients (Mitchell and Norton, 1989). While the plasma from non-cachectic patients appeared to contain a growth factor which acted synergistically with insulin to increase the rate of protein synthesis.

Limited information is therefore currently available regarding the existence of specific proteolytic factors and their role in the process of cachexia however, confirmation

of their contribution to the devastating depletion of lean body mass in some experimental models of cancer cachexia (Smith and Tisdale 1993 a & b) emphasises the importance of their further elucidation.

1.5. Aims and objectives

The aim of this project is to further elucidate the contributory role of lipolytic and proteolytic factors in the mediation of the cancer cachexia syndrome. The MAC 16 tumour model of cancer cachexia will be used as the source of cachectic factors and purification methods employed to date (Beck *et al*, 1987) will be modified by the application of FPLC and HPLC in order to obtain material purified to homogeneity for protein sequence analysis. N-terminal amino acid sequence data will then be used for isolation of the relevant gene from MAC 16 total cDNA by both the polymerase chain reaction using oligonucleotide probes based on the protein sequence, and by expression cDNA library screening using a polyclonal antibody made against a synthetic peptide based on the N-terminal sequence. Further information on the proteins and their genes should enable a greater understanding of the mechanisms involved in the development of cancer cachexia to be achieved and may provide new therapeutic targets for the prevention or treatment of this syndrome.

CHAPTER 2:

PURIFICATION AND CHARACTERISATION OF CACHECTIC FACTORS FROM THE MAC 16 TUMOUR

2.1. MATERIALS, SOLUTIONS AND METHODS

2.1.1. Materials.

Amersham Intl. Bucks., UK.

Protein rainbow markers

Amicon LTD, Gloucs., UK.

Stirred cells: 50ml, 200ml, 400ml capacities

YM membranes: 10K and 1K cut-off

Microcon concentrators

Banting and Kingman, Hull, UK.

BKW mice

Bio-Rad Laboratories Ltd, Herts., UK

Acrylamide

Ammonium persulphate

Bio-Rad protein reagent

N,N'-Methylene-Bis acrylamide (Bis-acrylamide)

N,N,N',N'-Tetramethylethylenediamine (TEMED)

Silver stain reagent

Costar, Cambridge, MA., USA

Polyvinylchloride ELISA plates

Fisons laboratory supplies, Loughborough, UK.

HPLC-grade water

Glacial acetic acid

Methanol

Sulphuric acid

Gibco BRL Life Technologies, Paisley, Scotland, UK.

RPMI 1640 Tissuue culture medium

Glutamine

Trypsin/EDTA

Phosphate buffered saline (without calcium and magnesium)

Bovine foetal calf serum

Hoefer Scientific Instruments, San Francisco, CA., USA.

Nitrocellulose membrane

Romil Chemicals Ltd., Loughborough, UK.

Acetonitrile 190

Sigma Chemical, Dorset, UK

Adenosine 5'- Phosphate

Bis Tris

Bovine serum albumin

Bromophenol blue

Calcium chloride

Citric acid

Collagenase type II

Coomassie Brilliant Blue R

Dithiothrietol (DDT)

Di-sodium hydrogen orthophosphate

Di-potassium hydrogen orthophosphate

Ethylene glycol-bis (β-aminoethyl ether)

Glycerokinase

Glycerol

Glycine

Hydrogen peroxide

Iminodiacetic acid

Lactate dehydrogenase

Magnesium sulphate

Mercaptoethanol

β-Nicotinamide adenine dinucleotide phosphate, reduced form (NADH)

o-Phenylenediamine

Phenylmethylsulfonylflouride

Phosphoenolpyruvate

Polyoxyethyle-sorbitan (Tween 20)

Potassium chloride

Potassium dihydrogen orthophosphate

Protein A peroxidase

Pyruvate kinase

Sodium bicarbonate

Sodium carbonate

Sodium chloride

Sodium dihydrogen orthophoshate

Sodium dodecyl sulphate

Triethanolamine

Trizma base

OXOID, Basingstoke, Hamps. UK

Phosphate buffered saline (PBS) tablets

Whatman Intl., Maidstone, Kent UK.

Cellulose nitrate filters (0.2μm)

2.1.2. Solutions and media

2.1.2.1. FPLC anion exchange: Mono Q.

2.1.2.1.1. Anion-exchange buffer 1.

Tris.HCl pH 8.0	10mM
DTT	1.0mM
PMSF	0.5mM
EGTA	0.5mM

2.1.2.1.2. Anion-exchange buffer 2.

Tris.HCl pH 8.0	10mM
DTT	1.0mM
PMSF	0.5mM
EGTA	0.5mM
NaCl	0.2M

2.1.2.2. FPLC gel-exclusion: Superose buffer.

Potassium phosphate buffer pH 8.0	50mM *
DTT	1.0mM
PMSF	0.5mM
EGTA	0.5mM
NaCl	0.3M

*Potassium dihydrogen orthophosphate (50mM) was added to dipotassium hydrogen orthophosphate (50mM) to obtain pH 8.0.

2.1.2.3. FPLC chromatofocussing: Mono P.

2.1.2.3.1. Determination of isoelectric points pH 9.0-6.0.

Start buffer: Tris.CH_3COOH pH 9.3 0.075M

Polybuffer : 10ml Polybuffer + 90ml deionised water pH 6.0 (glacial acetic acid)

2.1.2.3.2. Determination of isoelectric points pH 7.0-4.0.

Start buffer: Bis Tris pH 7.0 0.25M

Gradient : 10ml polybuffer + 90ml deionised water, pH 4.0 with saturated iminodiacetic acid

Buffers for FPLC were filtered through a 0.2μm cellulose nitrate membrane filter and degassed prior to use.

2.1.2.4. Reverse-phase HPLC (C8).

2.1.2.4.1. Buffer 1.

HPLC grade water + 0.06% triflouroacetic acid (TFA)

2.1.2.4.2. Buffer 2.

Acetonitrile 190 + 0.04% triflouroacetic acid (TFA)

Buffers for HPLC were filtered through a 0.2μm cellulose nitrate membrane filter and degassed prior to use.

2.1.2.5. Deionised/HPLC-Water/PBS + protease inhibitors.

DTT	1.0mM
PMSF	0.5mM
EGTA	0.5mM

2.1.2.6. Gycine buffer pH 10.4.

Glycine	50mM
5M NaOH	to pH 10.4

2.1.2.7. Sodium acetate buffer pH 4.5.

Sodium acetate	50mM / 100mM
Concentrated HCl	to pH 4.5

2.1.2.8. Affinity chromatography.

2.1.2.8.1. Equilibration buffer :

PBS	(1 tablet / 100ml deionised water)
DTT	1.0mM
PMSF	0.5mM
EGTA	0.5mM

2.1.2.8.2. Elution buffer :

Gycine	100mM
Concentrated HCl	to pH 2.5

2.1.2.9. Immunoreactivity determination by ELISA.

2.1.2.9.1. Sodium carbonate/bicarbonate buffer pH 9.5.

(A) Sodium carbonate	0.1M
(B) Sodium bicarbonate	0.1M

A was added to B to obtain pH 9.5

2.1.2.9.2. Sodium citrate/phosphate buffer pH 5.0 .

0.2M Di-sodium hydrogen orthophosphate	25.7ml
0.1M Citric acid (sodium salt)	24.3ml
Deionised water	50ml

2.1.2.9.3.Wash buffer :

PBS	1 tablet / 100ml deionised water
Tween 20	0.15%

2.1.2.9.4. Antibody dilution buffer .

PBS + 3% bovine serum albumin (BSA)

2.1.2.9.5. Substrate buffer .

Phosphate / citrate buffer pH 5.0 (2.2.6.2)

o-Phenylenediamine (OPD)	0.04%
Hydrogen peroxide	0.012%

2.1.2.10. Krebs Ringer bicarbonate buffer.

Sodium chloride	118mM
Potassium chloride	5.0mM
Calcium chloride	2.0mM
Potassium dihydrogen orthophosphate	1.0mM
Sodium bicarbonate	25mM
Magnesium sulphate	1.0mM

2.1.2.11. Krebs buffer.

Krebs Ringer bicarbonate solution + 3% BSA

2.1.2.12. Glycerol assay buffer.

Triethanolamine	100mM
Magnesium sulphate	2.0mM
Phosphenolpyruvate	0.4mM
α-Nicotinamide adenine dinucleotide (NADH)	0.25mM
Adenosine 5'triphosphate (ATP)	1.2mM
Pyruvate kinase	1.0 units / ml
Lactate dehydrogenase	7.0 units / ml

The constituents were mixed in deionised water and the pH was adjusted to 7.4 with concentrated hydrochloric acid. The reaction was initiated by the addition of 1 unit of glycerokinase and allowed to proceed for 15min.

2.1.2.13. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.1.2.13.1. Acrylamide stock 1.

Acrylamide	44% (w/v)
N,N'-Bis-methylene acrylamide	0.8% (w/v)

2.2.13.2. Acrylamide stock 2.

Acrylamide	30% (w/v)
N,N'-Bis-methylene arylamide	0.8% (w/v)

2.2.13.3. Sample buffer.

Tris.HCl pH 6.8	0.0625M
10% (w/v) SDS	1.0ml
b-Mercaptoethanol	5%
Glycerol	10% (w/v)
Bromophenol blue	0.01%

2.2.13.4. Running buffer (10x).

Trizma base	30.29g
Glycine	144.3g
10% (w/v) SDS	100ml
Distilled water	to 1 litre

2.2.13.5. Coomassie brilliant blue stain solution.

Coomassie Brilliant Blue R	0.1%(w/v)
Methanol	25% (v/v)
Glacial acetic acid	10% (v/v)

2.2.13.6. Destain solution.

Methanol	25% (w/v)
Glacial acetic acid	10% (w/v)

2.2.14. Immunoreactivity determination by western blot.

2.2.14.1. Running buffer (10x).

Trizma base	30.3g
Glycine	144g
Sodium dodecyl sulphate (SDS)	15g
Distilled water	to 1 litre

For use:	10x blotting buffer	100ml
	Methanol	200ml
	Distilled water	to 1 litre

2.2.14.2. Wash buffer .

PBS	1 tablet/100ml distilled water
Tween 20	0.15%(v/v)

2.2.14.3. Antibody diluent.

PBS	1 tablet/100ml distilled water
Tween 20	0.15%(v/v)
Marvel (fat free dried milk)	1.5%(w/v)

2.2.14.4. Blocking solution.

PBS	1 tablet /100ml distilled water
Tween 20	0.15%(v/v)
Marvel	5.0μl%(w/v)

2.1.3. Methods.

2.1.3.1. Anion-exchange batch extraction.

MAC 16 tumours were excised from cachectic NMRI mice and homogenised in 5.0ml/g tumour of anion-exchange buffer 1 (2.1.2.1.1.). Cell debris was removed by low speed centrfugation (4000rpm, 15 min) in a bench-top centrifuge and the supernatant was diluted with 3 x volumes of the same buffer. DEAE-cellulose (5g/g tumour) was equilibrated with 100 mM Tris.HCl (pH 8.0) then washed three times with 10mM Tris.HCl (pH 8.0). The resulting slurry was added to the diluted homogenate and the mixture stirred for 30 minutes at 4°C. The DEAE-cellulose was recovered by low speed centrifugation and the supernatant was disgarded. Bound material was eluted by resuspension of the matrix in 0.3M NaCl, followed by re-centrifugation to recover the supernatant. The DEAE-cellulose pellets were further resuspended in 0.3M NaCl and the slurry was gently stirred at 4°C for 30 min thus ensuring complete elution of bound material. After centrifugation, the supernatants were pooled and the concentration of sodium chloride reduced to less than 1.0mM by ultrafiltration through a YM 10 membrane (Amicon Ltd, 10K cut-off) in a 500ml capacity Amicon stirred cell using deionised water with protease inhibitors (2.1.2.5.) as the diluent. The sample volume was finally reduced to approximately 2.0ml, then freeze-dried and resuspended in anion-exchange buffer 1 to a concentration of not more than 15mg/500μl as determined by the Bio-Rad protein assay (section 2.1.3.8.).

2.1.3.2. FPLC anion-exchange Mono Q.

Material recovered by batch extraction was prepared for Mono Q chromatography by high speed centrifugation (13000 rpm, 10 min) to remove particulates. The supernatant was transferred to a fresh tube and 500μl was injected onto a Mono Q HR 5/5 column equilibrated with anion-exchange buffer 1 (2.1.2.1.1.). The column was run initially at a

flow rate of 0.5ml/min and 10 fractions were collected (1.0ml). Material bound to the column was eluted by a 50ml salt gradient (0-0.2M NaCl) using anion-exchange buffer 2 (2.1.2.1.2.) as the high salt buffer, at a flow rate of 1.0ml/min. In order to monitor protein recovery, material remaining on the column was eluted by the injection of 2M NaCl (0.5ml) and a further 10 x 1ml fractions were collected. All fractions were assayed for protein content and lipolytic activity as described in sections 2.1.3.8. and 2.1.3.6.

2.1.3.3. FPLC gel-exclusion: Superose.

Fractions identified as containing lipolytically-active material after purification by FPLC MONO Q were pooled, freeze-dried and dissolved in approximately 300μl Superose buffer (2.1.2.2.). Particulate material was removed by centrifugation (13000 rpm, 10 min), the supernatant (200 μl) was injected onto a Superose 12 pre-packed 10/30 gel-exclusion column (Pharmacia Biotech) and chromatographed in Superose buffer at a flow rate of 0.5 ml/min. Thirty x 1.0ml fractions were collected and assayed for lipid-mobilising activity and protein content (2.1.3.6 & 2.1.3.8).

2.1.3.4. Affinity chromatography.

Hybridomas producing monoclonal antibodies were produced by fusion of splenocytes from MAC 16 tumour-bearing mice with weight-loss and mouse BALB/C myeloma cells using PEG 1500. Clones were screened against material with lipid-mobilising activity (pooled and concentrated fractions after Superose purification), positive clones were single cell-cloned and maintained in RPMI 1640 medium containing 25mM HEPES, 5% (v/v) glutamine, and 10% (v/v) foetal bovine serum myoclone plus under a humid atmosphere of 5% CO_2 in air at 37°C (Todorov*et al*, in press). Monoclonal antibody was purified from hybridoma tissue culture supernatant by passage through protein A sepharose and elution with 100mM glycine HCl buffer (pH 3.0). An affinity column was then prepared by coupling monoclonal antibody to Affi-Gel Hz matrix

according to the manufacturers instructions, and pouring the mix into a Pharmacia C 10/10 (1.0 x 10cm) column. The column was finally equilibrated with phosphate buffered saline and stored in 0.05% sodium azide, 4°C (Dr. Cariuk, personal communication).

Solid MAC 16 tumours excised from NMRI cachectic mice were homogenised in anion-exchange buffer 1 as described (2.1.3.1). After low speed centrifugation (4000rpm, 15min), proteins were precipitated by the slow addition of ammonium sulphate (40%, w/v) to the stirred supernatant at 4°C. The resulting precipitate was pelleted by centrifugation (5000rpm, 20min) and the supernatant prepared for affinity column purification by reduction of ammonium sulphate concentration and equilibration with 3 x volume PBS + protease inhibitors (2.1.2.5.) in a 500ml capacity Amicon ultrafiltration stirred cell through a YM 10 membrane. The volume was then reduced to approximately 10ml, the sample was centrifuged to remove particulate material and the protein concentration was determined. The sample was circulated through the affinity column by a peristaltic pump (0.1ml/min) overnight at 4°C. Prior to elution, the column was washed for 2h with PBS + protease inhibitors (0.2ml/min) to remove unbound material and finally antibody-bound material was then eluted with 100mM glycine HCl buffer pH 2.5 (2.1.2.8.2.) into tubes containing 0.5ml Tris HCl (1.0M pH 8.0) for neutralisation. The flow rate was increased to 1.0ml/min for elution and 3.0ml fractions were collected. These were assayed by ELISA as described in section 2.1.3.7..

2.1.3.5. Reverse-phase HPLC.

2.1.3.5.1. HPLC after gel-exclusion chromatography.

Active fractions purified by Superose gel-exclusion were pooled and the salt concentration was reduced to approximately 30mM by Amicon ultrafiltration in a 50ml capacity stirred cell. A 10K cut-off membrane was used and HPLC grade water plus protease inhibitors (2.1.2.5.) was the diluent. The sample was reduced in volume to

approximately 500μl, particulate material was removed by centrifugation (13000rpm, 10 min), protein and lipolytic activity were determined in 5.0μl and 25μl respectively (2.1.3.8. & 2.1.3.6). The sample was injected onto a Brownlee Aquapore RP 300A 3mm C8 column (Applied Biosystems) attached to a Shimadzu HPLC system as a series of consecutive runs (50-150μg/50μl/run) and chromatographed at a flow rate of 0.2ml/min. The mobile phase was HPLC-grade water + 0.06% (v/v) TFA and acetonitrile + 0.04% (v/v) TFA (2.1.2.4.). After loading the column was run with 5% acetonitrile for 5min followed by two gradients of increasing acetonitrile concentration: 5% - 65% (30min) and 65% - 100% (15min). The column was prepared for the next run by washing with 100% acetonitrile for 10min followed by re-equilibration with a gradient of decreasing acetonitrile concentration: 100% - 5% (10min). Absorbance was monitored at 214nm and all peaks were collected and assayed for lipolytic activity and immunoreactivity (sections 2.1.3.6 and 2.1.3.7 respectively). Acetonitrile and TFA were removed by centrifugation under vacuum (Gyro-Vap) after the addition of 100μl Tris.HCl (100mM, pH 8.0) thus preventing the samples from evaporating to dryness. Samples were then neutralised with 20% (w/v) potassium hydroxide if required and the volumes equalised to 100-150μl prior to assay.

2.1.3.5.2. HPLC after affinity chromatography.

Neutralised immunoreactive fractions isolated by affinity chromatography were pooled and the glycine concentration was reduced to less than 30mM in a 50ml-capacity Amicon ultrafiltration stirred cell through a 10K cut-off YM10 membrane using HPLC-grade water plus protease inhibitors (3 x volume) as the diluent. The volume was reduced to 0.5ml and the protein concentration was determined (2.1.3.8). Samples requiring further volume reduction to obtain a protein concentration of 100-150μg/50μl were centrifuged in Amicon micro-concentrators (10K cut-off, 13000rpm, 10-30min, 4°C). Particulate material was removed prior to injection onto the column by centrifugation (13000rpm, 10min).

Prepared samples were injected (50μl) and fractionated as described in section 2.1.3.5.1. Peaks were collected in microfuge tubes containing 100μl carbonate/bicarbonate buffer (2.1.2.9.1.) and acetonitrile and TFA were removed under a stream of nitrogen until the volume had been reduced to 100μl. Samples were then immobilised on a polyvinylchloride plate and assayed by ELISA (2.1.3.7).

2.1.3.6. Measurement of lipid-mobilising activity.

The lipolytic activity of a sample was determined by its ability to cause the release of glycerol from isolated adipocytes, the number of glycerol molecules released was measured by an enzyme assay resulting in the conversion of NADH to NAD^+ and a concomitant decrease in absorbance at 340nm (Wieland *et al*, 1974).

Epididymal adipose tissue was excised from BKW mice killed by cervical dislocation. The tissue from one mouse provided adequate fat cell numbers for the assay of ten samples. The tissue was finely chopped in Krebs Ringer bicarbonate buffer + 3% (w/v) bovine serum albumin (2.1.2.11.) + 4.0mg/ml collagenase (1.0ml/ epididymal tissue from one mouse), gassed briefly with 95% O_2, 5% CO_2 and incubated with shaking for 30min. Collagenase was removed by three washes with Krebs Ringer bicarbonate buffer (2.2.10) and the isolated adipocytes were resuspended in Krebs Ringer bicarbonate buffer + 3% (w/v) bovine serum albumin (0.9ml/sample). Samples to be assayed (100μl) were mixed with 0.9ml of fat cell suspension gassed briefly and incubated for 2h at 37°C. The volume of fat cell suspension used in the incubations (0.9ml) represented approximately 10^5 - $2x10^5$ adipocytes as determined by counting the cells on a haemocytometer during earlier experiments). Control reactions included incubation of samples in the absence of fat cells, thus eliminating the possibility of false positives; incubation of fat cells without sample to determine endogenous glycerol release and incubation of fat cells in the presence of isoprenaline (1.0μm).

Release of glycerol was measured enzymatically after the samples (0.5ml) had been deproteinised with 10% (v/v) $HClO_4$ (0.5ml) and neutralised with 40% (w/v) KOH

(120μl). Glycerol reaction buffer (2.1.2.12.) (0.83ml) was added to 200μl sample, the reaction was started by the addition of 1.0 unit of glycerokinase (10μl, 2mg/ml) and allowed to proceed for 20min. Lipid-mobilising activity was expressed as micromoles of glycerol released per 10^5 adipocytes per 2h.

2.1.3.7. Determination of immunoreactivity by ELISA.

Fractions purified by FPLC Mono Q, Superose and affinity chromatography (2.1.3.2, 2.1.3.3, 2.1.3.4) were tested for immunoreactivity by the addition of 50μl carbonate/bicarbonate buffer (2.1.2.9.1) to 50μl fraction, immobilisation onto a polyvinylchloride plate followed by incubation overnight at 4°C. Fractions purified by HPLC were concentrated in 100μl carbonate/bicarbonate buffer prior to immobilisation. Following immobilisation, the contents were removed and the wells were washed x 3 with PBS+ 0.1% (v/v) Tween 20. Non-specific binding was reduced by blocking with PBS + 3% (w/v) BSA (200μl/well) for 1h at 37°C. After removal of blocking solution, the wells were washed as described and the samples were incubated for 1h at 37°C with either serum from a MAC 16 tumour-bearing mouse (1/100 dilution) or with monoclonal antibody (10μg/ml, section 2.1.3.4), the diluent in both cases being PBS + 3% (w/v) BSA. Following removal of the well contents and 5 washes, the presence of bound antibody was determined by incubation for 1h in PBS + 3% (w/v) BSA + protein A conjugated with horse radish peroxidase (250μg, final concentration). Well contents were removed and after 5 washes immunoreactivity was detected with a substrate solution containing 0.04% (w/v) o-Phenylenediamine (OPD) and 0.012% (v/v) hydrogen peroxide in phosphate/citrate buffer (2.1.2.9.5) (100μl/well). The colour was allowed to develop for 30min after which it was stopped by the addition of 50μl of 0.2M H_2SO_4 and the absorbance was measured at 492nm in a microtitre plate reader (Anthos Labtec Instruments).

Controls included incubation of samples with serum from a MAC 13 tumour-bearing mouse, incubation of samples in the absence of the first antibody and incubation of serum and monoclonal antibody in the absence of sample.

2.1.3.8. Measurement of protein concentration.

Protein concentration was monitored throughout all stages of the purification by the Bio-Rad protein assay according to the manufacturers instructions. Briefly, Bio-Rad reagent (200μl) was added to the sample after dilution in water to 800μl and absorbance was measured at 595nm. Protein concentration (μg/ml) was obtained from a BSA calibration graph from which the following calculation was derived:

$$\text{Protein } (\mu g/ml) = \frac{\text{absorbance 595nm}}{0.053^*}$$

* Gradient of the BSA calibration graph.

2.1.3.9. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein purification was monitored by SDS-PAGE (Laemmli, 1970) using a Mini-Protean 2 cell (Bio-Rad Laboratories Ltd), using a 15% denaturing polyacrilamide gel prepared as shown in Table 2.1.3.9.1.

Samples to be analysed were concentrated by centrifugation in Amicon microcon concentrators boiled 1:1 in sample buffer (2.1.2.13.3) prior to loading and were electrophoresed at 200V until the tracking dye had reached the bottom of the gel. Markers used were pre-stained Rainbow markers (Amersham Int.) and were a mix of the following proteins: Myosin (200K), phosphorylase b (97.4K), BSA (69K), ovalbumin (46K), carbonic anhydrase (30K), trypsin inhibitor (21.5K) and lysozyme (14.3K).

Table 2.1.3.9.1. Composition of running and stacking gels for 15% SDS - PAGE

Constituent	Running gel	Stacking gel
Stock 1 (2.1.2.11.1)	3.75	
Stock 2 (2.1.2.11.2)		2.0
SDS (10% w/v)	0.3	0.12
1.5M Tris HCl (pH 8.0)	3.7	
0.5M Tris HCl (pH 8.0)		3.0
Water	3.95	6.4
TEMED	0.03	0.032
APS (10% w/v)*	0.04	0.04

* Ammonium persulphate

2.1.3.10. SDS-PAGE protein visualisation.

2.1.3.10.1. Coomassie staining.

After electrophoresis, gels were stained for protein visualisation. Gels with more than 1μg protein per sample were stained by soaking with gentle agitation in 0.1% (w/v) Coomassie Brilliant Blue R-250 stain (2.1.2.13.5.) for 30-60min. The gel was destained in 25% methanol/10% acetic acid and then dried onto filter paper .

2.1.3.10.2. Silver staining.

Gels with less than 1μg protein per sample were visualised by silver staining according to the manufacturers instructions (Bio-Rad Laboratories Ltd). Briefly, the gels

were fixed in 40% (v/v) methanol/10% (v/v) acetic acid for 30min, they were then furthur fixed by submersion in two changes of 10% (v/v) ethanol/5% (v/v) acetic acid for 15min each. The proteins were oxidised for 5min then the gels washed until no yellow colour remained. After a rinse in deionised water, the gels were developed in 3 changes of developer solution (5min each) until the required level of visualisation had been obtained. The reaction was stopped by submersing the gels in 5% (v/v) acetic acid. Following a rinse in water, the gels were dried onto filter paper. Gels were gently agitated during all steps of the procedure.

2.1.3.11. Analysis of immunoreactivity by western blot.

After electrophoretic separation, proteins were transferred to nitrocellulose membrane using a mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories Ltd). Transfer was run for 1h at 100V (200mA) in blotting buffer (2.1.2.14.1). Transferred proteins were immunodetected using monoclonal antibody, protein A peroxidase and chemiluminescence. Blots were incubated in blocking solution (2.1.2.14.4) overnight at 4°C, they were then washed x 3 with wash buffer (2.1.2.14.2) and incubated with monoclonal antibody (10μg/ml) in antibody dilution buffer (2.1.2.14.3) at room temperature with gentle agitation. Following incubation in first antibody, the blots were washed (3x15min) and transferred to antibody diluent containing protein A peroxidase (0.5μg/ml) for 1h at room temperature. The blots were washed (3 x 15min) and bands were detected with an emission chemiluminescence system (ECL) according to the manufacturers instructions. Briefly, membranes were blotted onto filter paper to remove excess liquid, ECL reagents were mixed (1:1, v/v) and poured over the antibody-bound surface of the membranes (2ml/membrane). After incubation for 1min at room temperature, the solution was removed and the blots exposed to ECL film for 1min-30min.

2.1.3.12. Protein sequence analysis.

Immunoreactive peaks fractionated by HPLC were posted to the Molecular Toxicology group, University College London and Middlesex School of Medicine for amino acid sequence analysis by Dr B. Coles on an Applied Biosystems 470 A protein sequencer and 120 A PTH HPLC system. Samples were sent immediately after elution in acetonitrile without further modification.

2.1.3.13. FPLC chromatofocussing: Mono P.

Fractions with lipid-mobilising activity following fractionation on a Superose column (2.1.3.3) were pooled and equilibrated with either an alkaline start buffer (2.1.2.3.1) for determination of isoelectric point between pH 9.0 and pH 6.0, or a neutral start buffer (2.1.2.3.2)) for determination of isoelectric point between pH 7.0 and pH 4.0. Equilibration was by Amicon ultrafiltration in a 500ml capacity stirred cell with 5 x volumes of start buffer through a 10K cut-off YM 10 membrane. The sample volume was reduced to 0.5ml, and protein content and lipid-mobilising activity were determined in 5.0μl and 25μl respectively (2.1.3.8 & 2.1.3.6). Prior to loading, the sample was centrifuged (13000rpm, 10min) to remove particulate material. The sample (500μl) was injected onto a Mono P HR 5/5 FPLC column (Pharmacia Biotech) equilibrated with start buffer. The column was then washed with 30ml start buffer (flow rate: 0.5ml/min) and the sample eluted with 50ml of either Polybuffer 96 or 74 (2.1.2.3.1 or 2.1.2.3.2), thus producing a pH gradient 9-6 or 7-4 for the determination of alkaline or acid isoelectric points respectively. Polybuffer was loaded onto the column via a Superloop at a flow rate of 0.5ml/min. Finally, any material remaining on the column was eluted by the injection of 2M sodium acetate (1.0ml) and 10 further fractions were collected for assay. All fractions (1.0ml) were assayed for lipid-mobilising activity and protein was monitored by absorbance at 280nm.

Fractions with lipolytic activity were concentrated by freeze-drying to approximately 0.3ml and analysed by gel-exclusion chromatography (2.1.2.3).

2.1.3.14. Effect of sodium periodate on lipid-mobilising activity.

Material with lipid-mobilising activity was purified as described in sections 2.1.3.1, 2.1.3.2 & 2.1.3.3. Superose fractions were pooled and the salt concentration was reduced by ultrafiltration through a YM 10 membrane in 50ml capacity Amicon stirred cell with 5 x volumes of deionised water + protease inhibitors as the diluent (2.1.2.5). The volume was reduced to 500μl and protein content and lipid-mobilising activity determined in 5μl and 25μl respectively (2.1.3.8 & 2.1.3.6). The sample was divided into two equal volumes (200μl) and incubated overnight at 4°C in the dark as follows :

A + 200μl 0.1M sodium acetate buffer (pH 4.5) + 20mM sodium periodate.

B + 200μl 0.1M sodium acetate buffer (pH 4.5).

After incubation, the samples were fractionated by Superose chromatography (2.1.3.3.).

2.1.3.15. Effect of trypsin digestion on lipid-mobilising activity.

Material with lipid-mobilising activity, purified as described in sections 2.1.3.1, 2.1.3.2 and 2.1.3.3 was equilibrated with 50mM sodium phoshate buffer pH 8.0 (2.1.2.2) by ultrafiltration through a YM 10 membrane in a 50ml capacity Amicon stirred cell. The volume was reduced to 0.5ml and protein content and lipid-mobilising activity were determined in 5μl and 25μl respectively (2.1.3.8 and 2.1.3.6). The sample was divided into two equal volumes and incubated at 20°C for 2h, 37°C for 2h or 37°C for 8h as follows:

A + Trypsin (0.2μg/μg protein)

B: No enzyme.

After incubation the samples were analysed by Superose gel-exclusion chromatography.

2.1.3.16. Effects of phosphatases on lipid-mobilising activity.

Material with lipid-mobilising activity was purified as described in sections 2.1.3.1, 2.1.3.2 & 2.1.3.3. Superose fractions were pooled and divided into two equal volumes A & C. Aliquot A was equilbrated with 3 x volumes of 50mM glycine buffer, pH 10.4 (2.1.2.6) while C was equilbrated with 50mM sodium acetate buffer, pH 4.5 (2.1.2.7) by Amicon ultrafilration through a YM 10 membrane in a 50ml capacity stirred cell. The volumes were reduced to 0.5ml and divided into 2 x 250μl : A+B, C+D, assayed for lipid-mobilising activity (2.1.2.6) and incubated overnight at 37°C as follows:

A + 4 units alkaline phosphatase

B + No enzyme

C + 4 units of acid phosphatase

D + No enzyme

After incubation, the samples were assayed for lipid-mobilising activity before and after analysis by gel-exclusion chromatography (2.1.3.3).

2.1.3.17. Effect of MAC 16 monoclonal antibody on lipid-mobilising activity.

MAC 16 monoclonal antibody (20μg, 50μg, and 100μg) was mixed with superose-purified lipolytic material from the MAC 16 tumour (85μg and 65μg) in a total volume of 100μl in PBS. The mixtures were incubated overnight at 4°C with constant agitation, then assayed as described (2.1.2.6).

2.2. RESULTS

2.2.1. Purification of lipolytic material from the MAC 16 adenocarcinoma

2.2.1.1 Introduction

Lipolytic material was purified from MAC 16 tumour extract by a modification of the methods employed by Beck *et al* (1990). Modifications included addition of protease inhibitors to all the buffers used; anion-exchange batch-extraction prior to anion-exchange chromatography; ultrafiltration through Amicon membranes for the removal of small molecular weight material and buffer equilibration for subsequent purification steps; use of FPLC columns for the anion exchange and gel exclusion purification steps; and HPLC using a C8 column as the final stage.

Fractions containing lipolytic material were identified during the initial purification steps by bioassay as described (2.1.3.6), and by ELISA using polyclonal antibody from MAC 16 tumour-bearing mice (2.1.3.7) after HPLC due to the interference of the solvents used at this stage with the bioassay.

2.2.1.2. Results

Batch extraction of MAC 16 tumour extract with DEAE-cellulose followed by ultrafiltration of the eluate through a 10K cut-off membrane in an Amicon 500ml stirred cell resulted in the removal of approximately 80% of tumour proteins (table 2.2.1.2.1). Measurements of lipolytic activity for the calculation of recovery were commenced after this step which effectively removed materials present in the tumour extract that were observed to hinder accurate measurement of lipolysis.

Anion-exchange chromatography employing a Mono Q column with a sodium chloride gradient resulted in the elution of lipolytic material by 0.14M-0.15M NaCl (figure 2.2.1.2.1). This purification step was scaled up by use of a Q-Sepharose column

with a 10-fold increase in capacity from which lipolytic material was eluted by 0.14-0.18M NaCl as several peaks (figure 2.2.1.2.2). This stage of purification resulted in the removal of a further 99% of tumour proteins and represented a 116-fold increase in specific activity (table 2.2.1.2.1).

Active fractions from anion-exchange chromatography were pooled, concentrated and purified further by superose gel-exclusion chromatography. Fractionation in a buffer with low ionic strength resulted in resolution of lipolytic activity as high molecular weight material (figure 2.2.1.2.3a) while an increase in the salt concentration of the running buffer resulted in reduction in the rate of migration (figure 2.2.1.2.3b) until an apparent molecular weight of 9.64-47kD was observed with buffer containing 0.3M NaCl (figure 2.2.1.2.3c), as determined by the superose calibration information provided by Pharmacia Biotech. Purification by this step resulted in removal of a further 50% of tumour proteins and an increase in specific activity of 1.78-fold (table 2.2.1.2.1).

Fractions obtained by Q-Sepharose and superose chromatography were also assayed by ELISA using antisera from MAC 16 tumour-bearing mice in order to ascertain the correlation between lipolytic activity and antibody reactivity and hence, determine the suitability of this method for the detection of lipolytic material after fractionation by HPLC. Assay of fractions eluted from Q-Sepharose by both methods resulted in a similar activity profile (figure 2.2.1.2.4). Two areas of immunoreactivity were observed in fractions chromatographed by superose gel-exclusion (figure 2.2.1.2.5). The early major area containing material of molecular weight 80-136kD appeared to represent non-specific immunoreactivity, as it was also detected by antisera from mice bearing the non-cachexigenic MAC 13 tumour (figure 2.2.1.2.6a). Immunoreactivity detected by MAC 16 antisera was partially masked by the non-specific response due to the overlap in molecular weight, but was observed in fractions 16 and 17 (MW 9.64-16.4) (figure 2.2.1.2.6b).

SDS-PAGE analysis of lipolytic fractions 14-17 revealed the presence of major protein bands at 29kD, 24kD, 20kD and 13kD (figure 2.2.1.2.7).

Western blot analysis of lipolytic fractions 14-17 (MW 9.64-47kD) using MAC 16 antiserum resulted in the detection of a band with molecular weight approximately 24kD that was not detected by MAC 13 antisera (figure 2.2.1.2.8).

Superose fractions containing lipolytic material were pooled, concentrated and purified further by HPLC using a C8 column and acetonitrile + TFA as the mobile phase. Peaks absorbing at 214nm were assayed for lipid-mobilising activity and polyclonal antibody reactivity. Initially, two peaks of lipolytic activity and immunoreactivity were observed (figure 2.2.1.2.9). The first peak which was positive for both methods of assay, eluted at approximately 28min (51% acetonitrile) and represented a further 17.2-fold increase in specific activity after superose chromatography and comprised approximately 0.005% of the original total tumour protein (table 2.2.1.2.1). The second area eluted between 32-33min (59-61% acetonitrile) and comprised an immunoreactive peak at 32min (59% acetonitrile) and a peak with lipolytic activity at 33min (61% acetonitrile). No immunoreactivity was detected in these areas by antisera from MAC 13 tumour-bearing mice (figure 2.2.1.2.10), while non-specific immunoreactivity was observed at a retention time of 40min (83% acetonitrile) by both antisera. Western blot analysis of peaks at 28min and 32-33min with MAC 16 antisera resulted in detection of a 24kD band in both fractions while no immunoreactivity was detected by MAC 13 antisera (figure 2.2.1.2.11a&b).

The discrepancy in positive peak detection by the two assay systems at this stage may be due to the unsuitability of HPLC conditions for the bioassay, a phenomenon that was often observed during attempts to assay HPLC fractions during the purification procedure. HPLC peaks absorbing at 214nm were subsequently routinely assayed by ELISA and were observed to occur at 26min (47%), 28min (51%) and 32-33min (59-61%).

Sequence analysis by Dr. B. Coles (Molecular Toxicology group, University College and Middlesex School of Medicine, London) revealed a single N-terminal, 22 amino acid sequence for the peak with retention time 26min which showed no similarities to proteins in the Swissprot and Genbank databases (table 2.2.1.2.2 and 2.2.1.2.2.1.).

Analysis of peaks at 28min and 32-33min revealed the presence of this same sequence as a minor component of other sequences.

2.2.1.3. Conclusion

Purification of material from MAC 16 tumour extract by the methods described, using assay of activity and immunoreactivity in order to detect active fractions resulted in the isolation of a homogeneous 24kD protein with a unique amino acid sequence.

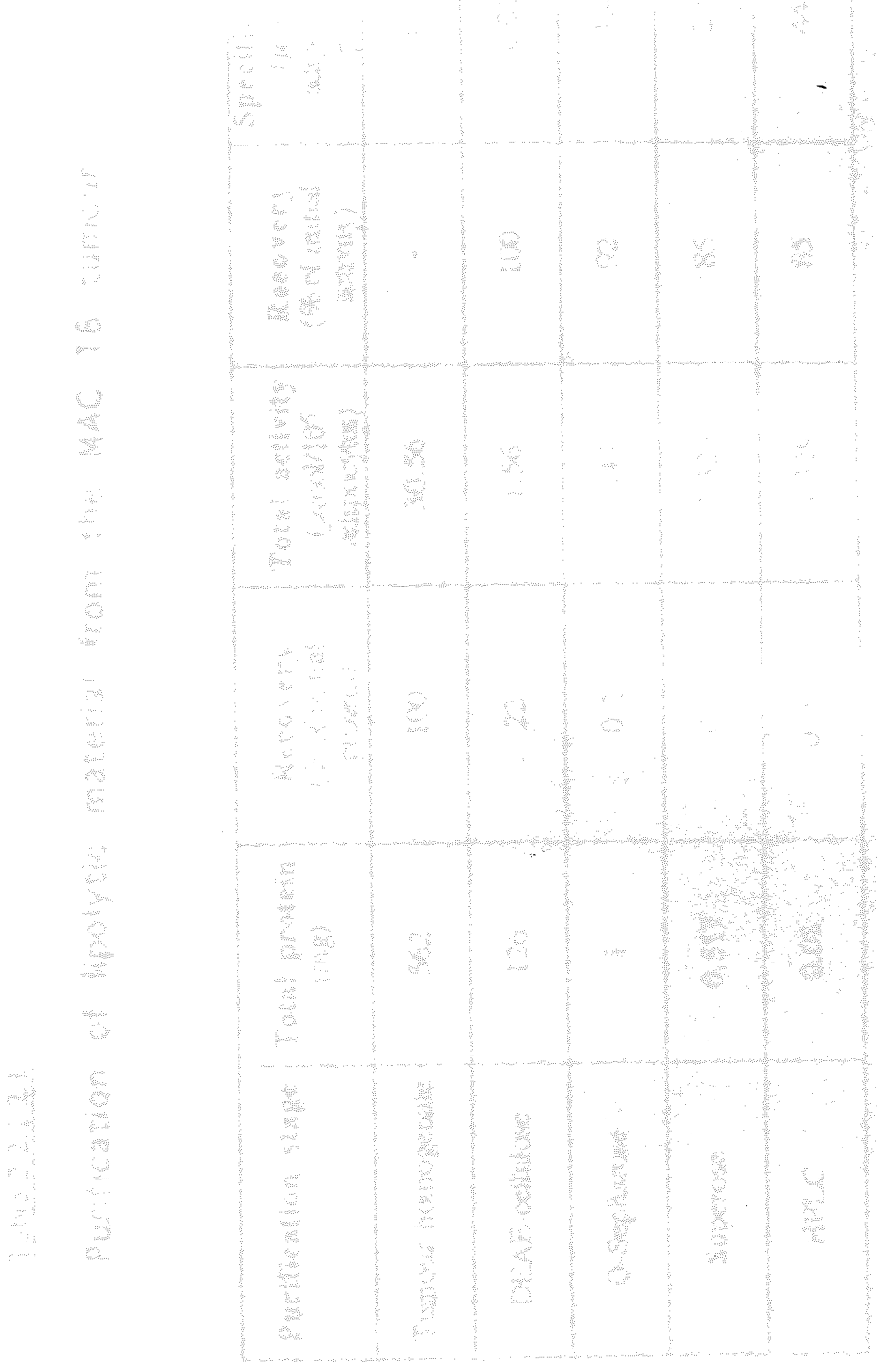


Table 2.2.1.2.1.

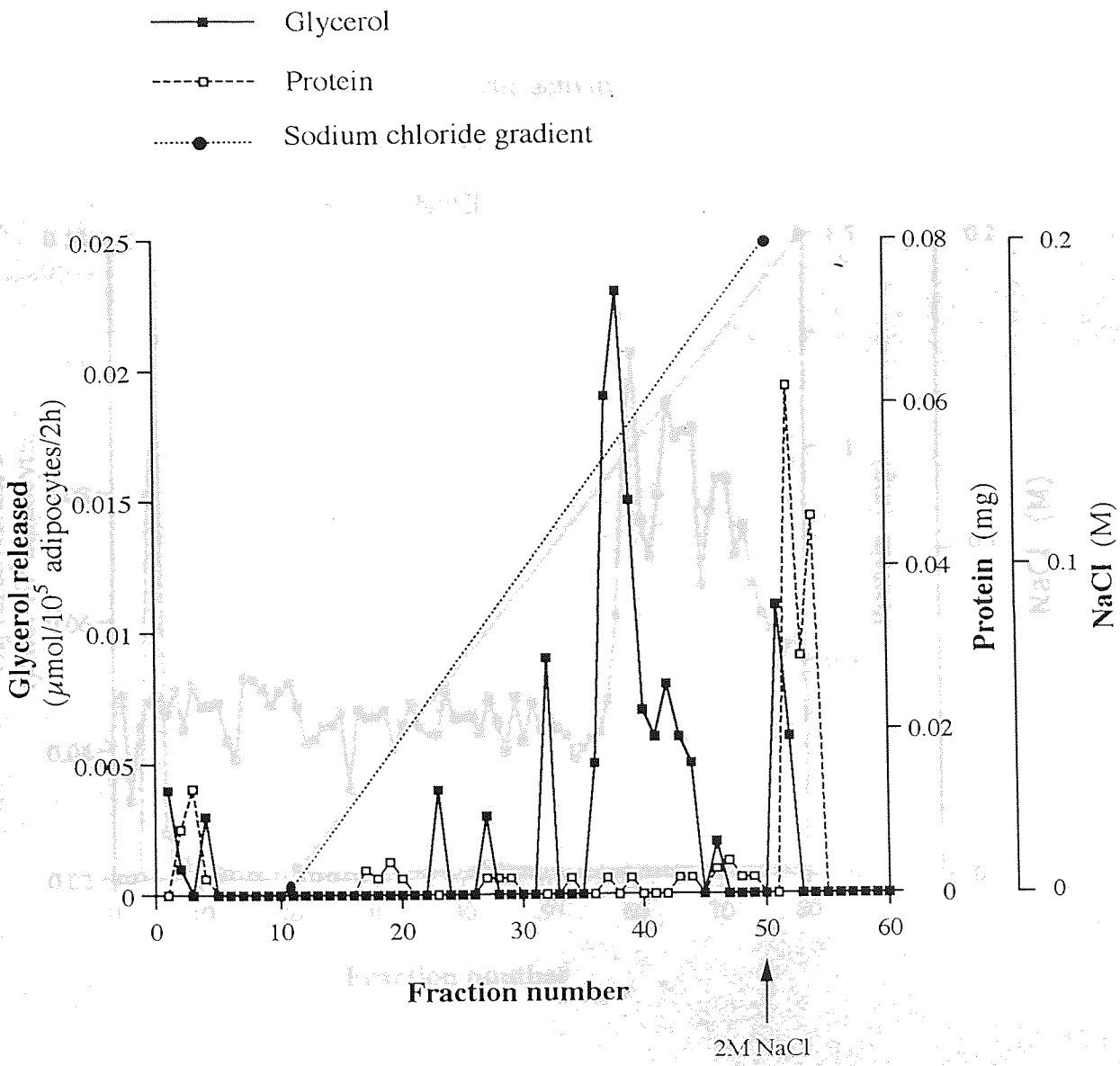
Purification of lipolytic material from the MAC 16 tumour

Purification stage	**Total protein** (mg)	**Recovery** (% of initial protein)	**Total activity** (μmol/10^5 adipocytes)	**Recovery** (% of initial activity)	**Specific activity** (μmol/10^5 adipocytes/mg protein)	**Purification fold**
Tumour homogenate	562	100	30.56	-	0.54	-
DEAE-cellulose	126	22	1.56	100	0.0124	1
Q-Sepharose	1	0.2	1.44	92	1.44	116
Superose	0.517	0.1	1.329	85	2.57	207
HPLC	0.03	0.005	1.329	85	44.2	3565

Figure 2.2.1.2.1.

Mono Q fractionation of lipolytic material from MAC 16 tumour after DEAE-cellulose batch extraction:

Analysis by lipolytic assay



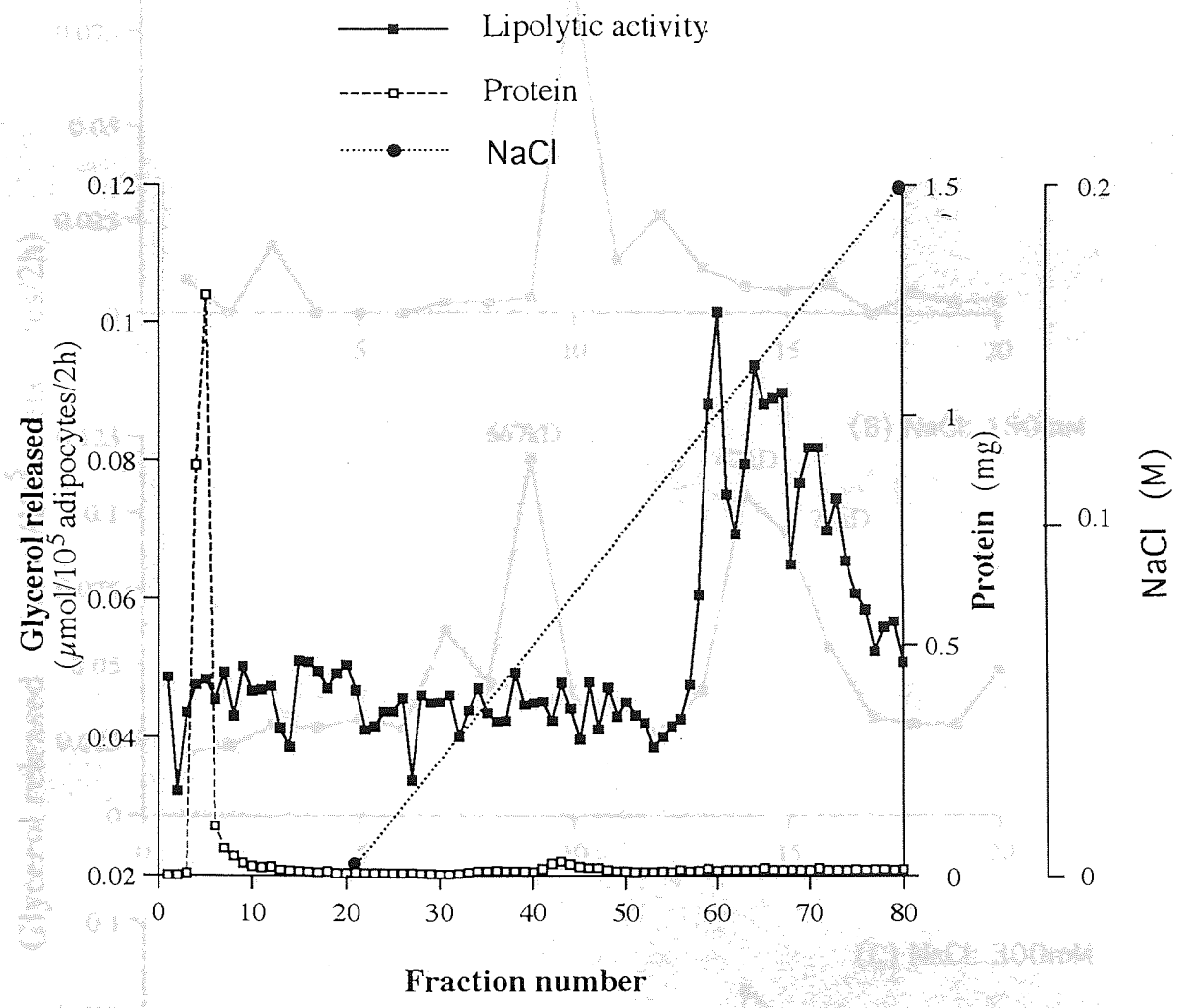
Sample parameters prior to loading:

Volume:	0.5ml
Total protein:	5.84mg
Lipolytic activity/50μl:	0.073μmol glycerol/10^5 adipocytes/2h
	(1.0μm isoprenaline: 0.1 ")

Figure 2.2.1.2.2.

Q-sepharose fractionation of MAC 16 tumour extract after DEAE-cellulose batch extraction:

Analysis by lipolytic assay



Sample parameters prior to loading:

Volume: 1.0ml
Total protein: 49mg
Lipolytic activity/50μl: 0.06μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.0927 ")

Figure 2.2.1.2.3.

Superose fractionation of MAC 16 tumour extract (43μg) after Mono Q chromatography: **Analysis by lipolytic assay**

Effect of increasing sodium chloride concentration.

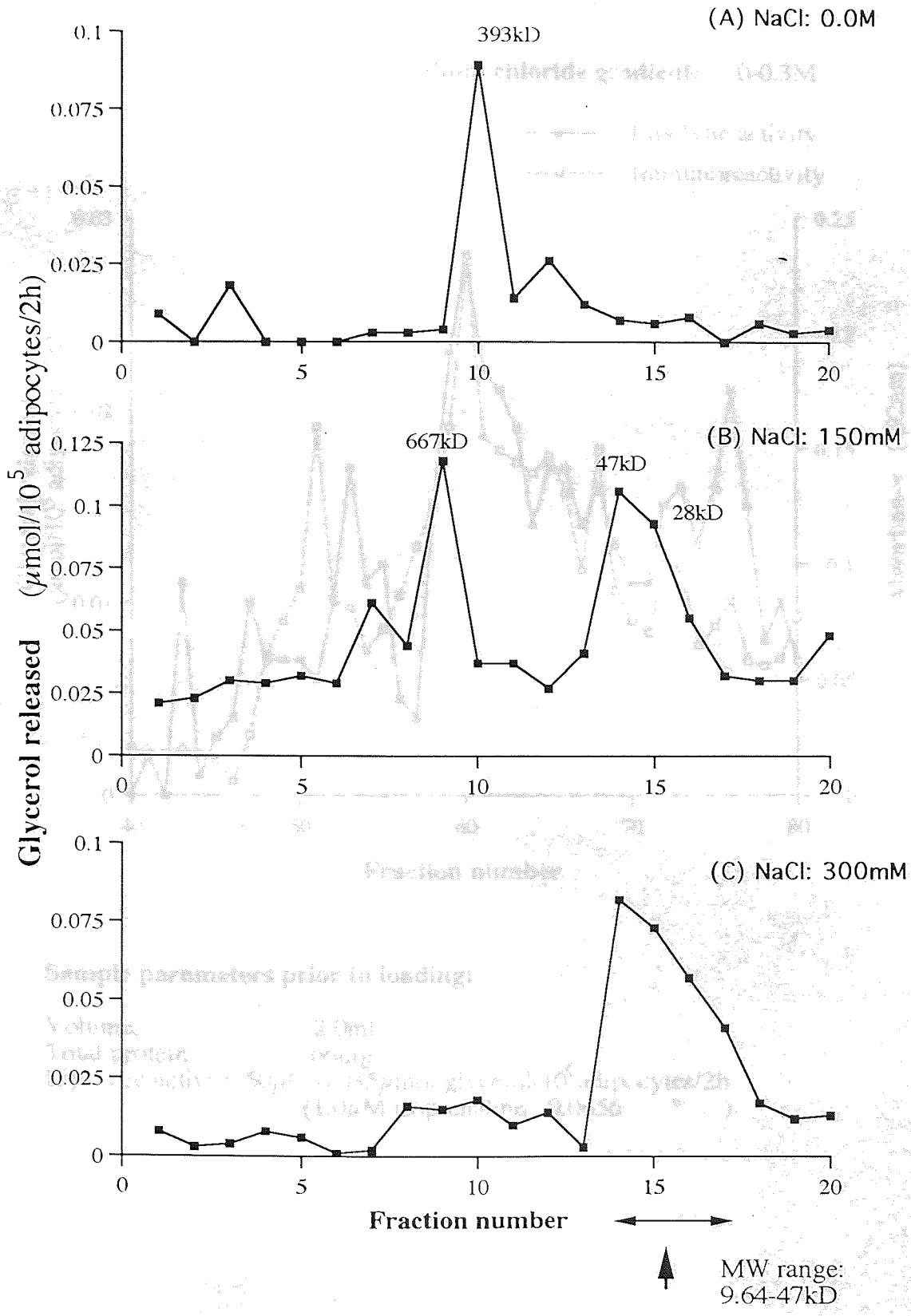
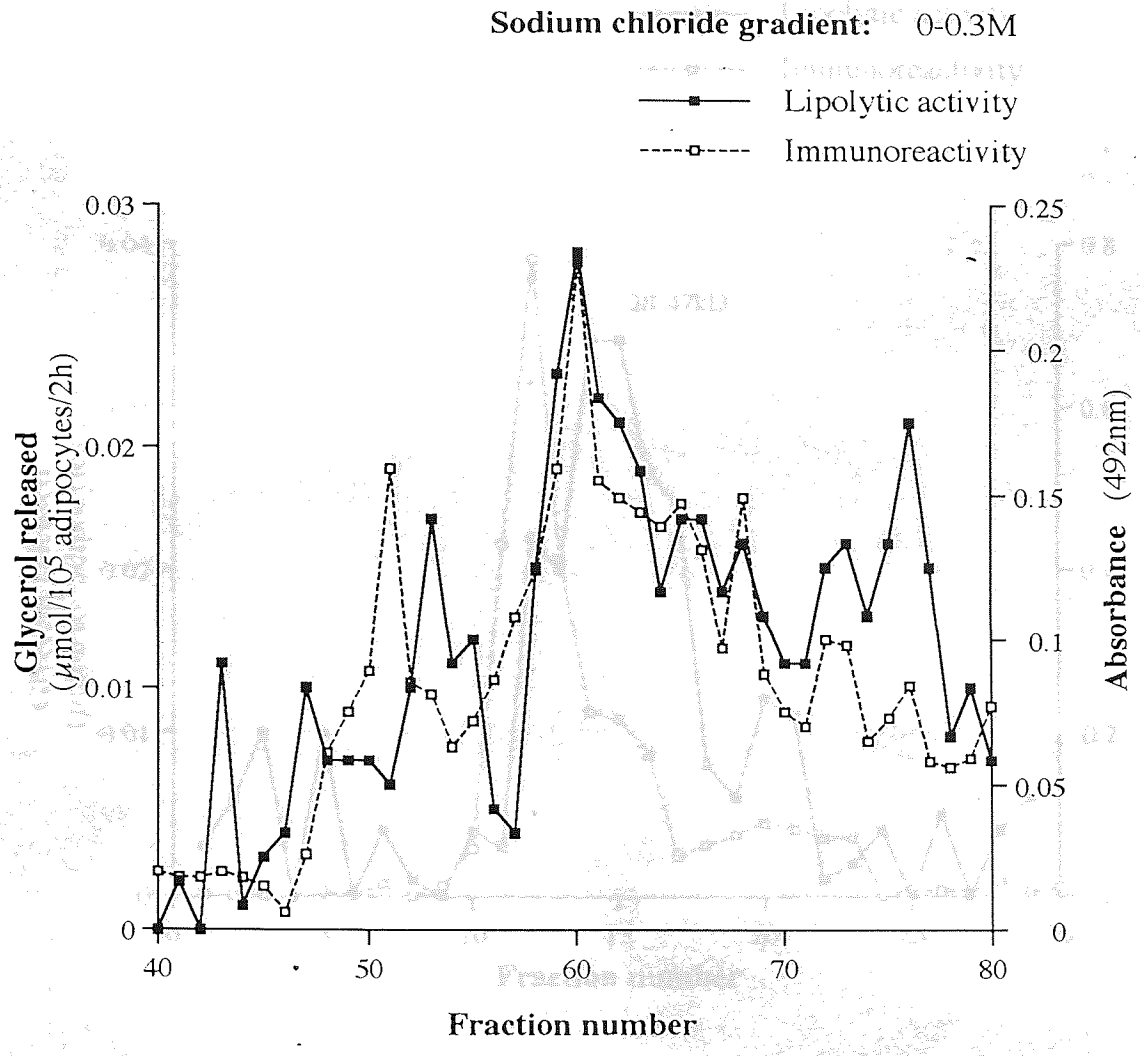


Figure 2.2.1.2.4.

Q-sepharose fractionation of lipolytic material from MAC 16 tumour:

Analysis by lipolytic assay and ELISA with MAC 16 antisera



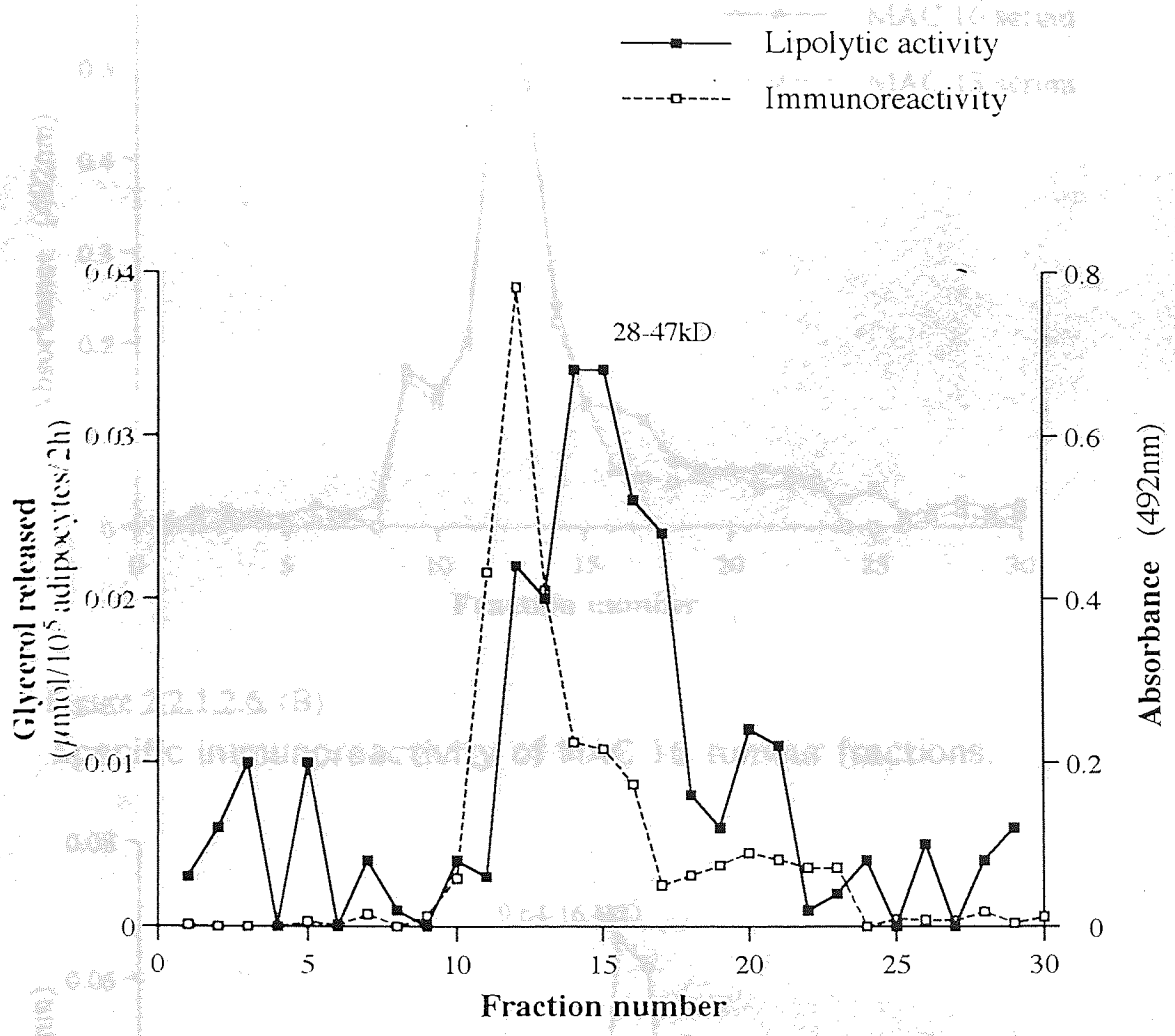
Sample parameters prior to loading:

Volume: 2.0ml
Total protein: 66mg
Lipolytic activity/50μl: 0.035μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.0656 ")

Figure 2.2.1.2.5.

Superose gel exclusion chromatography of lipolytic material from MAC 16 tumour:

Analysis by lipolytic assay and ELISA with MAC 16 antisera



Sample parameters prior to loading:

Volume: 200μl
Total protein: 600μg

Figure 2.2.1.2.6. (A)

Superose gel-exclusion chromatography of lipolytic material from MAC 16 tumour:

Analysis by ELISA using MAC 16 and MAC 13 antisera

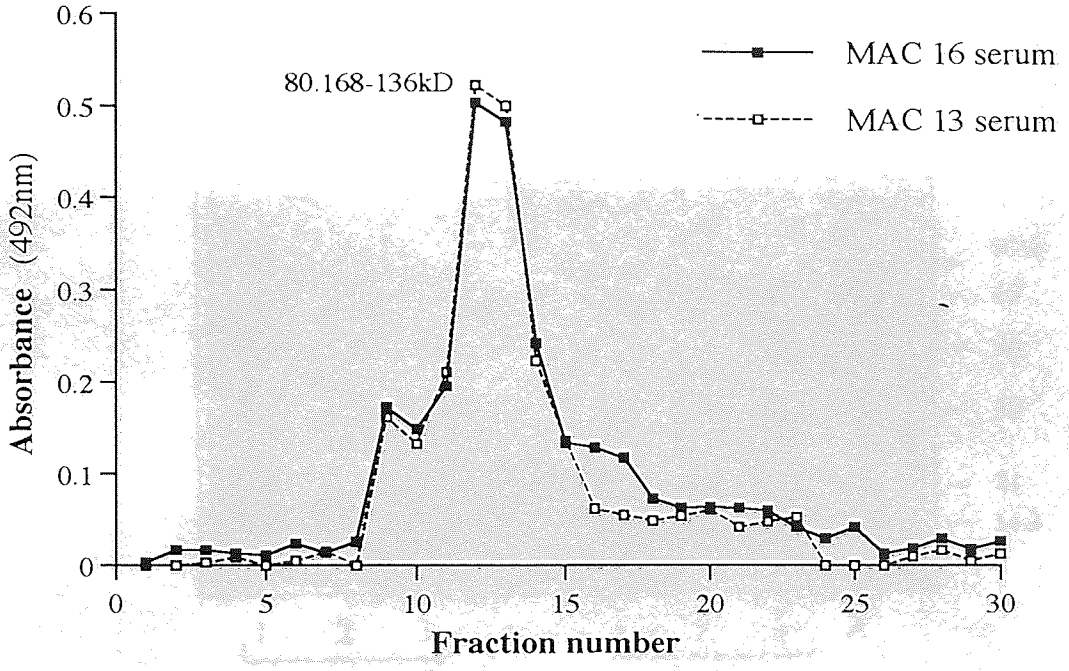


Figure 2.2.1.2.6. (B)

Specific immunoreactivity of MAC 16 tumour fractions.

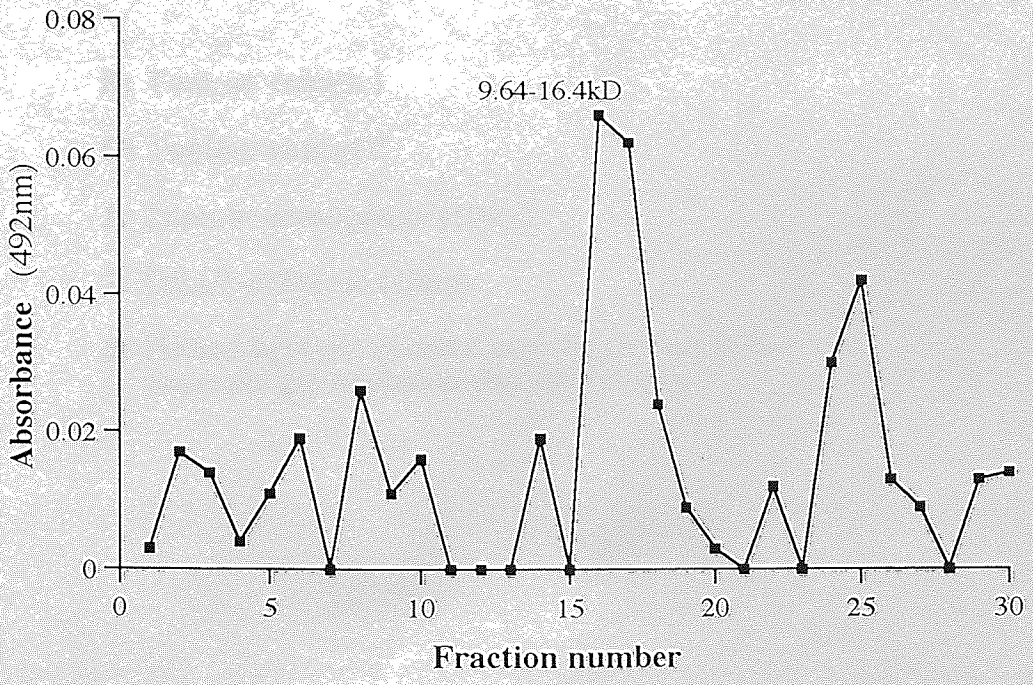
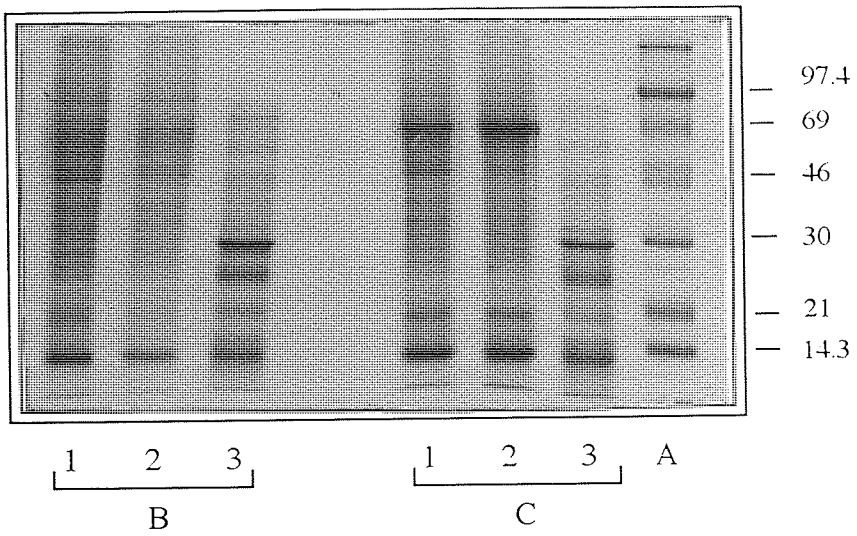


Figure 2.2.1.2.7

SDS-PAGE analysis of the purification of lipolytic material from MAC 16 tumour extract:

Visualisation by Coomassie brilliant blue stain



B: Tumour extract 1

C: Tumour extract 2

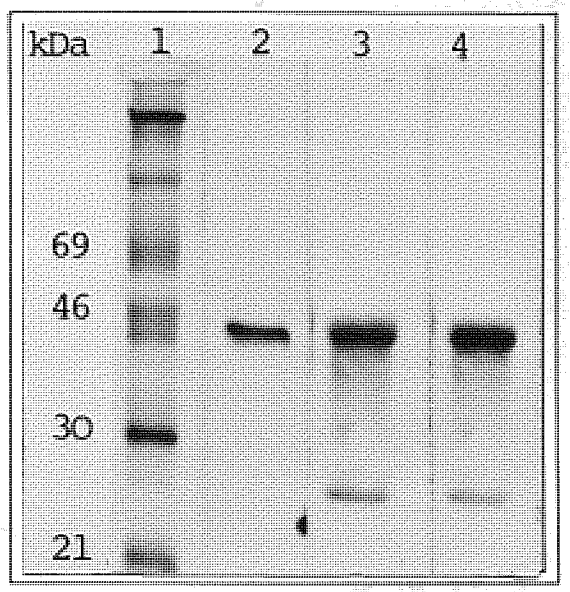
1: Tumour homogenate (10μg)

2: DEAE-cellulose (10μg)

3: Pooled lipolytic material after fractionation by superose gel-exclusion chromatography

Figure 2.2.1.2.8.

Western blot analysis of lipolytic material from the MAC 16 tumour after superose gel-exclusion chromatography using MAC 16 and MAC 13 antisera



1: Molecular weight markers (kD)

2: MAC 16 lipolytic material + antisera from MAC 13 tumour -bearing mice

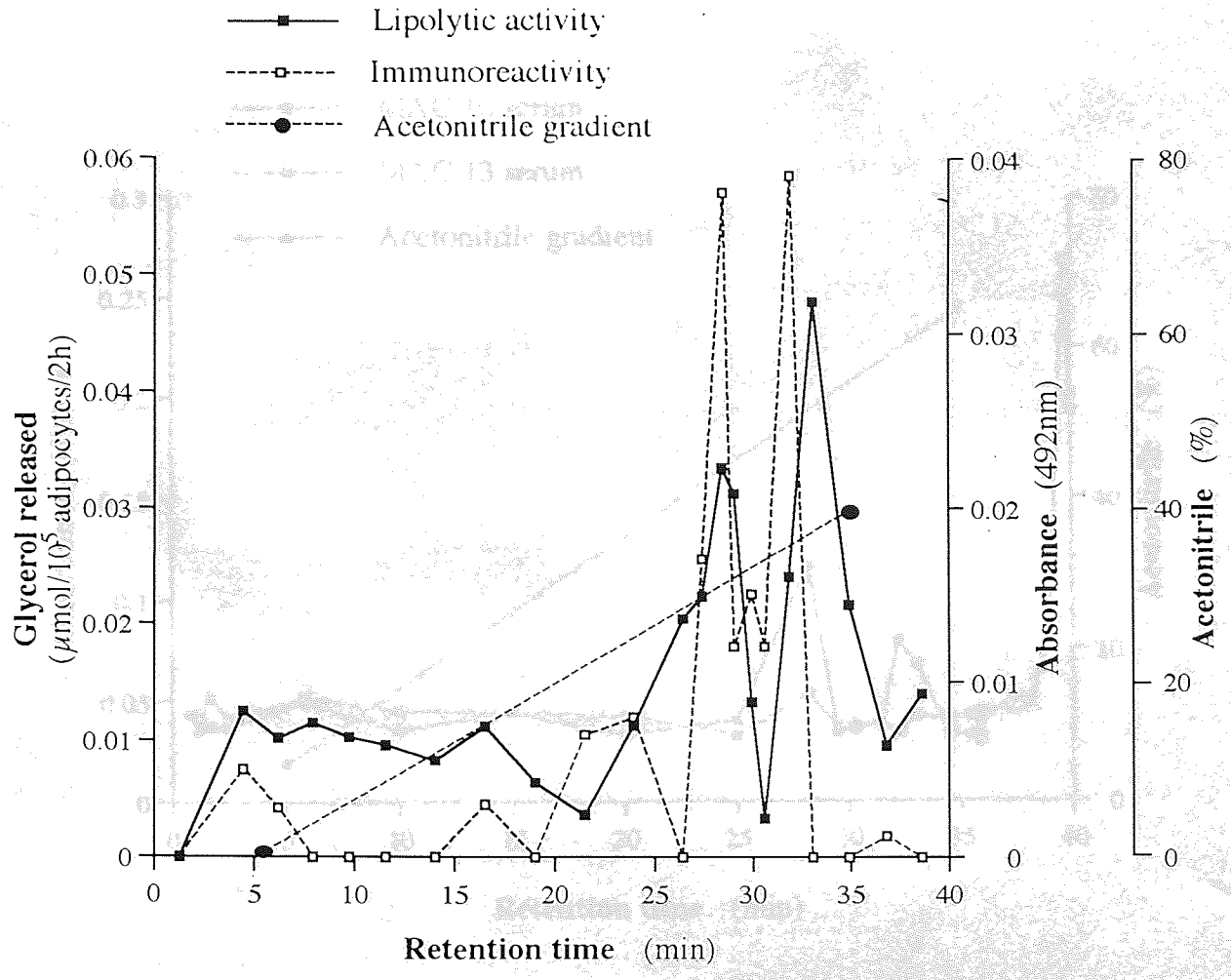
3 & 4: MAC 16 lipolytic material + antisera from MAC 16 tumour-bearing mice

(From: McDevitt *et al*, 1995)

Figure 2.2.1.2.9

Reverse-phase HPLC of MAC 16 tumour after superose fractionation

Analysis of peaks detected at 214nm by lipolytic assay and ELISA with MAC 16 antisera



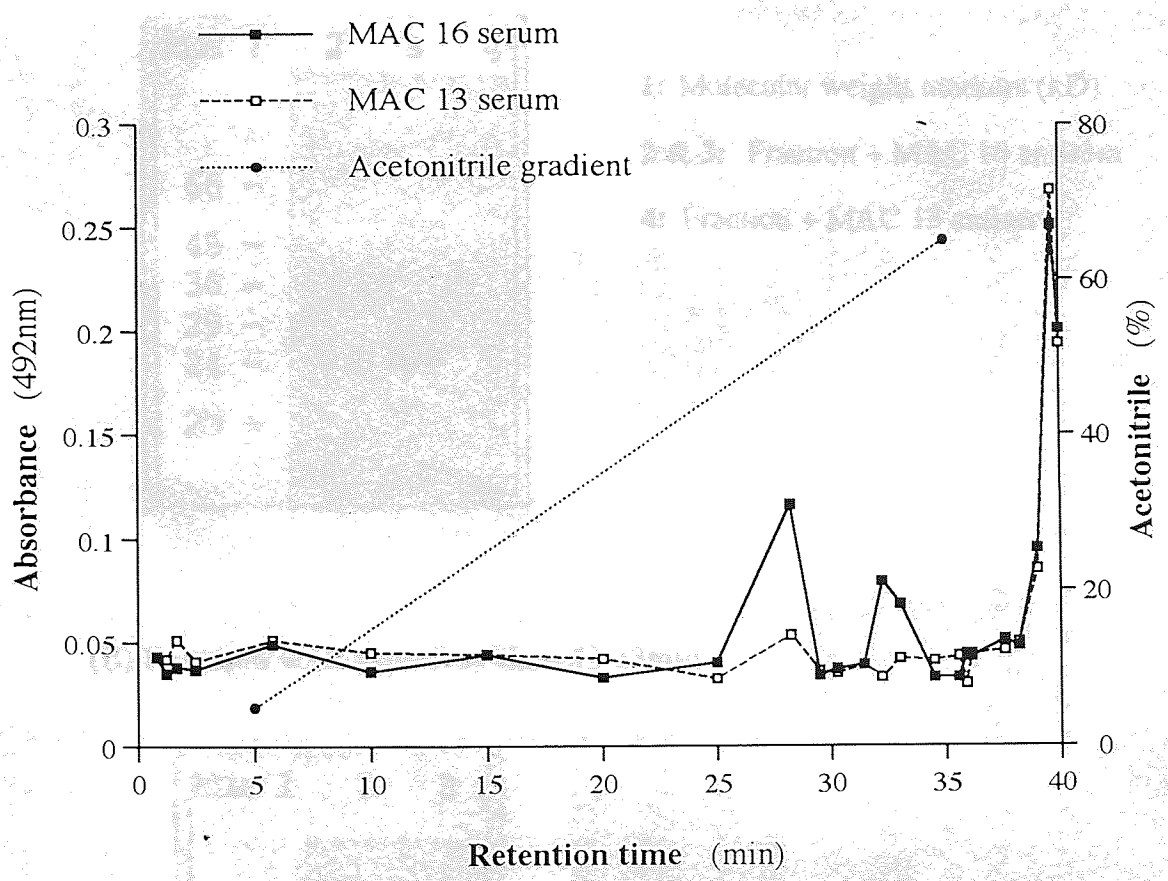
Sample parameters prior to loading:

Volume:	50μl
Total protein:	45μg
Lipolytic activity/30μl:	0.0448μmol glycerol/10^5 adipocytes/2h (1.0μM isoprenaline: 0.178 μmol ")

Figure 2.2.1.2.10.

Reverse-phase HPLC of MAC 16 tumour after superose fractionation:

Analysis of peaks detected at 214nm by ELISA with MAC 16 and MAC 13 antisera



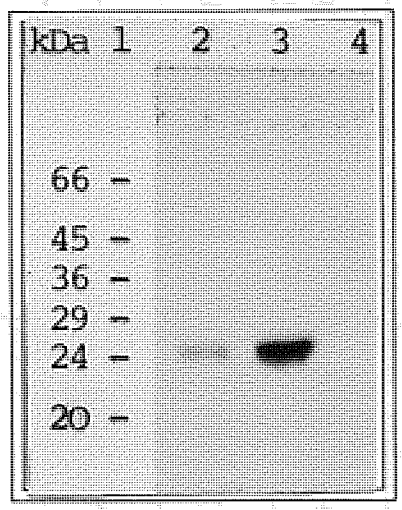
Sample parameters prior to loading:

Volume: 50μl
Total protein: 50μg
Lipolytic activity: 0.0632μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.169 ")

Figure 2.2.1.2.11. (A) and (B)

Western blot analysis of HPLC-purified lipolytic material from the MAC 16 tumour using MAC 16 and MAC 13 antisera

(A) Fraction with retention time 28min

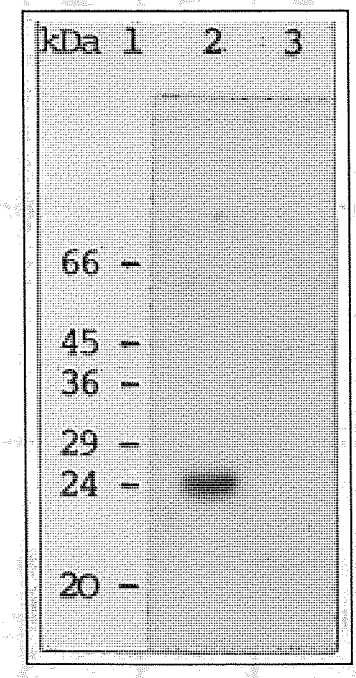


1: Molecular weight markers (kD)

2 & 3: Fraction + MAC 16 antisera

4: Fraction + MAC 13 antisera

(B) Fraction with retention time 32-33min



1: Molecular weight markers

2: Fraction with MAC 16 antisera

3: Fraction with MAC 13 antisera

(From: McDevitt *et al*, 1995)

Table 2.2.1.2.2. **Protein sequence analysis of lipolytic material from MAC 16 tumour:**

HPLC elution (% acetonitrile)	HPLC elution (min)	N-terminal sequence (major)	N-terminal sequence (minor)
47	26	YPPEAASAPGSGNSHHAA(S)AQQ	-
51	28	DEMA(E/P)QHANMYG(F/Y)PS	YPPEAASAPGSGNSHHAA(S)AQQ
59-61	32-33	VLHDEVKKIVLQKYHTINGHCAEEYK	YPPEAASAPGSGNSHHAA(S)AQQ

Table 2.2.1.2.2.1. **Database search for similar sequences:**

N-terminal sequence	Database sequence identity (Swissprot/Genbank)
YPPEAASAPGSGNSHHAA(S)AQQ	None
DEMA(E/P)QHANMYG(F/Y)PS	None
VLHDEVKKIVLQKYHTINGHCAEEYK	Bovine papillomavirus E7 protein

2.2.2. *Partial characterisation of lipolytic material*

2.2.2.1. *Introduction*

Superose fractions 14-17 were pooled, equilibrated with appropriate buffer and concentrated by ultrafiltration through a 10K cut-off membrane in a 50ml Amicon stirred cell. The concentrate was then analysed for the determination of isoelectric properties, the presence of carbohydrate moieties (sodium periodate treatment) and the effect of trypsin on bioactivity and molecular weight. Non-cachexigenic MAC 13 tumour extract was also purified by the same methods in order to ascertain the specificity of the lipolytic material to cachexia-inducing tumours. The immunoreactivity of the lipolytic material against a monoclonal antibody isolated from the serum of MAC 16 tumour bearing mice (Todorov *et al*, 1996, in press) was also tested during the anionic-exchange and gel-exclusion chromatography steps of the purification. The gel-exclusion step was scaled up by the use of a Superdex column during these analyses.

Lipolytic activity data obtained from these analyses were expressed as a proportion of the glycerol released from 1.0μM isoprenaline in order to facilitate comparisons between experiments:

Lipolytic activity is expressed as:

$$\frac{\text{Glycerol released } (\mu\text{mol}/10^5 \text{ adipocytes}/2\text{h}) / 100\mu\text{l sample}}{\text{Glycerol released } (\mu\text{mol}/10^5 \text{ adipocytes}/2\text{h}) / 1.0\mu\text{M isoprenaline}}$$

2.2.2.2. *Results*

The isoelectric properties of the lipolytic material were determined by fractionation on a Mono P chromatofocussing column under alkaline and acid conditions as described (2.1.3.13).

Using alkaline conditions, the lipolytic material remained retained on the column after completion of the pH gradient and was recovered by elution with 2M sodium acetate (figure 2.2.2.2.1). Fractionation of this material by superose gel-exclusion confirmed

that the bioactivity and apparent molecular mass of the material retained was unchanged by this procedure (figure 2.2.2.2.2.).

Chromatofocussing with acid conditions resulted in fractionation of lipolytic material into two peaks with different isoelectric properties (figure 2.2.2.2.3). The first peak eluted at pH 4.7 while the second, larger peak of bioactivity eluted at pH 4.0. Fractionation of these peaks separately by superose gel-exclusion chromatography revealed the first peak to have the same properties as material prior to chromatofocussing (MW: 9.64-47kD) (figure 2.2.2.2.4a), while the second appeared to comprise small molecular weight material, as determined by its elution with the bed volume (figure 2.2.2.2.4b). Analysis by mass spectrometry failed to yield further information on the nature of of this small molecular weight material (results not shown). Additional analyses were impeded by its unstable nature which resulted in loss of bioactivity during concentration steps, a phenomenon that is apparent by comparison of lipolytic activity obtained for the same material before and after concentration (figure 2.2.2.2.3 & 2.2.2.2.4b).

Complete loss of lipolytic activity was observed following incubation of lipolytic material with sodium periodate (figure 2.2.2.2.5) indicating the presence of carbohydrate moieties required for biological activity. However, treatment with deglycosylation enzymes endoglycosidase-F, endo-α-N-acetylgalactosaminidase and neuraminidase had no substantial effect on the biological activity suggesting the absence of N-linked glycans, O-linked glycans and sialic acid moieties, or that these are not required for lipolytic activity (McDevitt *et al*, 1995).

Incubation of the lipolytic material with 40μg trypsin for 2h at room temperature (figure 2.2.2.2.6a) or 37°C (figure 2.2.2.2.6b) did not substantially reduce its biological activity or molecular weight, as determined by superose gel-exclusion chromatography. Any apparent reduction in bioactivity was reversed by the inclusion of trypsin inhibitor in fractions to be assayed (figure 2.2.2.2.6c), suggesting possible impediment of the bioassay by trypsin present in the eluate (trypsin MW: 24kD).

Treatment to remove phosphate groups under acid and alkaline conditions gave inconclusive results. While acid phosphatase treatment had no effect on biological activity (figure 2.2.2.2.7), incubation of the lipolytic material under the conditions required for alkaline phosphatase resulted in complete inhibition of activity (figure 2.2.2.2.8). Thus, the material appeared to be susceptible to alkaline cleavage, and its structure may contain base-labile linkages that are required for bioactivity.

Purification of MAC 13 tumour extract by DEAE-cellulose batch extraction, anion-exchange FPLC chromatography and superose gel-exclusion resulted in the isolation of lipolytic material that was apparently indistinguishable from the material obtained from MAC 16 tumour extract (figure 2.2.2.2.9). However, lipolytic activity measured in sera from MAC 13 tumour-bearing mice was significantly reduced when compared to sera from MAC 16 tumour-bearing mice or non-tumour bearing controls (figure 2.2.2.2.10). Differences between the two groups were most apparent when 10μl of serum was measured, perhaps due to an increase in possible endogenous materials which interfere with the bioassay when larger volumes are used. In addition, immunoreactive peaks with retention times 26min, 28min and 32-33min obtained during HPLC-fractionation of MAC 16 superose fractions were absent from the MAC 13 HPLC profile, and fractions collected at these retention times during the MAC 13 run failed to show any immunoreactivity with MAC 16 antisera (figure 2.2.2.2.11), while non-specific immunoreactivity was observed for both samples at retention times: 40min-50min.

Analysis of the immunoreactivity of the lipolytic material against a monoclonal antibody purified from the serum of MAC 16 tumour-bearing mice, revealed minimal correlation between biological activity and immunoreactivity when lipolytic material was fractionated on Q-Sepharose (figure 2.2.2.2.12) or Superdex (figure 2.2.2.2.13 a&b) columns. In addition, biological activity was not inhibited by up to 100μg of monoclonal antibody (figure 2.2.2.2.14) and western blot analysis of proteins observed by SDS-PAGE of superdex fractions (figure 2.2.2.2.15 a&b) using the monoclonal antibody confirmed the lack of immunoreactivity of the lipolytic material (figure 2.2.2.2.16).

Immunoreactivity observed in high molecular weight material by ELISA (figure 2.2.2.2.13a&b) was probably due to the presence of antibodies as indicated by immunoblots incubated with protein A without monoclonal antibody (results not shown).

Fractionation of lipolytic material after ammonium sulphate precipitation through a monoclonal antibody affinity column as described (2.1.3.4) resulted in its non-retention by the column (figure 2.2.2.2.16). Analysis of the non-retained material by Q-Sepharose (figure 2.2.2.2.17a) and Superose (figure 2.2.2.2.17b) column chromatography confirmed that lipolytic material with the same properties as those described (2.2.1) had indeed failed to bind to MAC 16 monoclonal antibody immobilised on the affinity column.

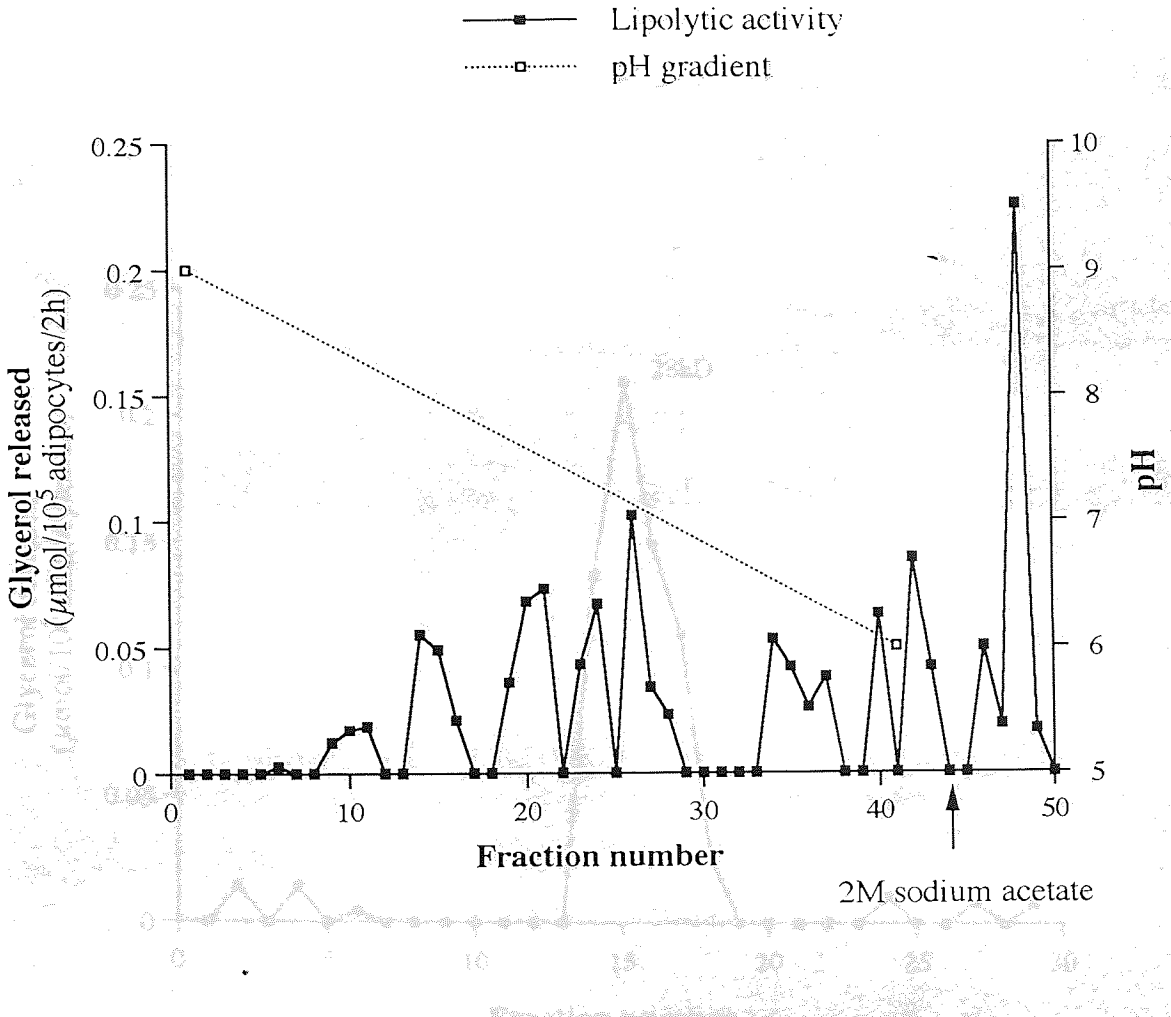
2.2.2.3. Conclusion

Partial characterisation studies suggest that material with lipolytic activity and apparent molecular mass 9.64-47kD has an isoelectric point at pH 4.7 with a possible sub-component of small molecular weight with an isoelectric point at pH 4.0. The biological activity was inhibited by incubation with periodate and unaffected by incubation with trypsin. Lipolytic material of the same molecular mass was purifiable from the non-cachexigenic MAC 13 tumour, though this material was not immunoreactive against MAC 16 antisera and appeared to be absent from the circulation of MAC 13 tumour-bearing mice. Monoclonal antibody purified from the serum of MAC 16 tumour-bearing mice was ineffective at inhibiting biological activity of the lipolytic material and also failed to bind to the material when analysed by western blot furthermore, lipolytic material was not retained by an affinity column containing immobilised monoclonal antibody.

Figure 2.2.2.2.1.

Isoelectric point determination of superose-fractionated lipolytic material from MAC 16 tumour by Mono P chromatography with a pH gradient 9-6:

Analysis by lipolytic assay



Sample parameters prior to loading:

Volume: 500μl
Total protein: 414μg
Lipolytic activity: 0.0705μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.1212 ")

Figure 2.2.2.2.2.

Superose fractionation of lipolytic material retained on the Mono P column after completion of pH gradient 9-6:

Analysis by lipolytic assay

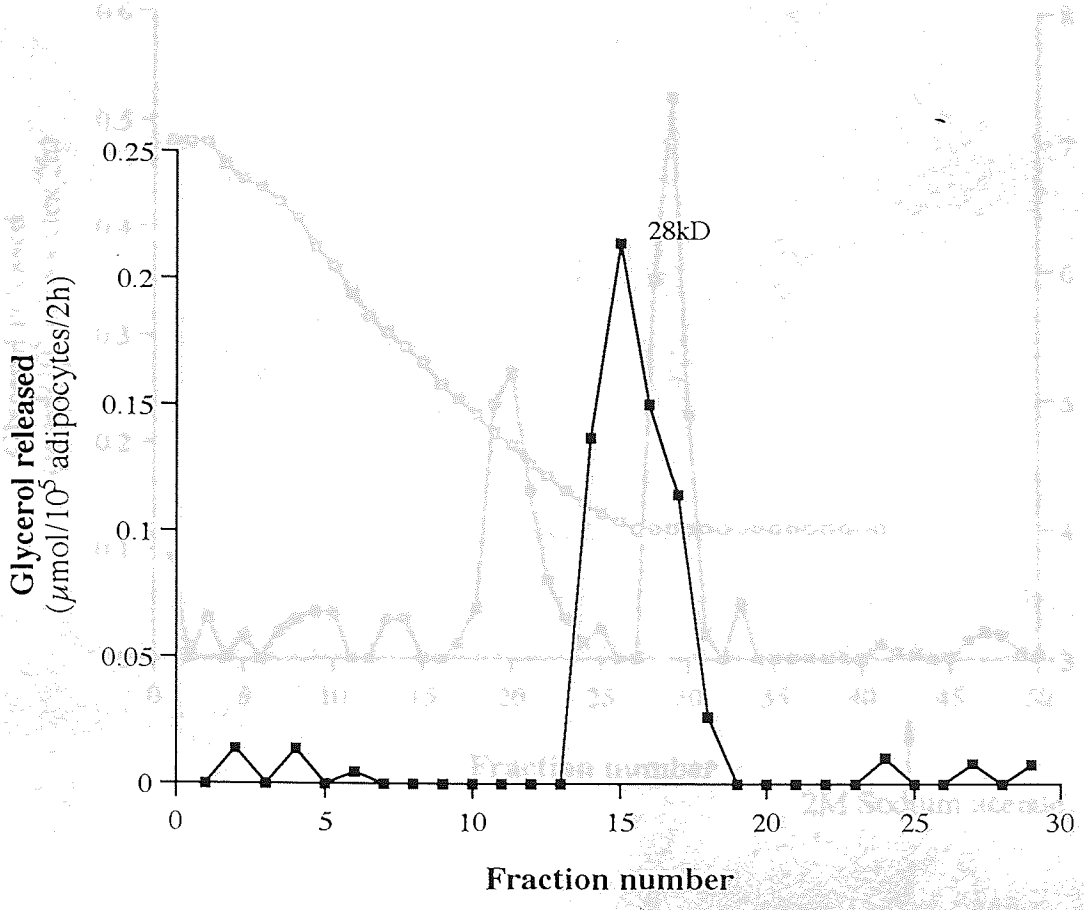
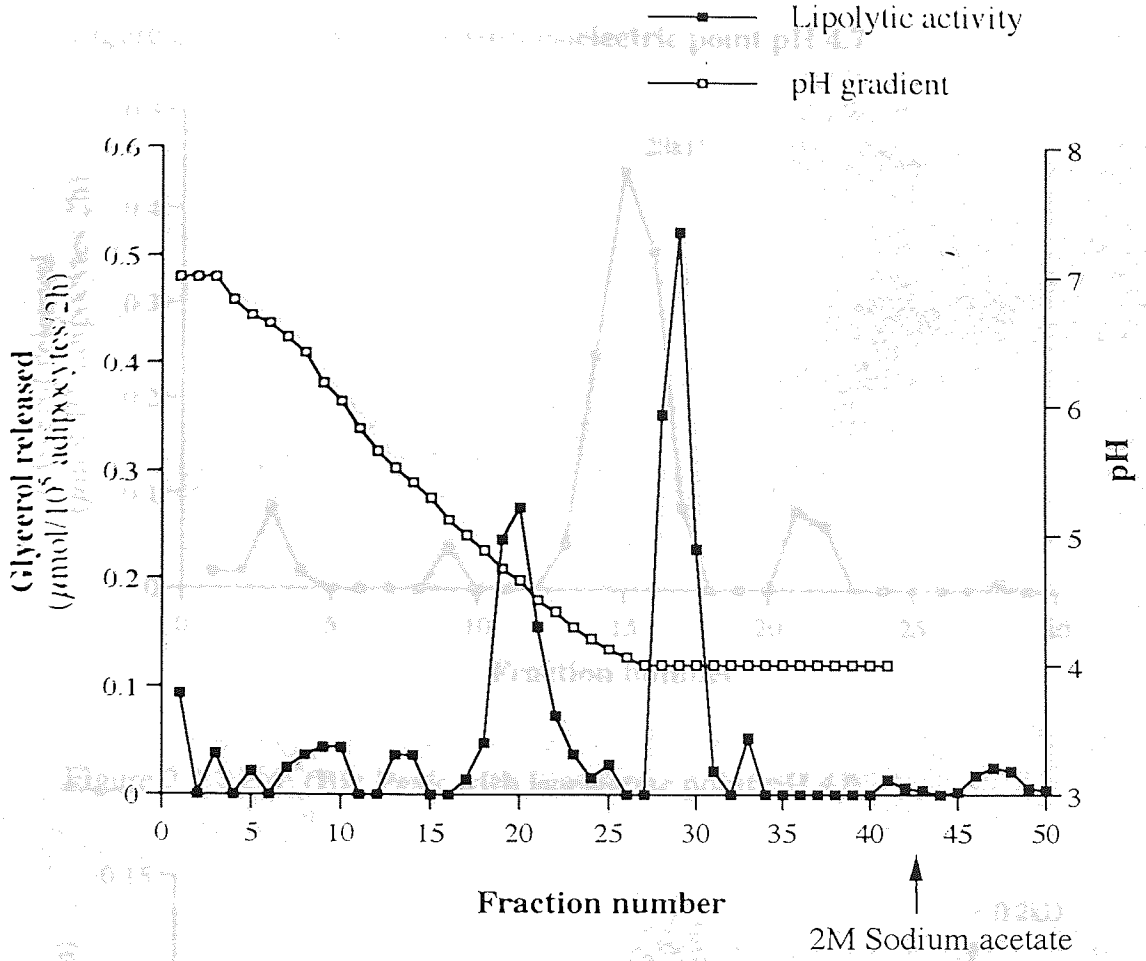


Figure 2.2.2.2.3.

Isolelectric point determination of superose-fractionated lipolytic material by Mono P chromatography with a pH gradient 7-4:

Analysis by lipolytic assay



Sample parameters prior to loading:

Volume: 500μl
Total protein: 251μg
Lipolytic activity: 0.1177μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.123 ")

Figure 2.2.2.2.4.

Superose fractionation of chromatofocussed lipolytic material eluted from the Mono P column by a pH gradient 7-4:

Analysis by lipolytic assay

Figure 2.2.2.2.4 (A) : **Peak with isoelectric point pH 4.7**

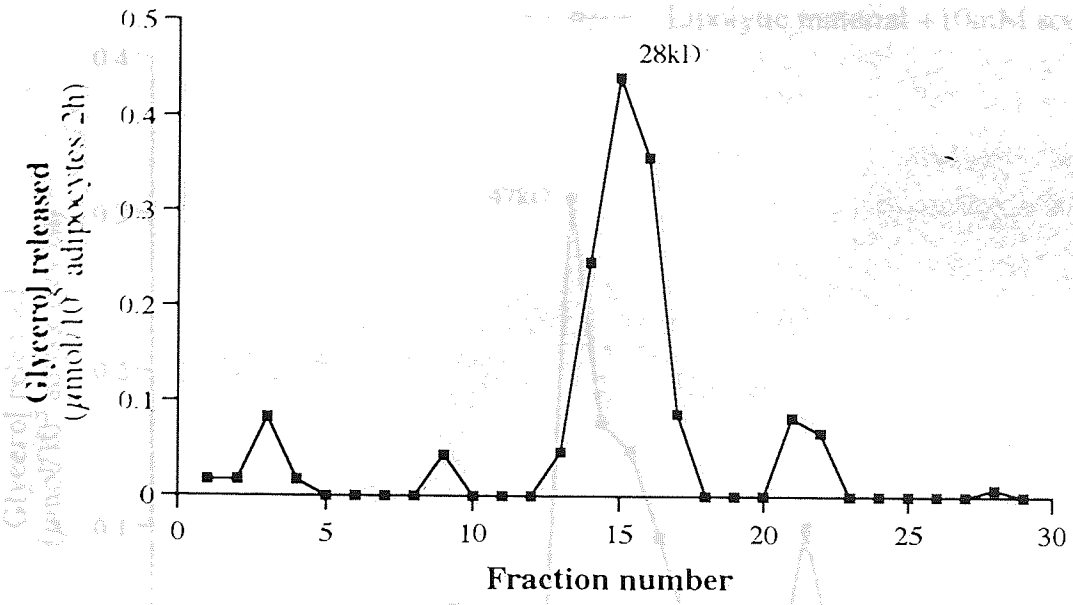


Figure 2.2.2.2.4 (B) : **Peak with isoelectric point pH 4.0**

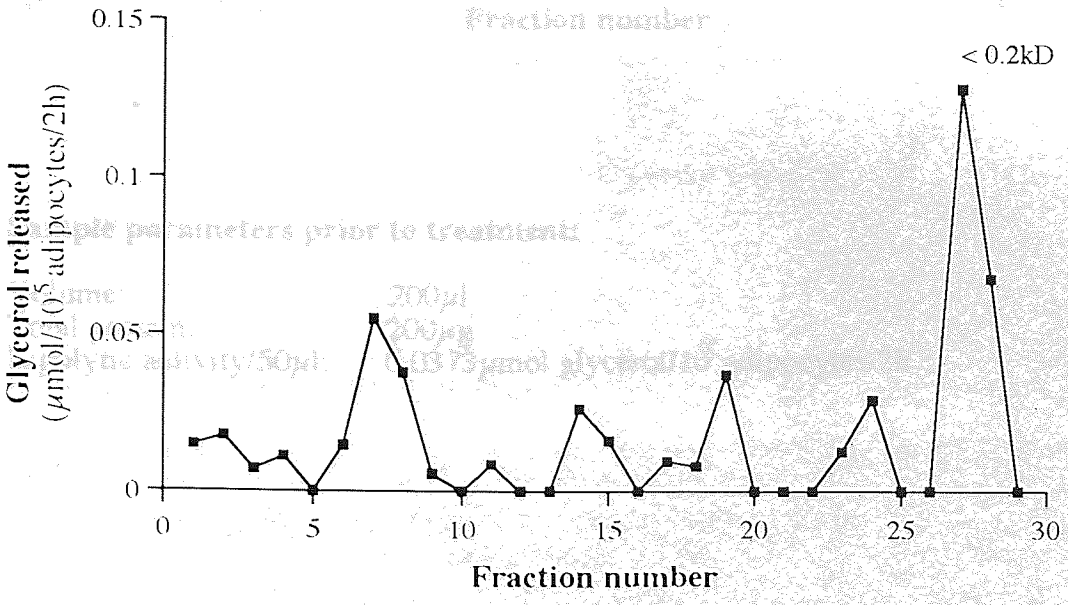
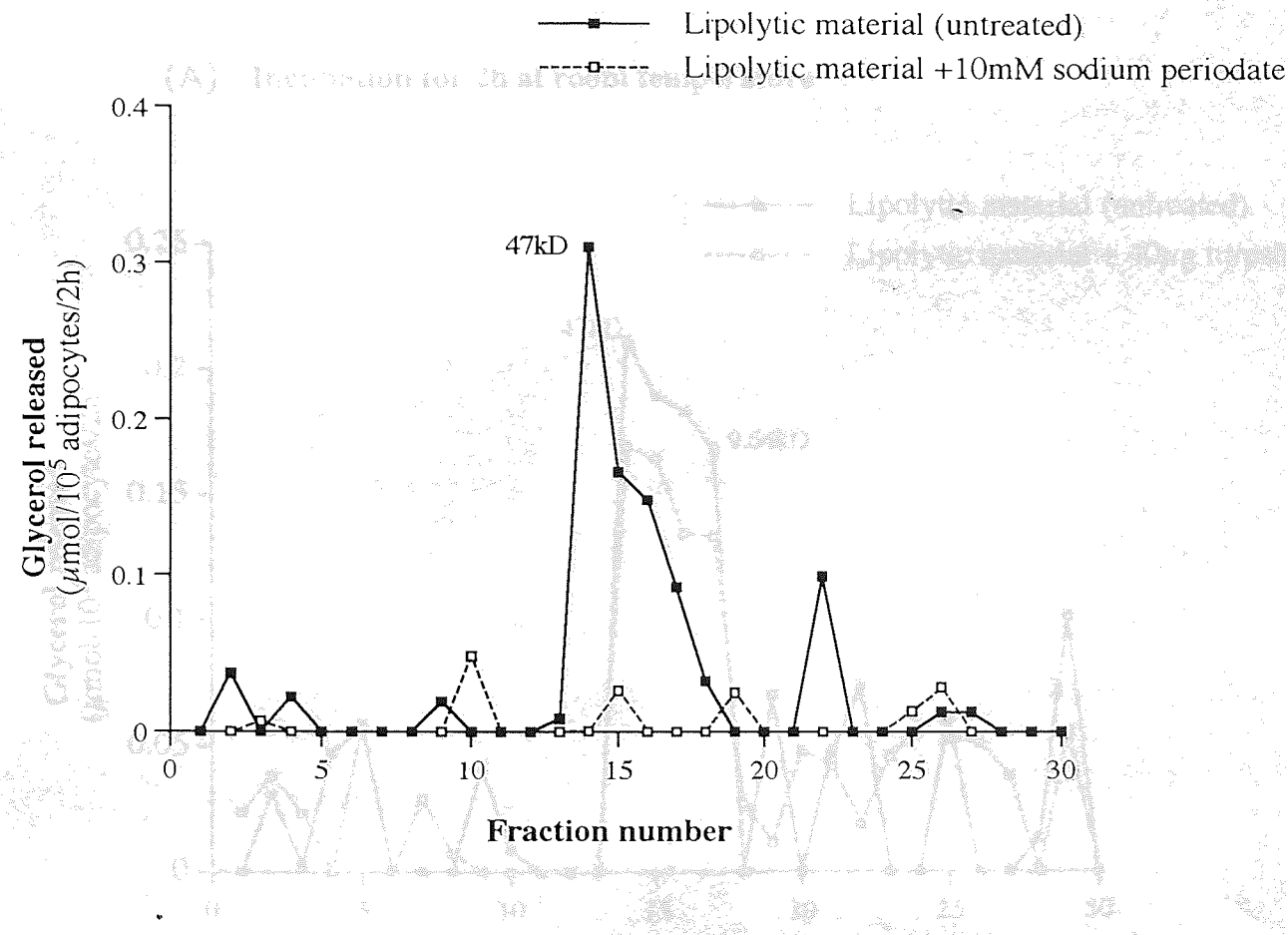


Figure 2.2.2.2.5.

Effect of sodium periodate on the biological activity of lipolytic material from the MAC 16 tumour:

Analysis by superose gel-exclusion chromatography and lipolytic assay of fractions



Sample parameters prior to treatment:

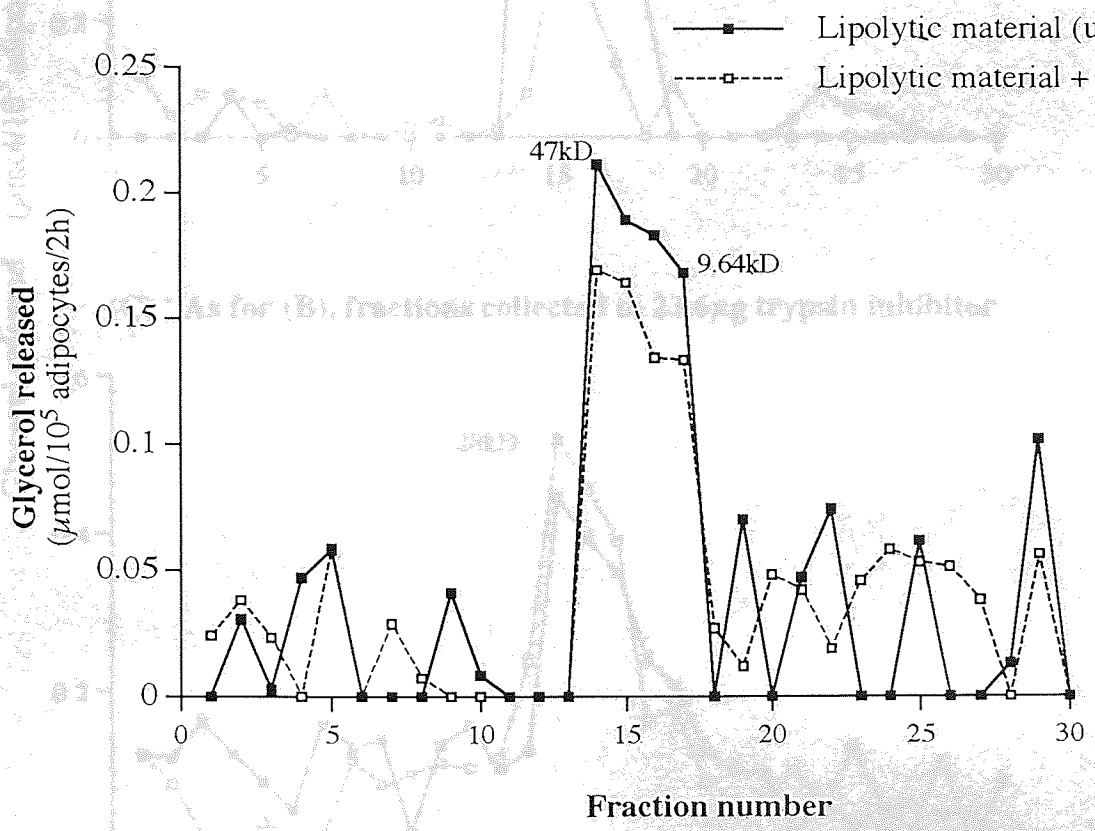
Volume:	200μl
Total protein:	200μg
Lipolytic activity/50μl:	0.0373μmol glycerol/10^5 adipocytes/2h

Figure 2.2.2.2.6 (A, B & C)

Effect of trypsin on the biological activity and molecular mass of lipolytic material from MAC 16 tumour:

Analysis by superose gel-exclusion chromatography and lipolytic assay

(A): Incubation for 2h at room temperature

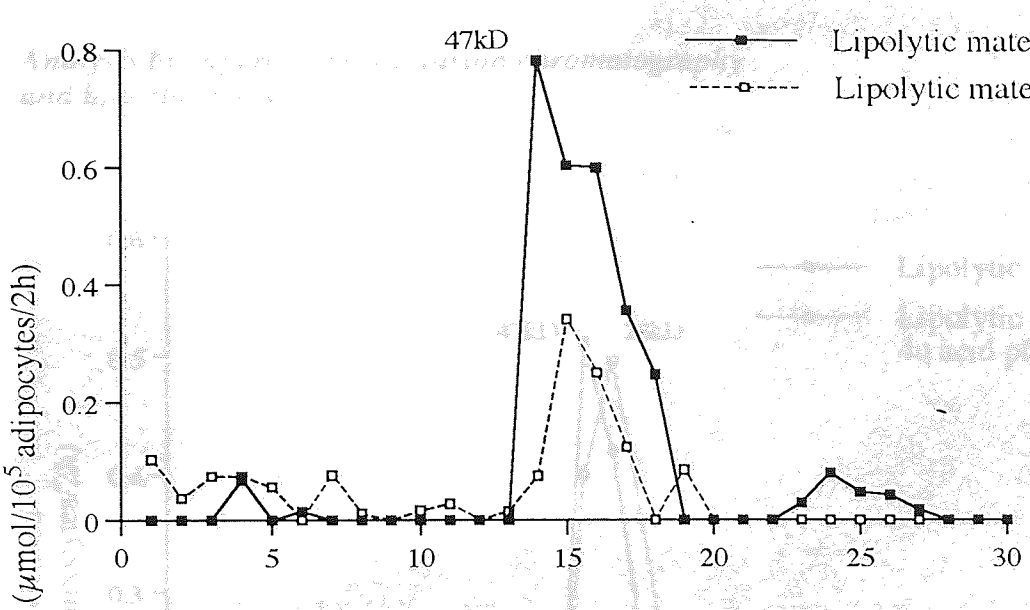


Sample parameters prior to loading :

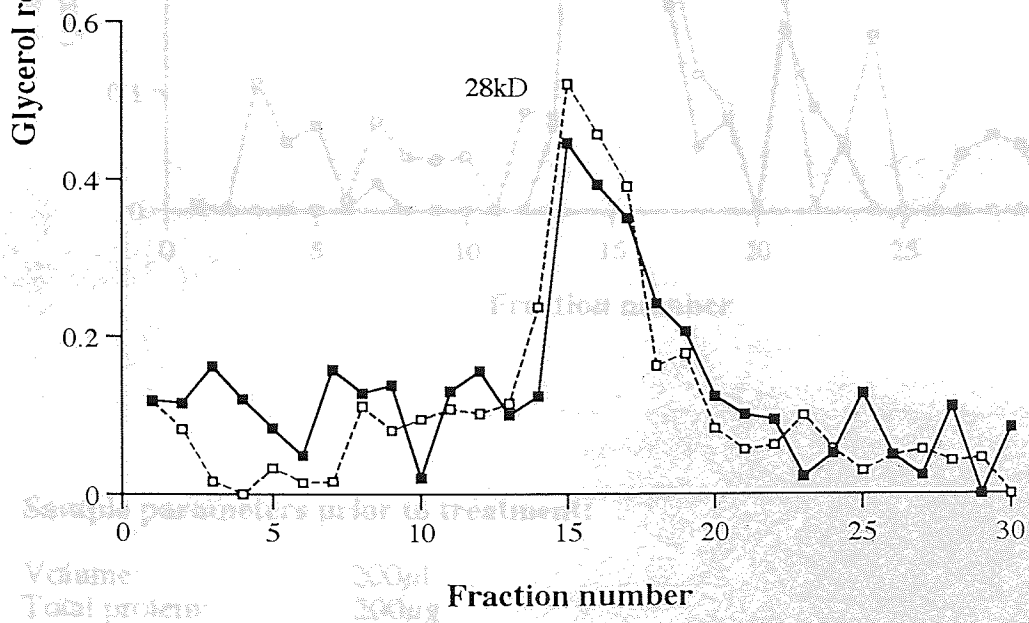
Volume: 200μl
Total protein: 200μg
Lipolytic activity/30μl: 0.0728μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.1129 ")

Figure 2.2.2.2.6 (B & C)

(B): Incubation at 37°C for 2h



(C) : As for (B), fractions collected in 23.6μg trypsin inhibitor



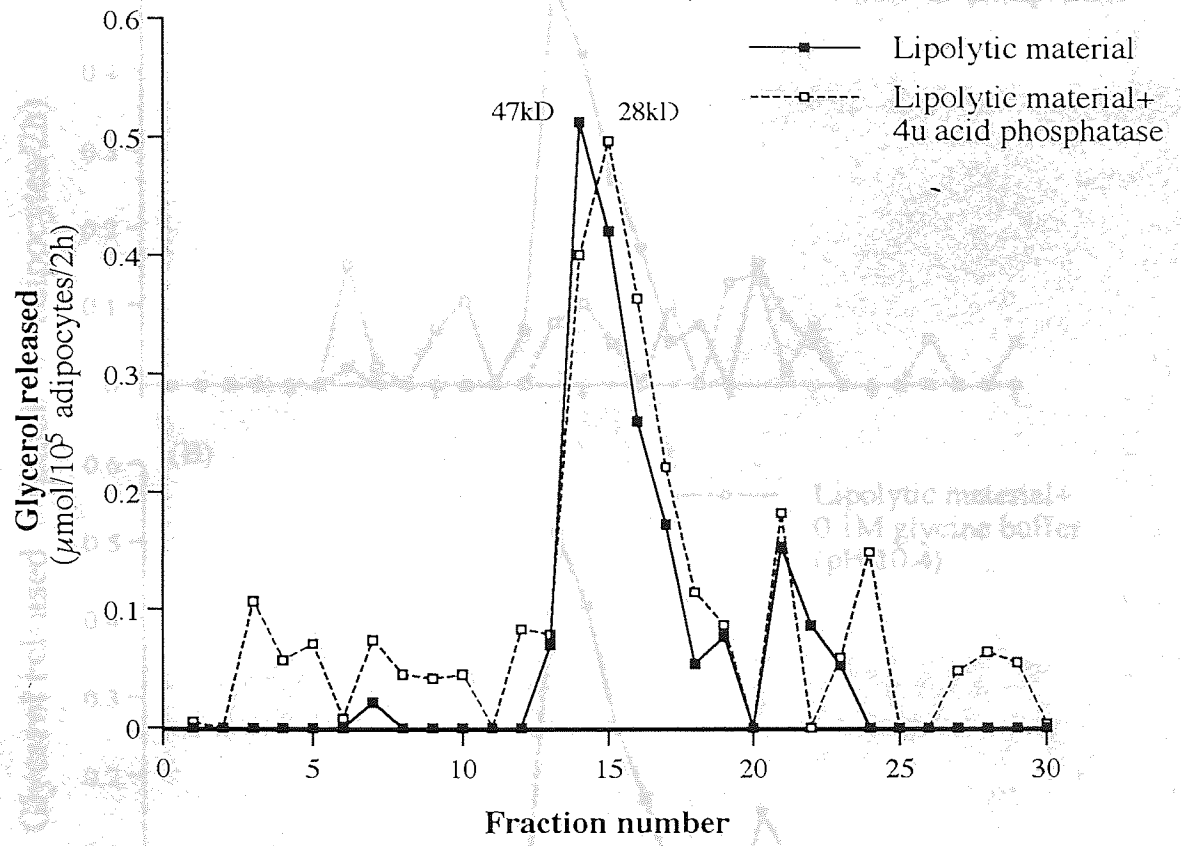
Sample parameters prior to treatment:

Volume: 200μl
Total protein: 200μg
Lipolytic activity/30μl: 0.0744μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.1158 ")

Figure 2.2.2.2.7.

Effect of acid phosphatase on the biological activity of lipolytic material from the MAC 16 tumour:

Analysis by superose gel-exclusion chromatography and lipolytic assay



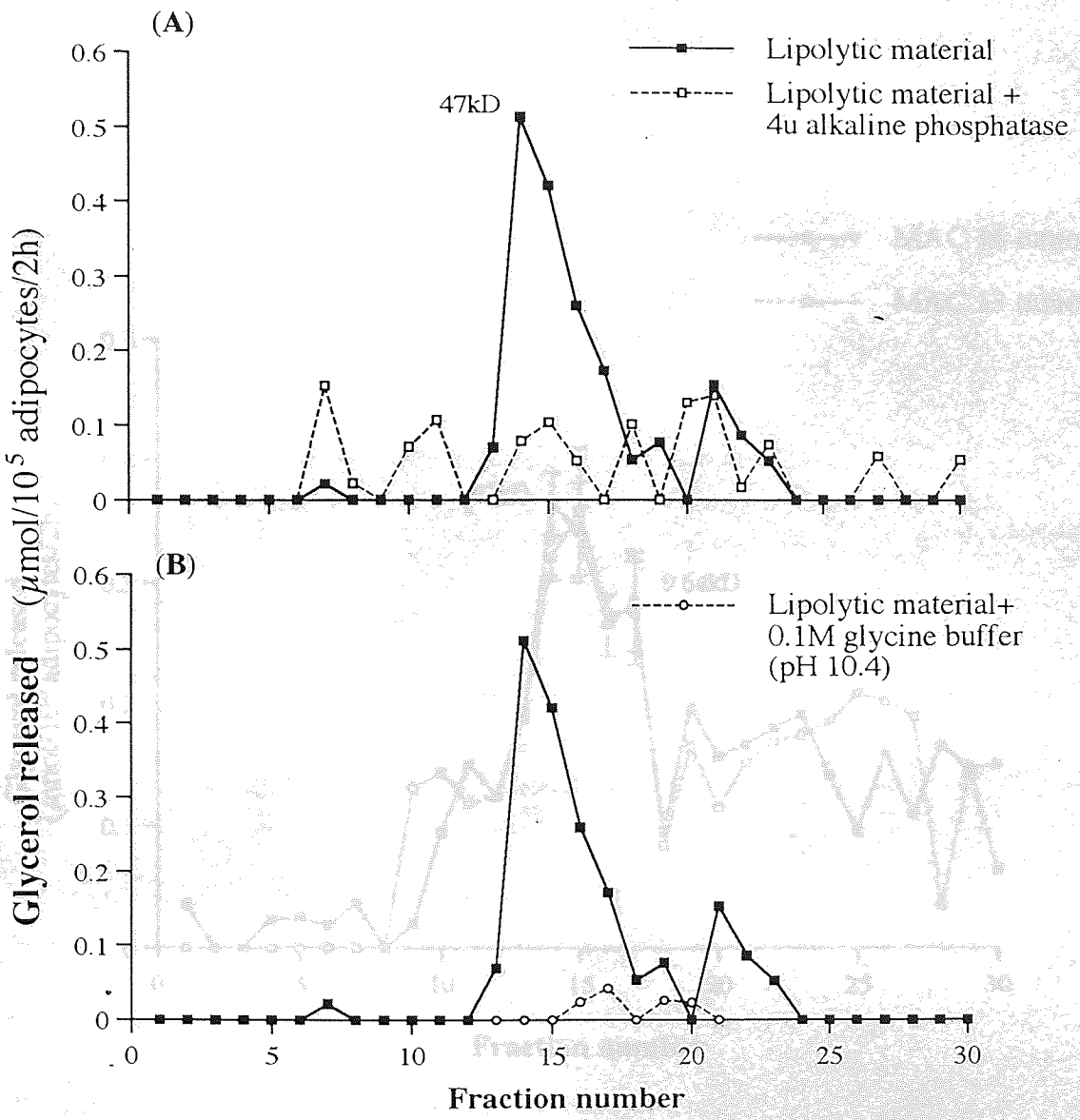
Sample parameters prior to treatment:

Volume: 200μl
Total protein: 200μg
Lipolytic activity/50μl: 0.0367μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline:0.0653 ")

Figure 2.2.2.2.8.

Effect of alkaline phosphatase on the biological activity of lipolytic material from the MAC 16 tumour:

Analysis by superose gel exclusion chromatography and lipolytic assay

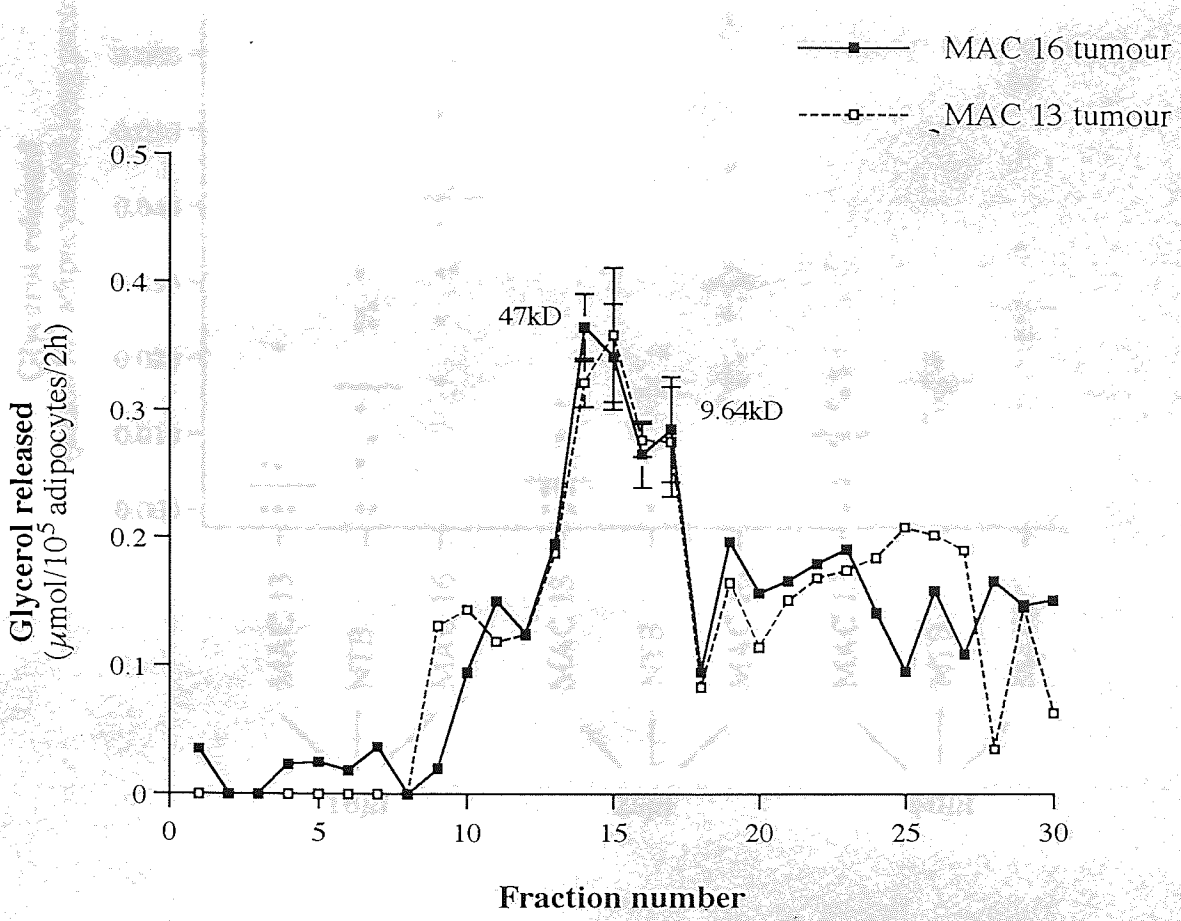


Sample parameters prior to treatment:
Volume: 200μl
Total protein: 200μg
Lipolytic activity/50μl: 0.0367μmol glycerol/10^5adipocytes/2h
(1.0μM isoprenaline: 0.0653 ")

Figure 2.2.2.2.9.

Superose gel-exclusion chromatography of lipolytic material from MAC 16 and MAC 13 tumours:

Analysis by lipolytic assay

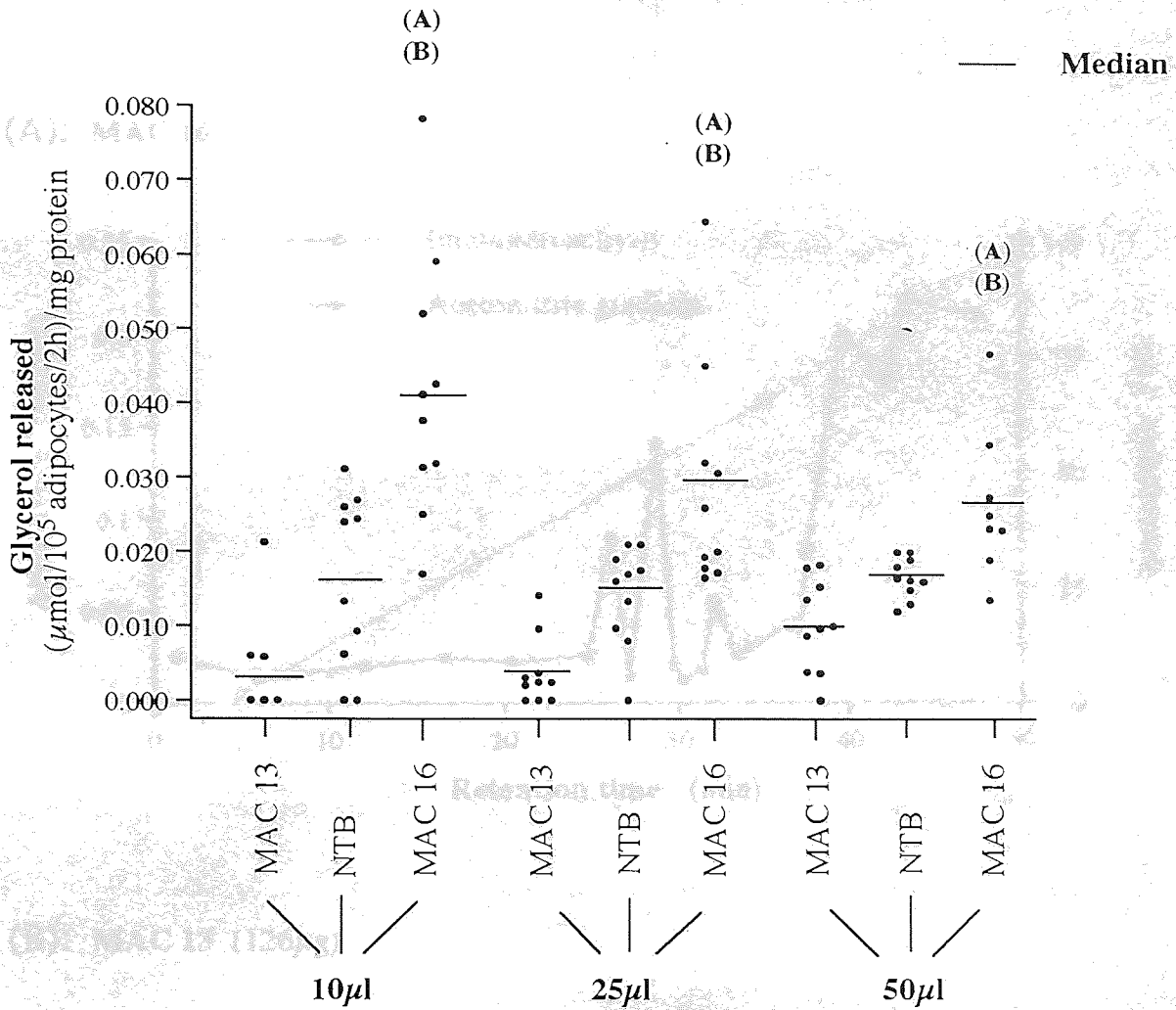


Sample parameters prior to loading:

Volume: 200μl
Total protein: 300μg

Figure 2.2.2.2.10.

Analysis of lipolytic activity of serum from MAC 16, MAC 13 and non tumour-bearing (NTB) mice



Each point represents lipolytic activity of serum from one mouse.
Data was analysed by ANOVA with Scheffes test.

10μl: 10 data points analysed
(A) Significant difference between MAC 16 and MAC 13 sera $p < 0.0001$
(B) Significant difference between MAC 16 and NTB sera $p < 0.001$

25μl: 10 data points analysed
(A) Significant difference between MAC 16 and MAC 13 sera $p < 0.0001$
(B) Significant difference between MAC 16 and NTB sera $p < 0.05$

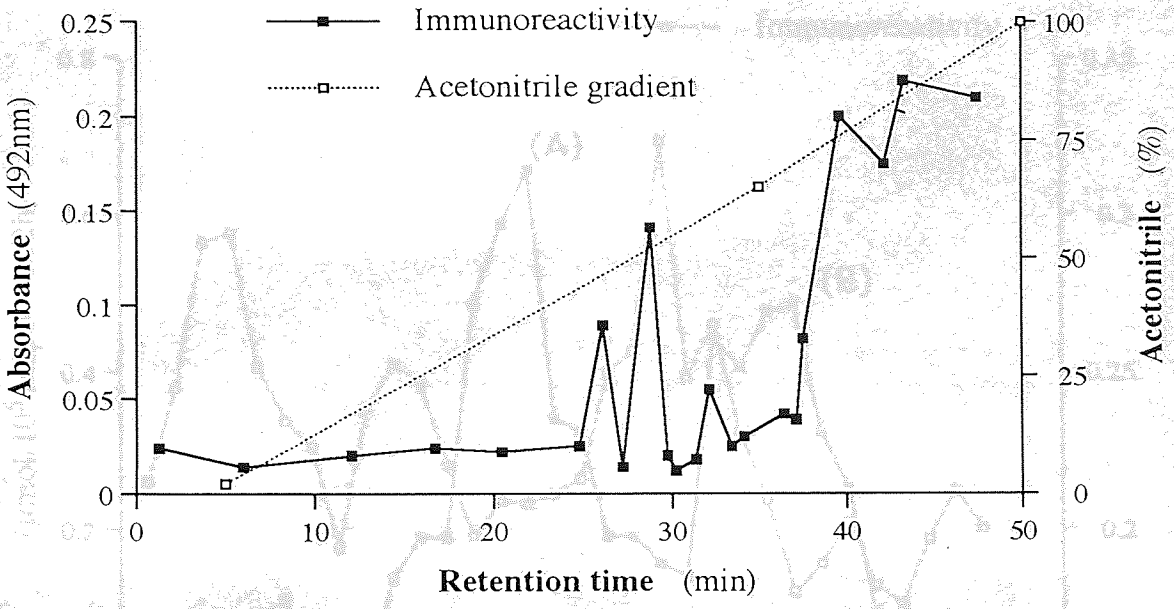
50μl: 8 data points analysed
(A) Significant difference between MAC 16 and MAC 13 sera $p < 0.001$
(B) Significant difference between MAC 16 and NTB sera $p < 0.05$

Figure 2.2.2.2.11.

Reverse-phase HPLC of superose-fractionated lipolytic material from MAC 16 and MAC 13 tumours:

Assay of peaks detected at 214nm by ELISA using MAC 16 antisera

(A): **MAC 16** (126μg)



(B): **MAC 13** (126μg)

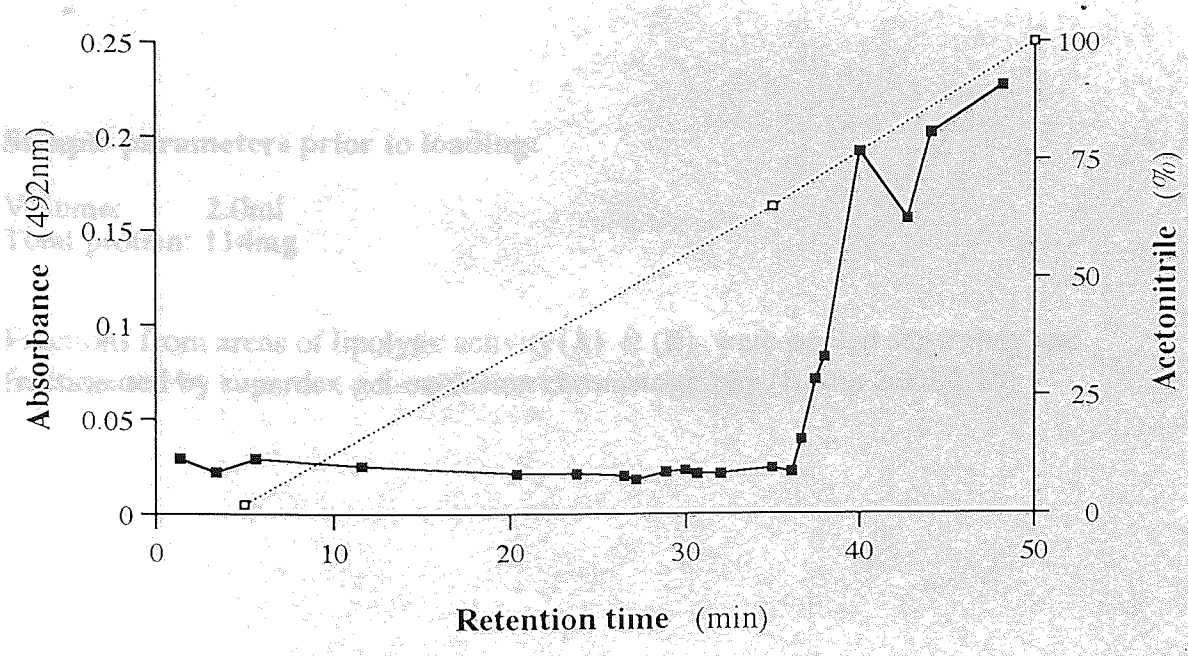
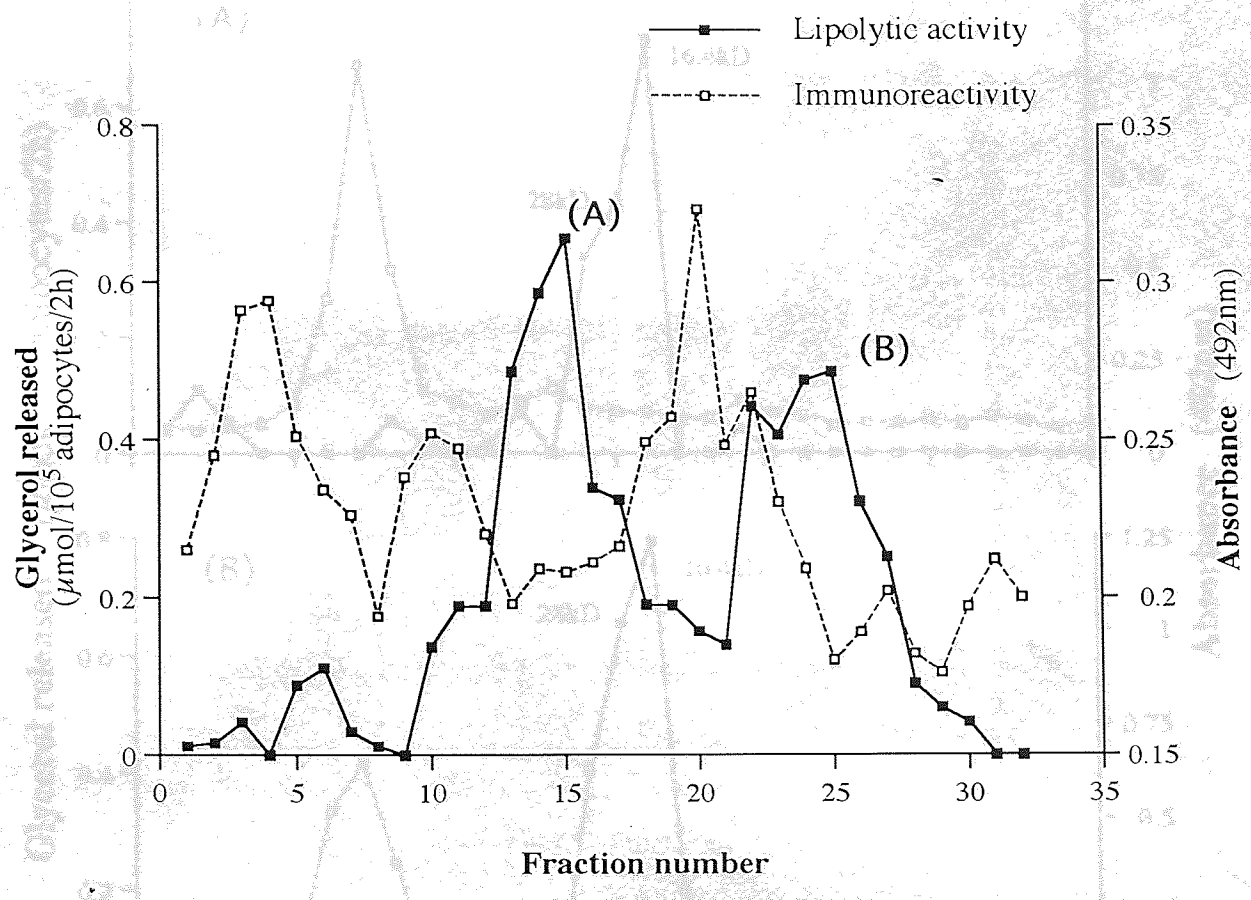


Figure 2.2.2.2.12.

Q-sepharose fractionation of MAC 16 tumour extract after DEAE-cellulose batch extraction:

Analysis by lipolytic assay and ELISA using MAC 16 monoclonal antibody



Sample parameters prior to loading:

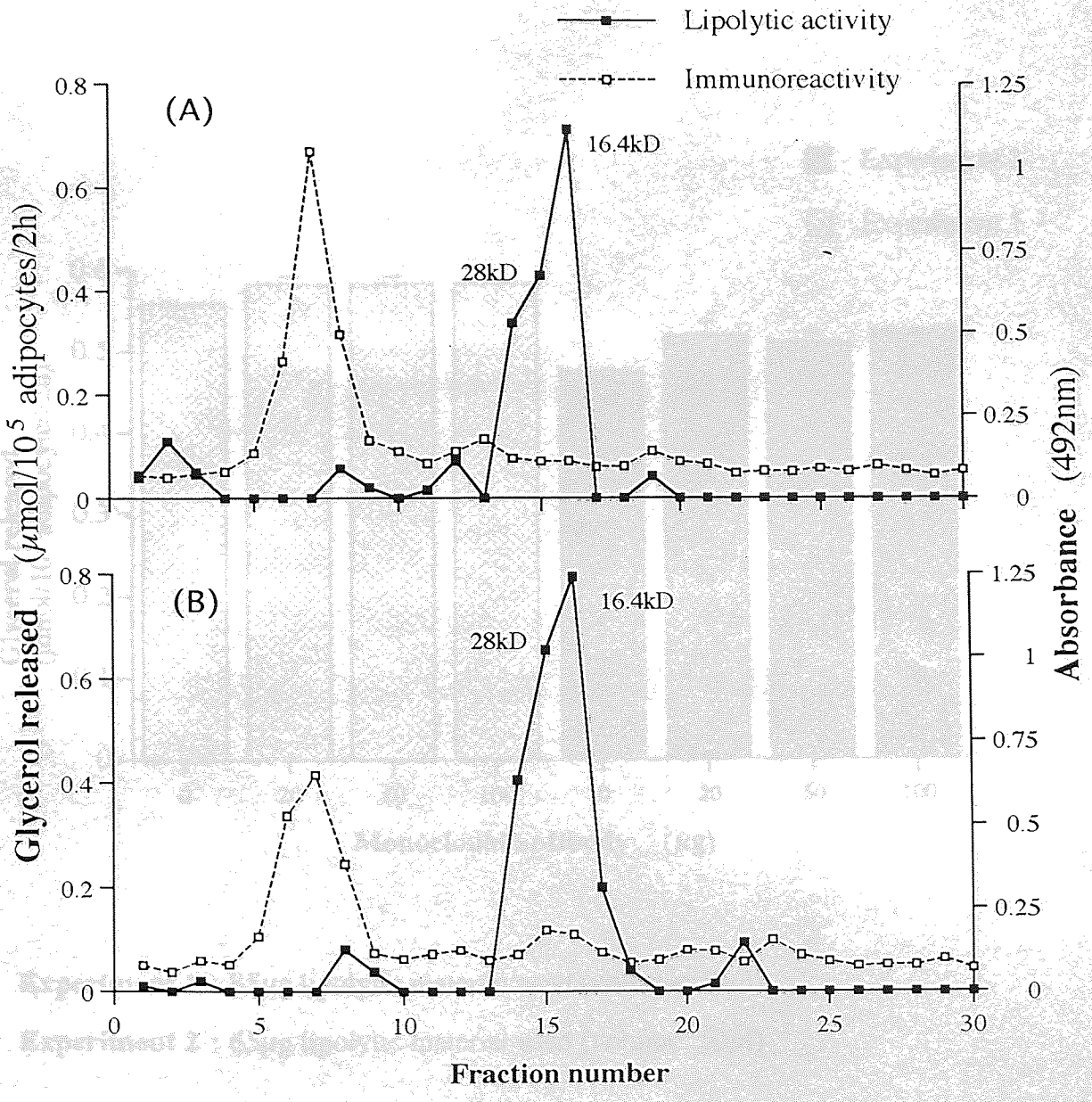
Volume: 2.0ml
Total protein: 114mg

Fractions from areas of lipolytic activity (**A**) & (**B**) were pooled separately and fractionated by superdex gel-exclusion chromatography (figure 2.2.2.2.13)

Figure 2.2.2.2.13.

Superdex gel-exclusion chromatography of lipolytic material (3mg) after Q-sepharose fractionation:

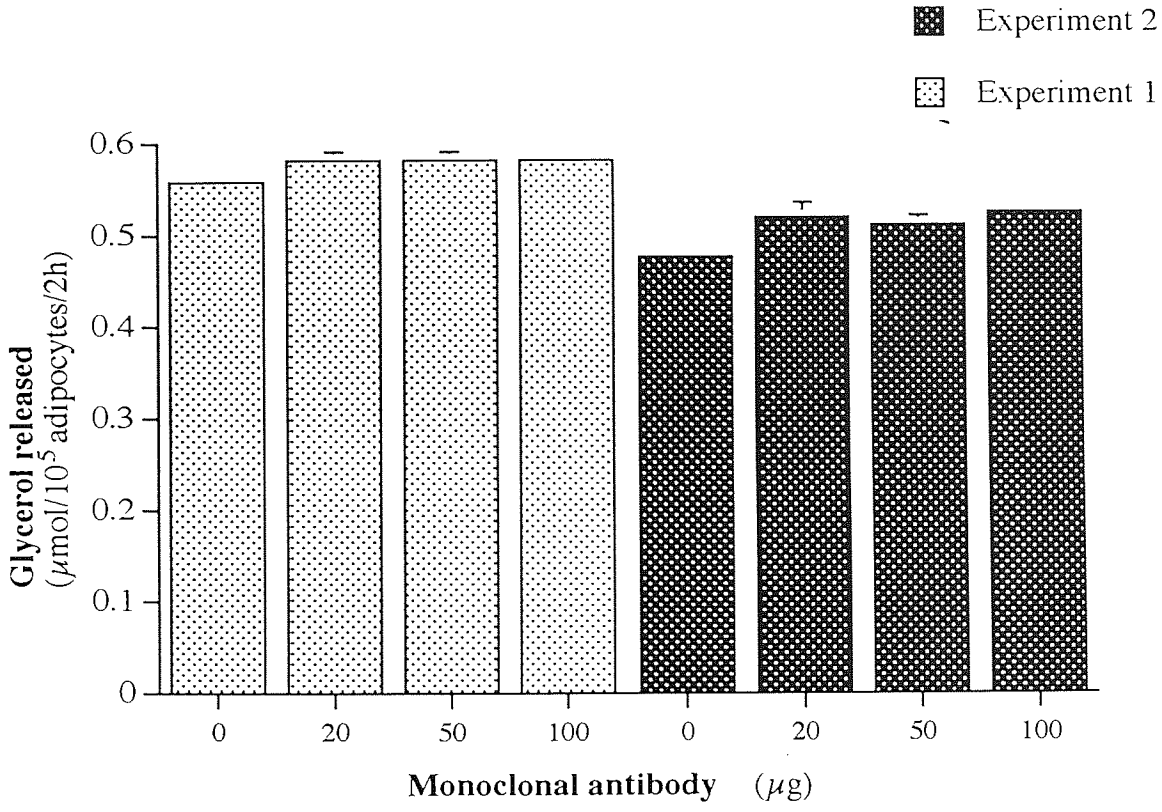
Analysis by lipolytic activity and ELISA using MAC 16 monoclonal antibody



(**A**) : Fractions 13-17 from Q-sepharose (NaCl: 0.15M), (figure2.2.2.2.12)
(**B**) : Fractions 22-27 from Q-sepharose (NaCl: 0.225M), (figure 2.2.2.2.12)

Figure 2.2.2.2.14.

Effect of monoclonal antibody on the biological activity of lipolytic material from MAC 16 tumour after superose gel-exclusion chromatography



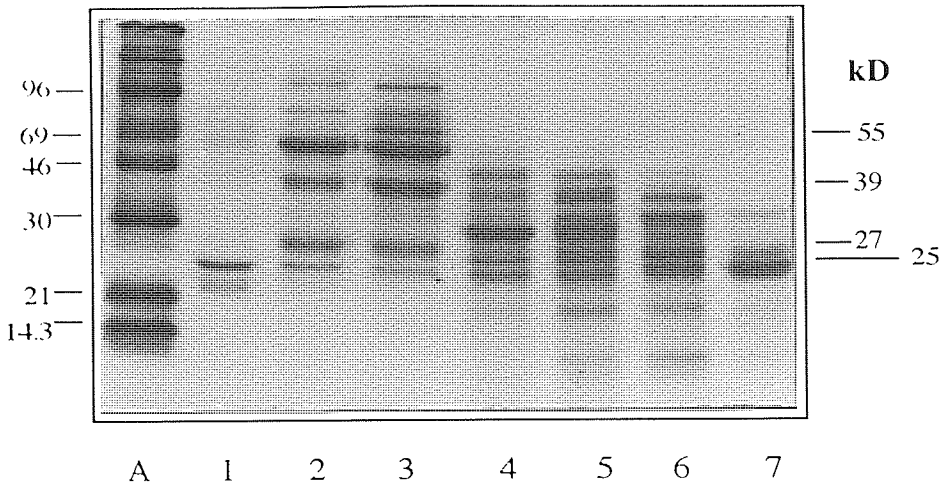
Experiment 1 : 85μg lipolytic material used (volume: 50μl)

Experiment 2 : 65μg lipolytic material used (volume: 50μl)

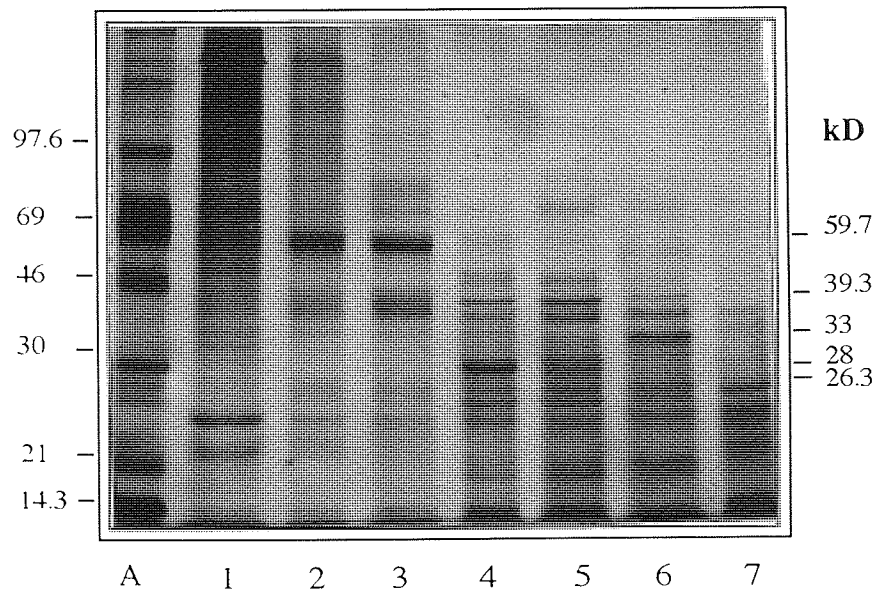
Figure 2.2.2.2.15.

SDS-PAGE of immunoreactive and lipolytic fractions after superdex gel-exclusion chromatography

(A) Coomassie brilliant blue stain (10μg/lane)



(B) Silver stain (1μg/lane)



1: Fraction 6
2: Fraction 7
3: Fraction 8
] Immunoreactive fractions

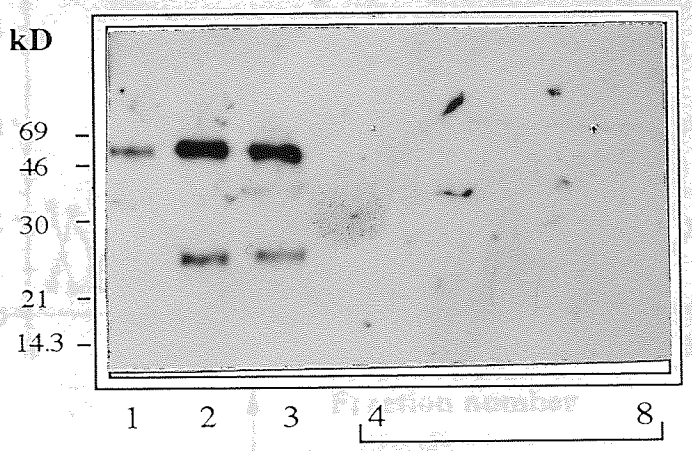
4: Fraction 14
5: Fraction 15
6: Fraction 16
7: Fraction 17
] Lipolytic activity

A: Molecular weight markers (kD)

Figure 3.1.2.2.16.

Western blot analysis of fractions (5μg/lane) after superdex gel fractionation:

Analysis of immunoreactivity using MAC 16 monoclonal antibody



1: Fraction 6
2: Fraction 7
3: Fraction 8

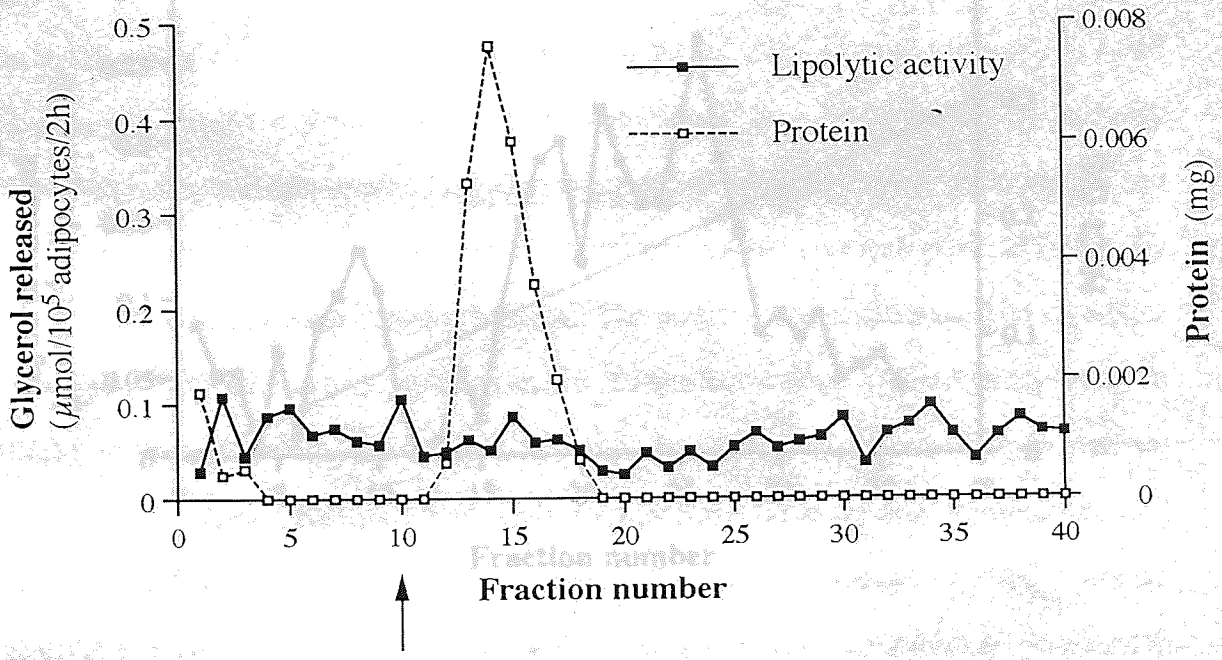
Immunoreactive

4-8: Fractions 14-17 (lipolytic)

Figure 2.2.2.2.17.

Affinity purification of MAC 16 tumour extract after ammonium sulphate precipitation (38%):

Analysis by lipolytic assay



100mM Glycine.HCl (pH 2.5)

Sample parameters prior to loading:

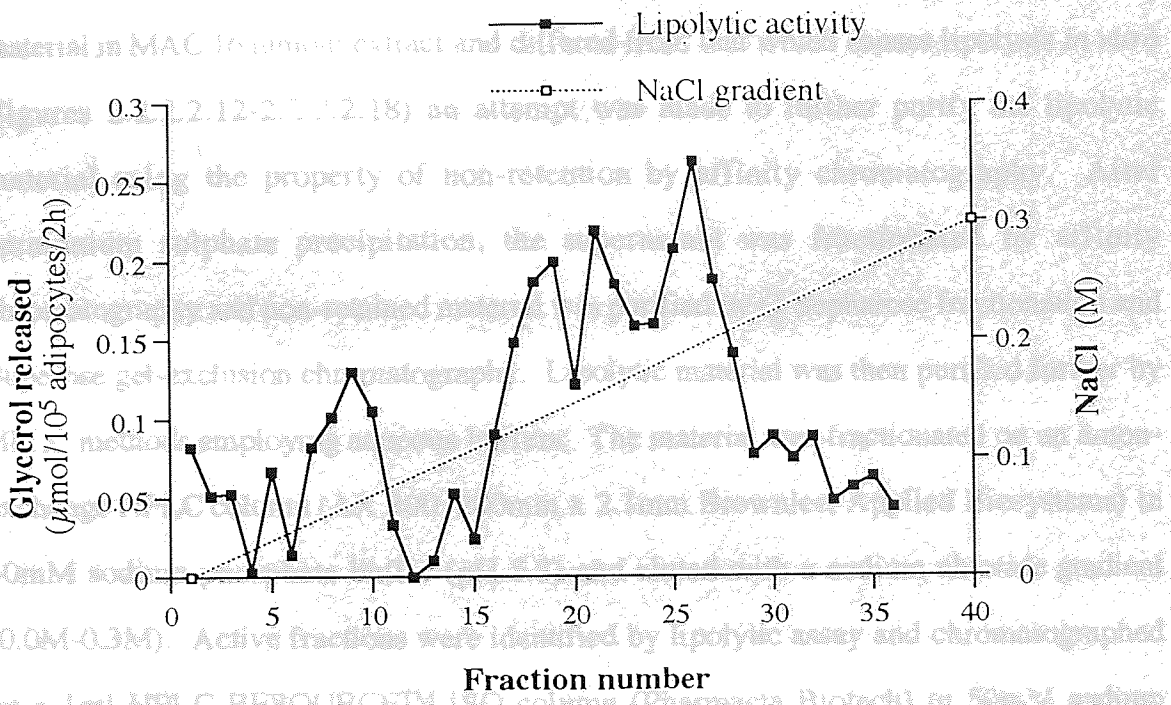
Total protein: 44.2mg
Lipolytic activity/50μl: 0.0984μmol glycerol/10^5 adipocytes/2h
(1.0μM isoprenaline: 0.1562 ")

Figure 2.2.2.2.18.

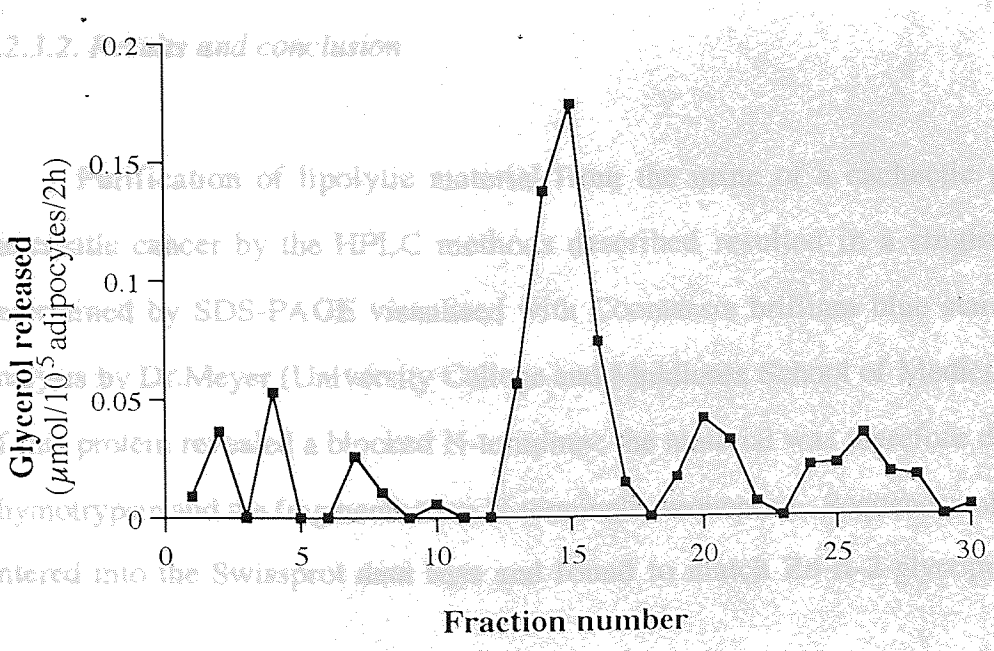
Affinity purification of MAC 16 tumour extract:

Analysis of non-retained material by lipolytic assay

(A): *Q-sepharose fractionation of non-retained material (13mg)*



(B): *Superose fractionation of non-retained material after Q-sepharose chromatography*



2.2.3. Purification of lipolytic material from human urine and the MAC 16 tumour to homogeneity (Todorov et al, unpublished results)

2.2.3.1. Introduction

As a result of observations that the monoclonal antibody appeared to be specific to material in MAC 16 tumour extract and differed from that which causes lipolysis *in vitro* (figures 2.2.2.2.12-2.2.2.2.18) an attempt was made to further purify the lipolytic material using the property of non-retention by affinity chromatography. After ammonium sulphate precipitation, the supernatant was fractionated by affinity chromatography and non-retained material was purified by Q-Sepharose fractionation and Superose gel-exclusion chromatography. Lipolytic material was then purified further by HPLC methods employing aqueous buffers. The material was fractionated on an anion-exchange HPLC column (AX 300, 200mm x 2.1mm Brownlee, Applied Biosystems) in 10mM sodium phosphate buffer (pH 5.3) and eluted with a sodium chloride gradient (0.0M-0.3M). Active fractions were identified by lipolytic assay and chromatographed on a 1ml HPLC RESOURCE™ ISO column (Pharmacia Biotech) in 50mM sodium phosphate buffer (pH 7.0) + 1.5M ammonium sulphate and eluted with an ammonium sulphate gradient (1.5M-0.0M).

2.2.3.2. Results and conclusion

Purification of lipolytic material from the urine of a cachectic patient with pancreatic cancer by the HPLC methods described resulted in a single protein, as determined by SDS-PAGE visualised with Coomassie brilliant blue stain. Sequence analysis by Dr.Meyer (University College and Middlesex School of Medicine, London) of this protein revealed a blocked N-terminus; the material was therefore digested with chymotrypsin and the fragments were N-terminally sequenced. Sequences obtained were entered into the Swissprot data base and found to match Zn-α-2-glycoprotein (tables

2.2.3.2.1 & 2.2.3.2.2; figure 2.2.3.2.1). SDS-PAGE analysis of the lipolytic material (figure 2.2.3.2.2a), western blot analysis using Zn-α-2-glycoprotein polyclonal antibody (figure 2.2.3.2.2b) and fractionation on a non-denaturing polyacrylamide gel (figure 2.2.3.2.3) revealed the material to be associated with a protein of apparent molecular mass 40kD with identical gel-migratory and antibody reactivity properties as Zn-α-2-glycoprotein. In addition, purification of murine lipolytic material from the MAC 16 tumour to homogeneity by the same procedure also resulted in isolation of a protein of apparent molecular mass 40kD (figure 2.2.3.2.4).

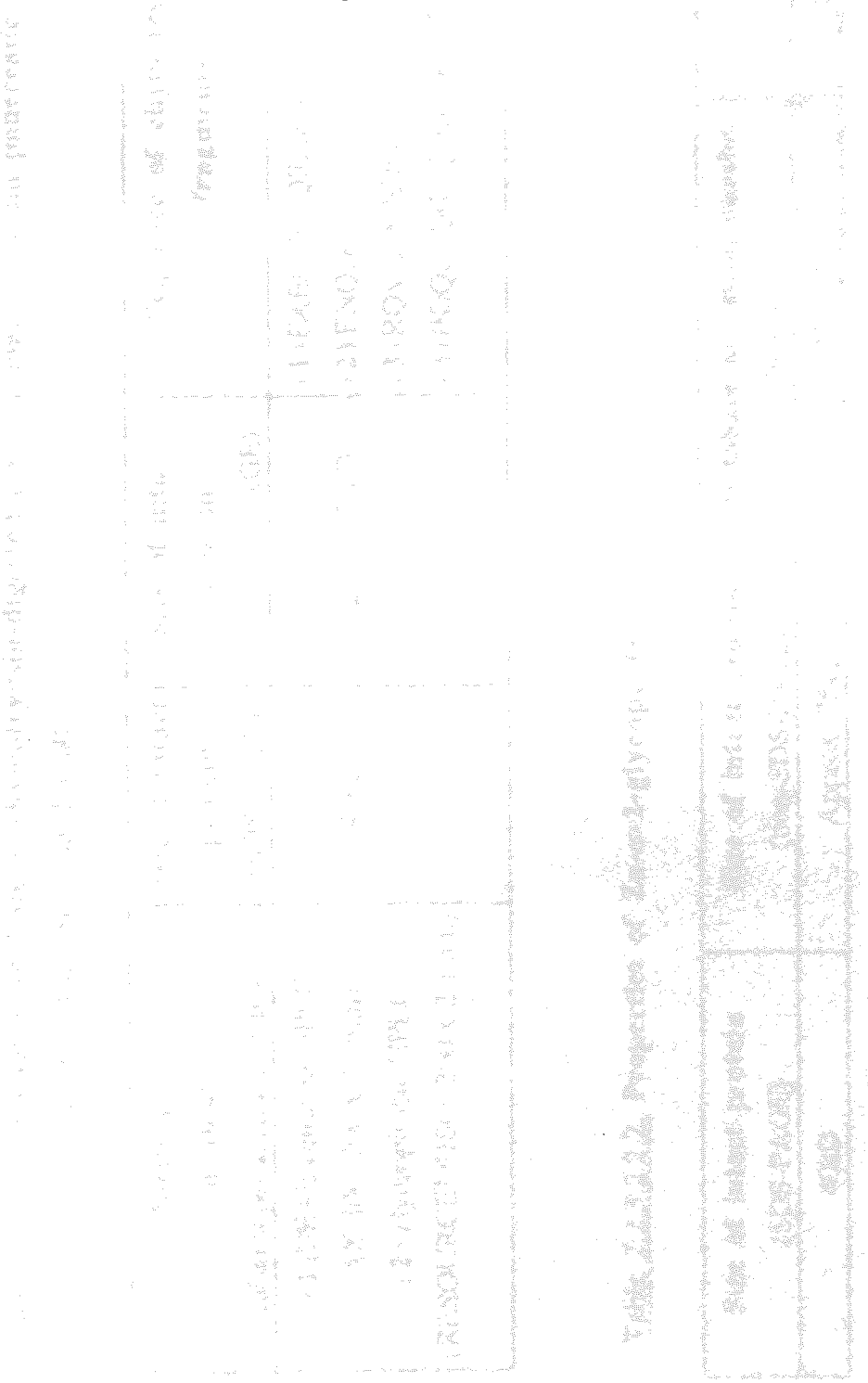


Table 2.2.3.2.1. **Sequence analysis of chymotrypsin-digested lipolytic material from pancreatic cancer patient urine** (Todorov et al, unpublished results)

Purification method (after superdex fractionation)	**Size of intact protein** (SDS-PAGE)	**Size of intact protein** (non-SDS PAGE)	**Sequence of chymotrypsin fragments**	**Database sequence identity**
(**1**)DEAE-cellulose HPLC (AX 300, 200 x 2.1mm) (**2**) Hydrophobic HPLC (RESOURCE^{TM}ISO, 200 x 2.1mm)	40kD	Approx. 75kD	(**1**) EAEPVYVQRAKAYLEEE(C)PAT (**2**) ENQDG (**3**) RQVEGMED(W) (**4**) (K)QDSQ(L)Q(K)A(R)E(DI)FMETL	Human Zn-α-2-glycoprotein (Swissprot)

Table 2.2.3.2.2.2. **Properties of Zn-α-2-glycoprotein:**

Size of intact protein (SDS-PAGE)	**Size of intact protein** (non-SDS-PAGE)	**Database accession number** (Swissprot)	**Reference**
40kD	Approx. 75kD	P25311	Ueyama et al (1991)

Figure 2.2.3.2.1

Comparison of human lipolytic material and human Zn-α-2-glycoprotein amino acid sequences

ENQDG
1 MVPVLLSLLLLLGPAVPQENQDGRYSLTYIYTGLSKHVEDVPAFQALGSLNDL

RQVEGMED QDSQ- Q - A -E-- FMETL
QFFRYNSKDRKSQPMGLWRQVEGMEDWKQDSQLQKAREDIFMETLKDIVEYYN

DSNGSHVLQGRFGCEIENNRSSGAFWKYYYDGKDYIEFNKEIPAWVPFDPAAQI

EAEPVYVQRAKAYLEEE-PAT
TKQKWEAEPVYVQRAKAYLEEECPATLRKYLKYSKNILDRQDPPSVVVTSHQAP

GEKKKLKCLAYDFYPGKIDVHWTRAGEVQEPELRGDVLHNGNGTYQSWVVVA

VPPQDTAPYSCHVQHSSLAQPLVVPWEAS **297**

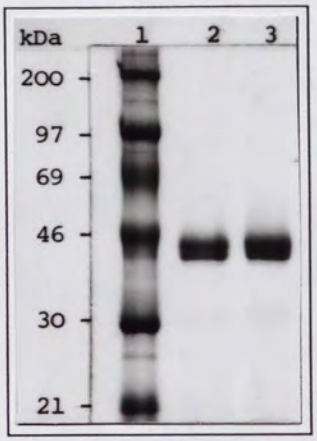
——	Database sequence of Zn-alpha-2-glycoprotein
——	N-terminal sequence of chymotrysin - digested fragments of lipolytic material from the urine of a pancreatic cancer patient

Figure 2.2.3.2.2.

Analysis of lipolytic material from human pancreatic cancer patient urine by SDS-PAGE and western blot:

Comparison with Zn-alpha-2 glycoprotein

(A) SDS-PAGE: Visualisation by Coomassie brilliant blue

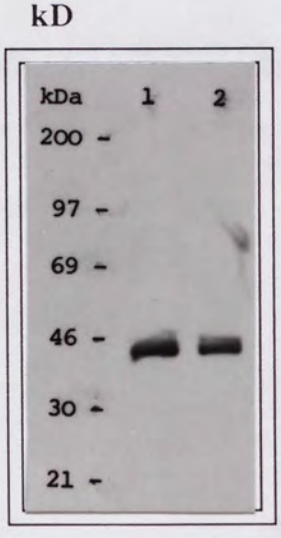


1: Molecular weight markers (kD)

2: Zn-α-2-glycoprotein

3: Lipolytic material

(B) Western blot using polyclonal antibody against Zn-alpha-2-glycoprotein



1: Zn-α-2 glycoprotein

2: Lipolytic material

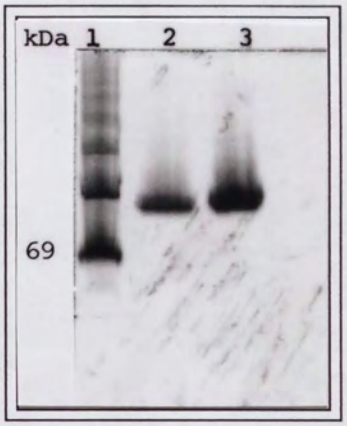
Zn-α-2-glycoprotein purified from human plasma and Zn-α-2-glycoprotein polyclonal antibody were kindly donated by Dr Iwao Ohkubo (Department of Medical Biochemistry, Shiga University of Medical Science, Seta, Otsu, Shiga, 520-21, Japan)

(From: Todorov *et al*, unpublished results)

Figure 2.2.3.2.3.

Analysis of lipolytic material from human pancreatic cancer patient urine, by non-denaturing PAGE:

Comparison with Zn-alpha-2-glycoprotein



1: Molecular weight markers (kD)

2: Lipolytic material from human urine

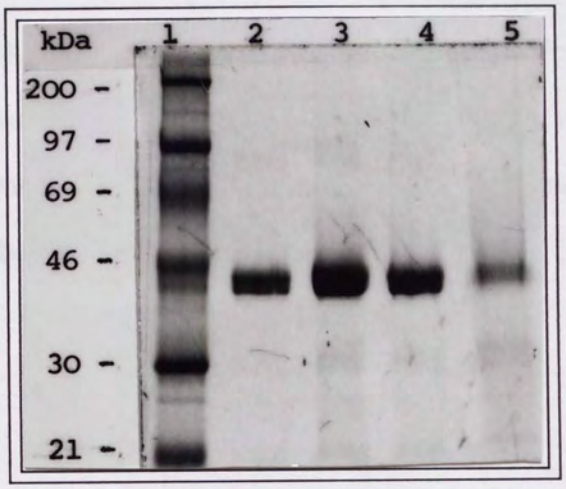
3: Zn-α-2-glycoprotein

(From: Todorov *et al*, unpublished results)

Figure 2.2.3.2.4

SDS-PAGE analysis of lipolytic material from the MAC 16 tumour and human pancreatic cancer patient urine:

Comparison with Zn-alpha-2-glycoprotein



1: Molecular weight markers (kD)

2: Lipolytic material from human urine

3 & 4: Zn-α-2-glycoprotein

5: Lipolytic material from the MAC 16 tumour

(From: Todorov *et al*, unpublished results)

2.2.4. Purification of MAC 16 monoclonal antibody-reactive material from the MAC 16 tumour

2.2.4.1. Introduction

Material with monoclonal antibody reactivity was purified from MAC 16 tumour extract by ammonium sulphate precipitation, fractionation through an affinity column as described (2.1.3.4), and HPLC (C8 column, 2.1.3.5.2). Fractions containing antibody-reactive material were identified by ELISA and analysed by western blot. The specificity of the immunogenic material to a cachexia-inducing tumour was also determined by comparison with the non-cachexigenic MAC 13 tumour.

2.2.4.2. Results

Ammonium sulphate precipitation of a MAC 16 tumour extract resulted in removal of 94.7% of tumour proteins (table 2.2.4.2.1). Fractionation of the supernatant on an affinity column containing monoclonal antibody immobilised to Affi-Gel Hz matrix resulted in the retention of immunoreactive material which was eluted by 100mM glycine buffer (pH 2.5) with the major protein peak (figure 2.2.4.2.1). Material at this stage represented 0.012% of the tumour proteins and had a specific activity of 168 ELISA units/mg (table 2.2.4.2.1). Specific activity was calculated from this stage due to the effect of contaminants present in the tumour homogenate and ammonium sulphate supernatant, which made the interpretation of ELISA results difficult for these steps. SDS-PAGE analysis of affinity-purified immunoreactive fractions visualised by silver stain revealed the presence of proteins of apparent molecular mass 70kD, 57.4kD, 44.9kD and 24.8kD (figure 2.2.4.2.2). Analysis of these proteins by western blot using MAC 16 monoclonal antibody and protein A resulted in the detection of immunoreactivity in proteins of apparent molecular mass close to 69kD and 24kD (figure 2.2.4.2.3). More recently, immunogenic fractions after affinity purification were analysed by western blot

using biotinylated MAC 16 monoclonal antibody, thus eliminating the possibility of detection of antibody leaking from the column by protein A. Using this method, proteins of similar apparent molecular mass were detected (figure 2.2.4.2.4a).

Determination of the presence of carbohydrate moieties in affinity-purified material by digoxigenin glycan detection revealed that these immunogenic proteins were glycosylated (figure 2.2.4.2.5).

HPLC fractionation of affinity-purified material resulted in the elution of immunogenic material at retention times 31.5-33.5min (57.5-62% acetonitrile) and 42min (90% acetonitrile) (figure 2.2.4.2.6). The first peak represented 40 parts per billion of the protein originally present in the tumour extract and fractionation of this material by HPLC resulted in a 427-fold purification (table 2.2.4.2.1). When the peak with retention time 31.5-33.5min from eight HPLC runs were pooled and analysed by SDS-PAGE with silver staining, a single protein with an apparent molecular mass of 24kD was observed (figure 2.2.4.2.7). Western blot analysis of these fractions resulted in detection of a 24kD protein for the peak with retention time 31.5-33.5min, and a protein with molecular mass close to 69kD for the peak with retention time 42min (figure 2.2.4.2.4b). Sequence analysis of the 24kD protein after HPLC revealed the material to have a single 18 amino acid N-terminal sequence, while the material with apparent molecular mass 69kD appeared to contain mouse albumin as the major sequence in addition to the sequence observed for the 24kD protein (table 2.2.4.2.2). Database searches in Swissprot and Genbank for sequences with similarity to that for the 24kD protein failed to produce a match. However a search in the Pir 3 database into which uncloned proteins are entered yielded a match with *Streptococcal* preabsorbing antigen (PA-Ag) (table 2.2.4.2.2.1.). Thirteen amino acids of the N-terminus of this antigen have been sequenced and entered into the database and a match with the 24kD material in eleven of these residues was observed.

Attempts to obtain internal sequence information for the 24kD protein by digestion with trypsin resulted in no additional sequences. Indeed, incubation with trypsin was observed to have no effect on the 24kD material when analysed by western blot (figure

2.2.4.2.8). Digestion with pronase, chymotrypsin or V8 protease also failed to yield further sequence information.

Affinity chromatography of 70mg of the non-cachexigenic MAC 13 tumour extract after ammonium sulphate precipitation resulted in the elution of fractions that were four-fold less immunoreactive than those obtained for 70mg of MAC 16 tumour extract (figure 2.2.4.2.9). In addition, serum from MAC 16 tumour-bearing mice appeared to be more immunoreactive by a factor of 2.6-fold than serum from MAC 13 tumour-bearing mice as determined by affinity column fractionation of 50mg of serum (figure 2.2.4.2.10). However, no differences in protein profile were apparent when immunoreactive fractions from both sources were analysed by SDS-PAGE and visualised by silver stain (figure 2.2.4.2.11). Comparison with affinity-purified MAC 16 tumour extract also revealed a similarity in protein profile with the exception of the absence of a band of apparent molecular mass 57kD (possibly antibody heavy chain) in the latter sample. In addition, western blot analysis of immunoreactive fractions from affinity-purified MAC 16 and MAC 13 serum also appeared to produce identical results (figure 2.2.4.2.12, lanes 1 and 2). Results obtained by incubation of the same blot with protein A alone (figure 2.2.4.2.12, lanes 3 and 4) suggest that interpretation may be complicated by the presence of antibodies in the sample.

2.2.4.3. Conclusion

Purification of MAC 16 tumour extract by 38% ammonium sulphate precipitation, affinity column fractionation and HPLC (C8 column) resulted in the isolation of a single glycosylated, protease-resistant protein with an apparent molecular mass of approximately 24kD as determined by SDS-PAGE and western blot. The material was purified 427-fold and represented approximately 40ppb of the original tumour proteins Homogeneity was further confirmed by sequence analysis which yielded a single 18 amino acid N-terminal sequence. Eleven of these residues were matched to the N-terminal sequence of *Streptococcal* preabsorbing antigen (PA-Ag) involved in the pathogenesis of acute post-

Streptococcal glomerulonephritis. However, this antigen showed no cross-reactivity with the MAC 16 monoclonal antibody (Todorov *et al*, unpublished results) and the MAC 16 tumour was found to be free of any microbiological contamination (Lambert, personal communication).

Immunogenicity of MAC 13 tumour extract and serum of MAC 13 tumour-bearing mice was found to be minimal as determined by ELISA of affinity-purified fractions. Furthermore, recent metabolic labelling experiments during which MAC 16 and MAC 13 cells were cultured in medium containing [^{35}S] sodium sulphate and subsequently affinity-purified, confirmed that there was negligible production of 24kD material by MAC 13 cells (Todorov *et al*, unpublished results). However, results obtained by immunoblot of affinity-purified MAC 13 serum fractions are difficult to interpret due to possible contamination of the sample with antibodies. The presence of antibody heavy chain is implicated by the binding of protein A in the absence of monoclonal antibody. Protein A does not bind to antibody light chain (Harlow and Lane, 1988), therefore the '24kD' band observed (figure 3.2.2.1.2., lanes 1 and 2) may be a consequence of the binding of free antibody heavy chain fragments in the monoclonal antibody incubation mix, to antibody light chains immobilised on the blot.

Table 2.2.4.2.1

Purification of immunoreactive material from the MAC 16 tumour

Purification stage	Protein (mg)	Protein recovery (%)	ELISA units (A492nm)	ELISA units recovery (%)	Specific activity (ELISA units/mg)	Purification fold
Tumour homogenate	3160	-	-	-	-	-
Ammonium sulphate (38%)	113	3.6	-	-	-	-
Affinity column	0.256	8×10^{-3}	43	-	168	-
HPLC (C8) 57.5-62% acetonitrile	1.2×10^{-4}	3.8×10^{-6}	8.6	20	71667	427
HPLC (C8) 90% acetonitrile	0.124	4×10^{-3}	3.8	8.8	31	0.18

Figure 2.2.4.2.1.

Affinity purification of MAC 16 tumour extract (56mg) after ammonium sulphate precipitation (38%):

Analysis by ELISA using MAC 16 monoclonal antibody

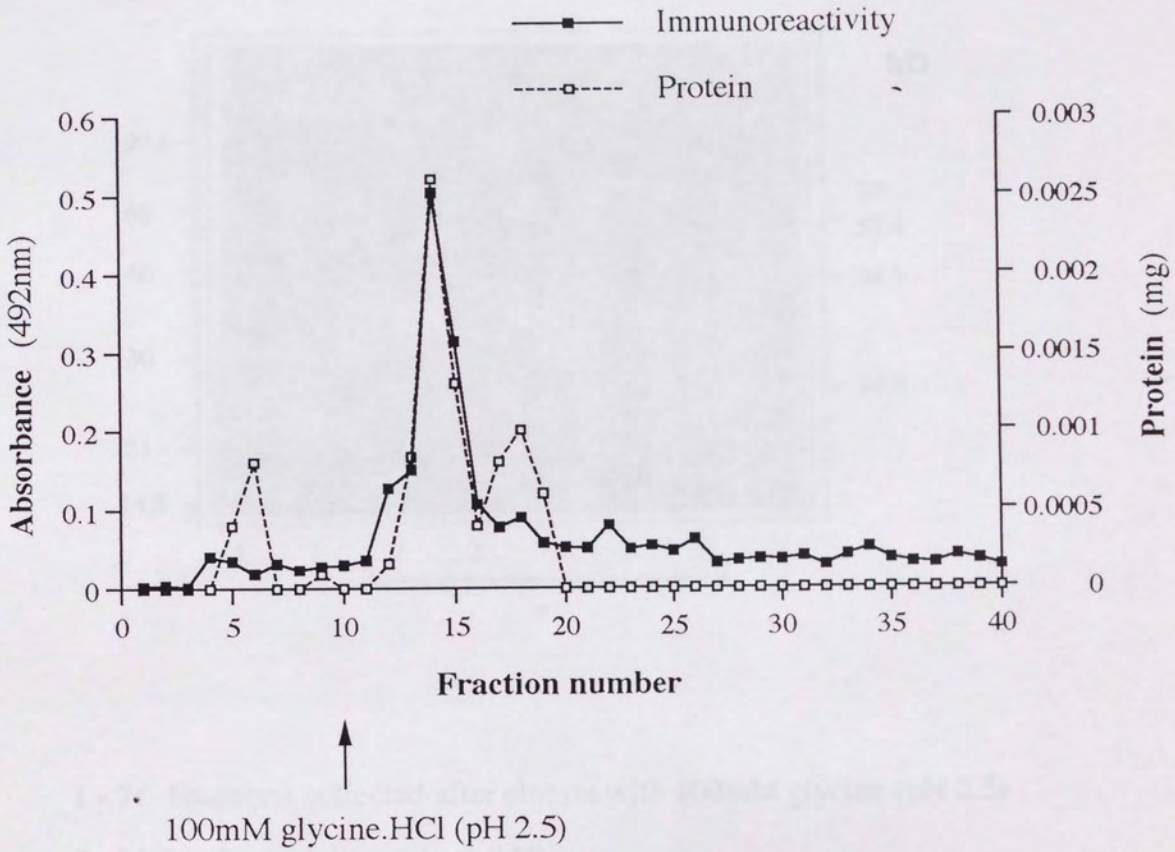
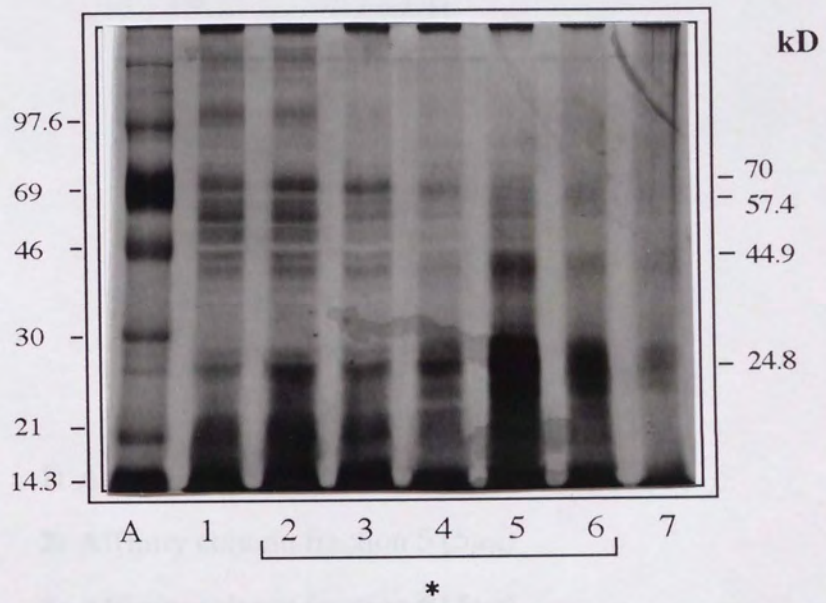


Figure 2.2.4.2.2.

Affinity purification of MAC 16 tumour extract after ammonium sulphate (38%) precipitation:

Analysis by SDS-PAGE and visualisation by silver stain



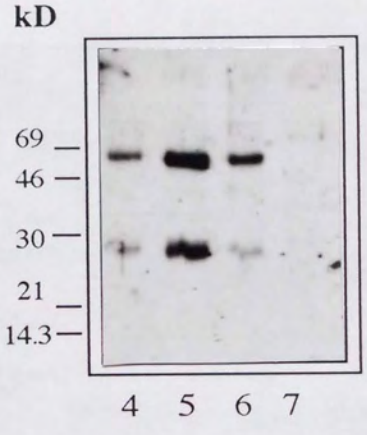
1 - 7: Fractions collected after elution with 100mM glycine (pH 2.5)

A: Molecular weight markers (kD)

***** Area of immunoreactivity

Figure 2.2.4.2.3.

Analysis of affinity-purified MAC 16 tumour extract by western blot using MAC 16 monoclonal antibody



1: Affinity column fraction 4 (5μg)

2: Affinity column fraction 5 (5μg)

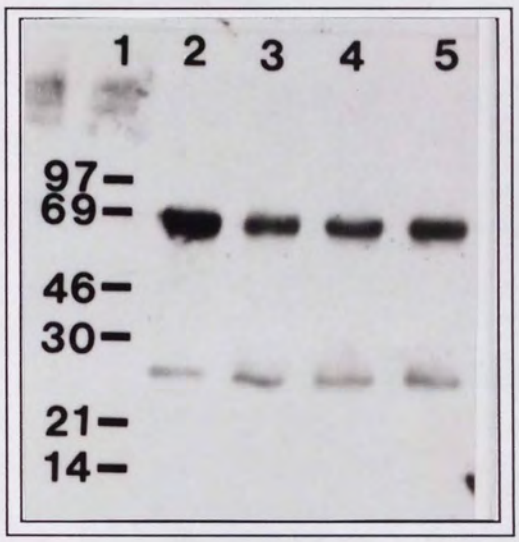
3: Affinity column fraction 6 (5μg)

4: Affinity column fraction 7 (5μg)

Figure 2.2.4.2.4 (A) & (B)

Analysis of MAC 16 tumour extract after affinity and HPLC purification, by western blot with biotinylated MAC 16 monoclonal antibody

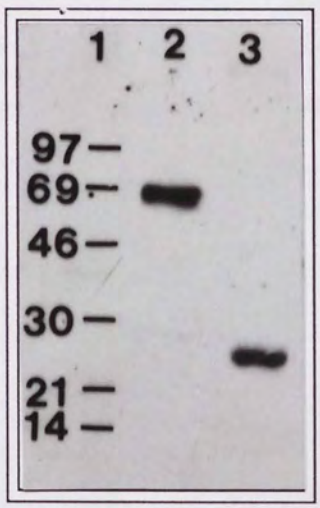
(A) Affinity column purification



1: Molecular weight markers (kD)

Lanes 2 - 4: Immunoreactive fractions

(B) HPLC (C8 column)



1: Molecular weight markers (kD)

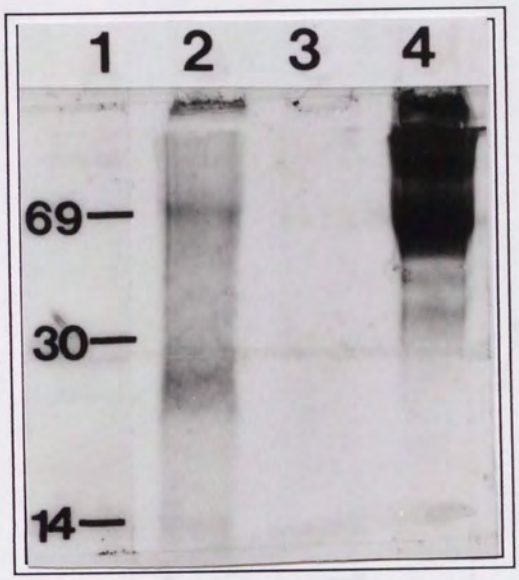
2: Immunoreactive material eluting at 90% acetonitrile

3: Immunoreactive material eluting at 57.5-62.% acetonitrile

(From: Todorov *et al*, 1996a)

Figure 2.2.4.2.5.

Detection of sugars in glycoconjugates in affinity purified MAC 16 tumour extract by digoxigenin glycan detection



1: Molecular weight markers

2: Affinity - purified immunoreactive fractions

3: Non-glycosylated control protein (creatinase)

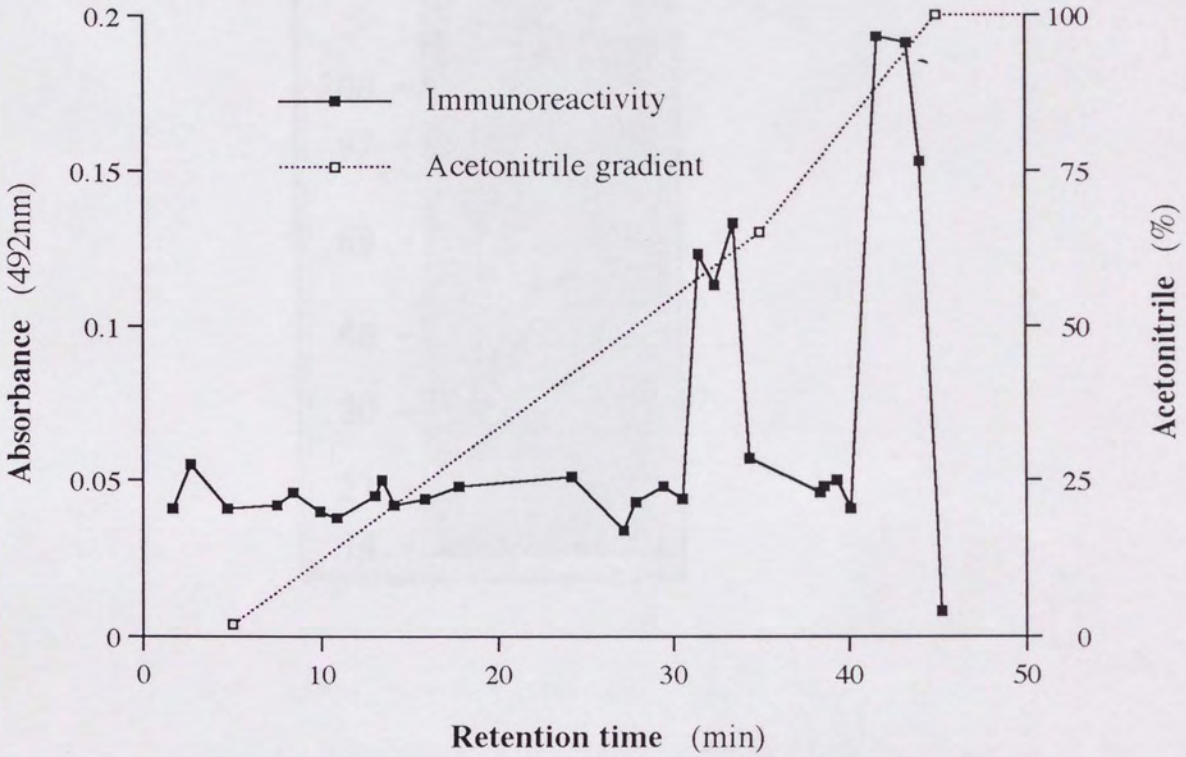
4: Control glycosylated protein (transferrin)

(From: Todorov *et al*, 1996a)

Figure 2.2.4.2.6.

Reverse-phase HPLC of affinity-purified MAC 16 tumour extract

Analysis of peaks detected at 214nm by ELISA using MAC 16 monoclonal antibody



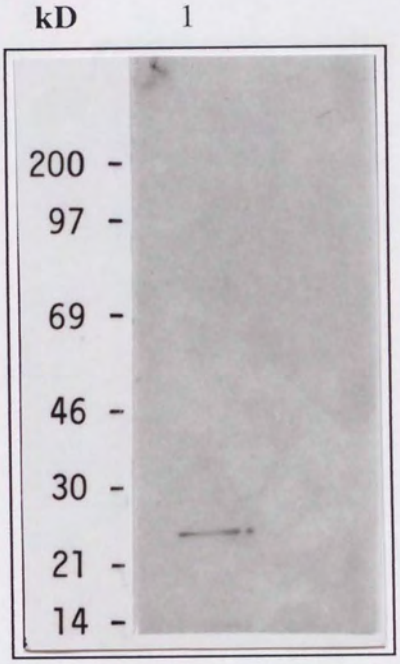
Sample parameters prior to loading :

Volume: 50μl
Tota protein: 50μg

Figure 2.2.4.2.7.

Analysis of the fraction eluting at 57.5-62% acetonitrile by SDS-PAGE and silver stain:

Fractions from eight HPLC runs pooled



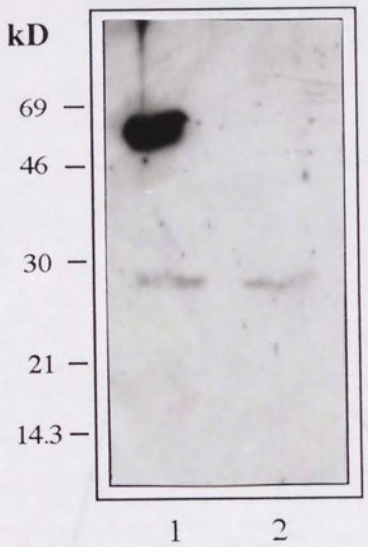
1: Immunoreactive fraction eluting at 57.5-62% acetonitrile

(From: Todorov *et al,* 1996a)

Figure 2.2.4.2.8.

Effect of trypsin on the size and immunoreactivity of affinity-purified MAC 16 tumour extract

Analysis by Western blot using MAC 16 monoclonal antibody



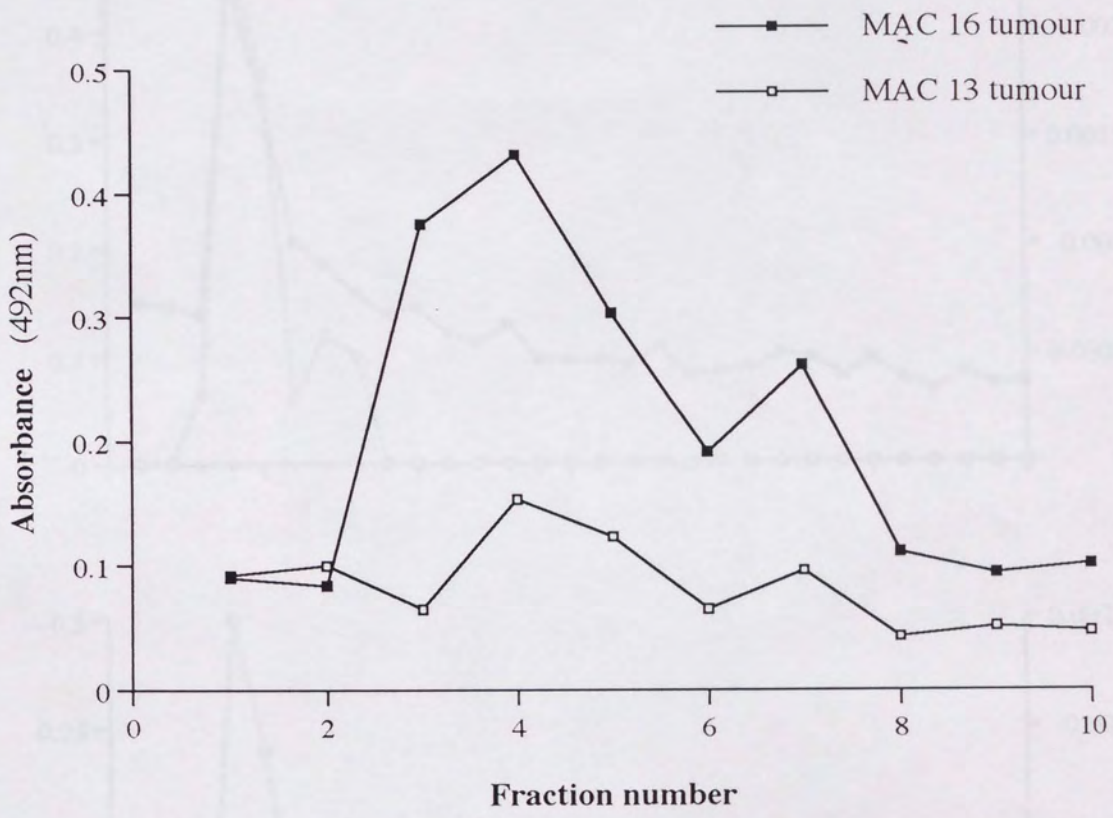
1: Pooled immunoreactive fractions (5μg)

2: Pooled immunoreactive fractions (5μg) + 1μg trypsin (12h, room temperature)

Figure 2.2.4.2.9.

Affinity purification of MAC 16 and MAC 13 tumour extracts after ammonium sulphate (38%) precipitation:

Analysis by ELISA using MAC 16 monoclonal antibody



Sample parameters prior to loading:

Volume: 2.0ml
Total protein: 70mg

Figure 2.2.4.2.10.

Affinity purification of serum (50mg) from MAC 16 and MAC 13 tumour-bearing NMRI mice:

Analysis by ELISA using MAC 16 monoclonal antibody

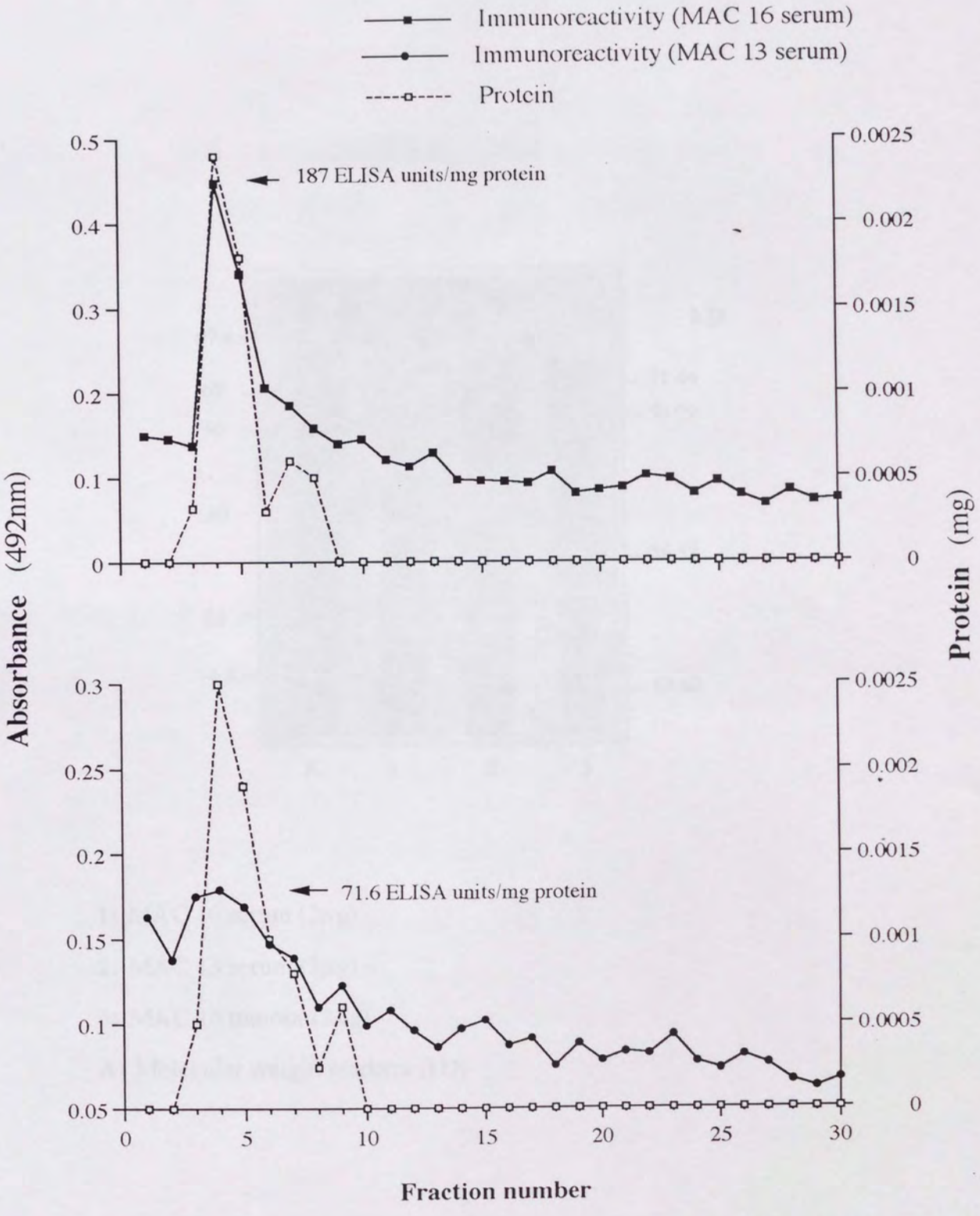
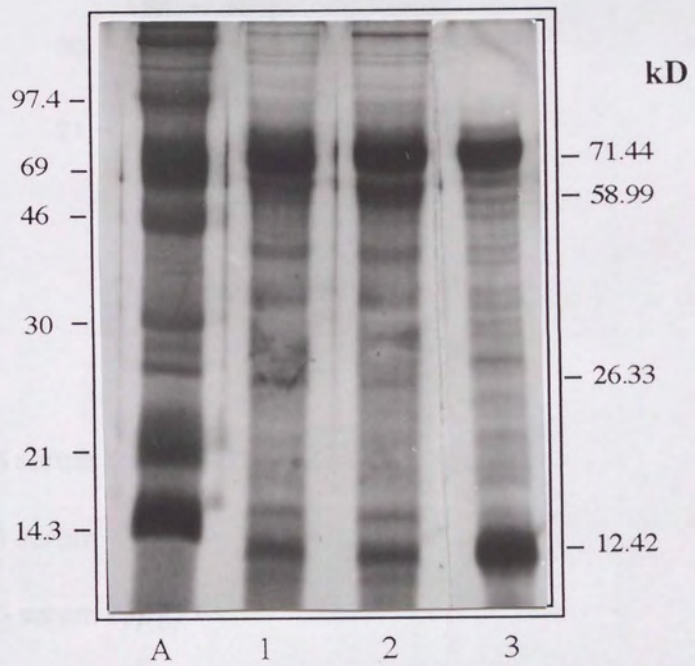


Figure 2.2.4.2.11.

Affinity purification of serum from MAC 16 and MAC 13 tumour-bearing mice:

Analysis by SDS-PAGE and visualisation by silver stain



1: MAC 16 serum (2μg)

2: MAC 13 serum (2μg)

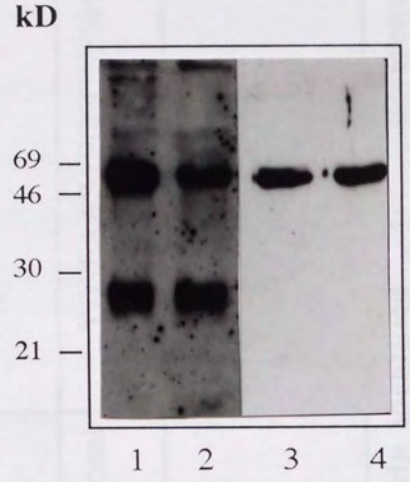
3: MAC 16 tumour (2μg)

A: Molecular weight markers (kD)

Figure 2.2.4.2.12.

Affinity purification of serum (50mg)from MAC 16 and MAC 13 tumour-bearing mice:

Analysis by Western blot using MAC 16 monoclonal antibody



1: MAC 16 serum (5μg) *

2: MAC 13 serum (5μg) *

3: MAC 16 serum (5μg) **

4: MAC 13 serum (5μg) **

* Detection by monoclonal antibody and protein A

** Detection by protein A

Table 2.2.4.2.2

Sequence analysis of immunoreactive material from the MAC 16 tumour extract

Elution (% acetonitrile)	**Elution time (min)**	**N-terminal sequence (major)**	**N-terminal sequence (minor)**
57.5-62	31.5-33.5	YDPEAASAPGSGNPSHEA	-
90	42	Mouse serum albumin (Coles, personal communication)	YDPEAASAPGSGNPSHEA

Table 2.2.4.2.2.1. **Database search for similar sequences:**

N-terminal sequence	**Database sequence identity**	**Protein identified (Accession number)**	**Reference**
YDPEAASAPGSGNPSHEA	YDPEAASAPGDGD (Pir 3)	Streptococcal preabsorbing antigen (PA-Ag) (A46463)	Yoshizawa *et al* (1992)

2.3. DISCUSSION

2.3.1. Purification and partial characterisation of lipolytic material from the MAC 16 tumour

2.3.1.1. Structural analysis

Lipolytic material was purified 207-fold from the cachexia-inducing MAC 16 tumour by anion-exchange batch-extraction, FPLC anion-exchange and gel-exclusion chromatography. The resultant non-homogeneous mix had a specific activity of 2.57 μmol glycerol/10^5 adipocytes/2h/mg protein representing an increase of approximately 207-fold from the crude tumour extract. Further purification was obtained by reverse-phase HPLC fractionation but due to the non-reproducibility of the yield of biological activity at this stage, various characterisation experiments were performed on material that was purified 207-fold.

The acidic nature of the lipolytic material reported previously (Beck *et al*, 1990) was confirmed by results obtained during this study. Thus the material was retained by anion-exchange matrices and was found to have an isoelectric point pI: 4.5-4.7. These weakly acidic properties may reflect the presence of a greater abundance of amino acid residues aspartic and glutamic acid as opposed to basic residues lysine and arginine in the amino acid composition of the material. In addition, the presence of sulphate groups as revealed by the observed dose-dependent inhibition of biological activity by sulphatase (McDevitt *et al*, 1995), may also confer a net negative charge.

Lipolytic activity purified from the MAC 16 tumour was previously attributed to three species with apparent molecular mass <3kD (Beck *et al*, 1990). Material with a low apparent molecular mass was also observed during the present study when it appeared to be separable from the larger molecular weight species on the basis of its more acidic

nature (pI: 4.0). The properties of this material are unknown. However, the possibility that it may be a product of proteolytic cleavage due to the absence of protease inhibitors during all purification steps in the former study and during isoelectric point determination in the present study cannot be ruled out. Production of small molecular weight material by chemical cleavage may also be a possibility, as active fractions were routinely concentrated by vacuum dialysis prior to the gel-exclusion step during previous purification procedures. The occurrence of proteolytic and/or chemical cleavage is further supported by the appearance of species' with apparent molecular mass <3kD when lipolytic activity associated with proteins eluting with the void volume of the gel-exclusion column was concentrated and re-chromatographed (Beck, 1989). During initial attempts to adapt the purification scheme for FPLC, MAC 16 tumour extract was found to contain small molecular weight material which interacted with reagents in the bioassay in the absence of fat cells to give 340nm absorbance readings that were in excess of ten-fold greater than those expected for true lipolytic activity. Prior to the employment of ultrafiltration through a 10K cut-off membrane, it was found that this material was a persistent contaminant during subsequent purification steps thus, further complicating interpretation of results from earlier studies.

Lipolytic activity purified 207-fold from the MAC 16 tumour during the present study was attributable to material with apparent molecular mass between 9.64 and 47kD, as determined by Superose gel-exclusion in buffer containing 0.3M sodium chloride. The bulk of activity was associated with material eluting in fractions 15 and 16, with a mean apparent molecular mass of approximately 22kD. Analysis by SDS-PAGE revealed the presence of four major proteins in this mix with apparent molecular masses 29, 24, 20 and 13kD, although it was not clear which of these was associated with lipolytic activity. Further analysis revealed that biological activity and size were unaffected by incubation with trypsin suggesting an absence of amino acid sequences Arg-X and Lys-X, or that these were inaccessible due to the tertiary structure of the material or the presence of carbohydrate side chains. Lipolytic material was found to be heat-labile as revealed by

complete loss of biological activity after incubation at 60°C for 20min (McDevitt *et al*, 1995), thus maintenance of tertiary structure may be important for optimum bioactivity. This was confirmed by additional observations including loss of activity after repeated freeze-thaw cycles (results not shown). The presence of cysteine residues in the amino acid composition of the material was implicated by an increase in biological activity observed following the addition of dithiothrietol (results not shown), and its susceptibility to oxidation, as evidenced by complete loss of activity after treatment with periodate. Thus, the formation of disulphide bridges may cause an alteration in conformation with a concomitant inhibition of bioactivity. Lipolytic activity did not appear to be dependent on phosphorylation of amino acid residues, as revealed by the absence of inhibition after incubation with acid phosphatase. Incubation with alkaline phosphatase produced inconclusive results with respect to phosphorylation, though the susceptibility of biological activity to inhibition by alkali was revealed by this procedure and hence the possibility that base-labile glycosidic linkages may be important. However, glycosylation did not appear to be necessary for bioactivity as specific removal of potential N-linked glycan chains by endoglycosidase F, O-linked glycan chains by O-glycanase, and removal of terminal sialic residues by neuraminidase had no substantial effect on biological activity (McDevitt *et al*, 1995). It is often necessary to remove peripheral sialic acid residues on the O-glycan core disaccharide prior to cleavage of O-glycosidic linkages (Gerard, 1990) hence, it is possible that loss of activity may have been observed following incubation with neuraminidase prior to O-glycanase.

The gel-exclusion elution profile for the lipolytic material and its subsequent apparent molecular mass appeared to depend on the ionic properties of the mobile phase. Thus, in buffer with low ionic strength a tendency to aggregate or interact with other proteins was observed resulting in an increase in apparent size. The inclusion of sodium chloride to a final concentration of 0.3M in the running buffer resulted in disaggregation or disassociation from other proteins perhaps due to displacement by chloride ion in a similar manner to that observed during anion-exchange.

Lipolytic material which was apparently identical with respect to purification properties and molecular mass, was purifiable from the non-cachexigenic MAC 13 tumour. However absence of this material from the circulation of tumour-bearing mice suggests that it may differ from that produced by the MAC 16 tumour in terms of structural modification for secretion. Thus, MAC 13 lipolytic material may not undergo various post-translation events such as glycosylation to enable it to be secreted from the cell. Further analysis of the amino acid composition and carbohydrate moieties of material from both sources is required to examine this possibility.

2.3.1.2. Comparison with other tumour-derived lipolytic factors isolated to date

The material purified from the MAC 16 tumour appeared to share some similarities with other lipolytic factors purified to date by three other groups, as summarised in table 2.3.1.2.1. Thus, Kitada and co-workers demonstrated that a component in the tumour tissue and serum of thymic lymphoma-bearing cachectic AKR mice, and in the supernatant of tumour tissue culture cells responsible for inducing extensive lipid mobilisation (Kitada *et al*, 1980), was a small acid-stable protein of apparent molecular mass <10kD, as determined by gel-exclusion chromatography (Kitada *et al*, 1981). Aggregation, and subsequent 100-fold increase in bioactivity was observed after standing for several days at 4°C. This was demonstrated by gel-exclusion chromatography in buffer with a low ionic strength and resultant elution of lipolytic activity in the column void volume (Kitada *et al*, 1982). Inclusion of dithiothrietol in the purification buffers was also found to increase biological activity, thus suggesting that the conformation conferred by reduction of disulphide residues resulted in optimal interaction between the material and its receptor. In contrast to MAC 16 lipolytic material, the aggregated form of the factor isolated by Kitada and co-workers was found to be relatively heat-stable and trypsin-sensitive, suggesting that variations in the composition of amino acids or subsituted side chains between the two may exist.

Table 2.3.1.2.1. Comparison of the structural properties of lipolytic factors isolated to date

Factor characteristics	Kitada *et al* (1981&1982)	Masuno *et al* (1981&1984)	Taylor *et al* (1992)	McDevitt *et al* (1995)
Source	Mouse thymic lymphoma	Ascites fluid: Mouse sarcoma 180 Human hepatoma Human ovarian carcinoma	Human A375 melanoma cells (culture supernatant)	Mouse adenocarcinoma of the colon (MAC 16)
Size (Method of assessment)	5 - 10kD (Gel-exclusion: 10mM phosphate buffer, pH7.2)	65.2 - 75 kD (Gel-exclusion: 50mM phosphate buffer, pH 8.0; SDS-PAGE; Sedimentation analysis)	6kD (HPLC-based gel-exclusion: PBS)	16 - 28kD (FPLC superose gel-exclusion: 50mM phosphate buffer + 0.3M NaCl)
Charge properties	-	pI: 4.7	Negatively charged	pI:4.5-4.7
Stability (% original activity)				
Heat	75% (80°C, 5min)	0% (100°C, 10min)	100% (65°C, 10min)	0% (60°C, 20min)
Acid	100% (pH 5.0)	-	pH-sensitive, (no further information)	100% (pH 4.5) 0% (pH 2.0)
Alkali	-	-		0% (pH 10.4)
Oxidation	Increased activity with DTT	-	-	Stabilisation with 1mM DTT
Glycosylation status (% original acitivity with 10mM periodate)	-	-	96%	0%
Trypsin sensitivity (% original activity)	0% (0.05μg/μg)	30% (0.01μg/μg, 37°C, 2h)	91% (10μg/μg, 37°C, 2h)	100% (0.2μg/μg, 37°C,2h)

A lipolytic factor has been purified to homogeneity from the ascites fluid of sarcoma-180 tumour-bearing mice in which tumour growth was associated with a reduction in total body triglycerides and a concomitant elevation in plasma free fatty acids (Masuno *et al,* 1981). Charge properties of this material were comparable to those observed for the MAC 16 factor, as observed by the similar concentration of sodium chloride required for elution from anion-exchange matrix and the similar isoelectric focussing values obtained for both. Material isolated by Masuno and co-workers (1981) appeared to be associated with a heat-labile protein with apparent molecular mass 75kD as determined by gel-exclusion chromatography with low ionic strength mobile phase. Further analysis by disc polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol, and analytical ultrafiltration for the determination of sedimentation equilibrium, revealed a homogeneous protein with apparent molecular mass 70kD - 75kD and 65.2kD respectively, to which the authors assigned the name Toxohormone-L. Material with similar properties was also purified from ascites fluid obtained from patients with hepatoma and ovarian carcinoma. Trypsin-incubation resulted in the production of a dialysable (<10kD) trypsin-resistant species with 30% of the bioactivity exhibited by the intact molecule.

A factor responsible for inducing massive lipid mobilisation in nude mice inoculated with human malignant melanoma cells (A-375) was shown during preliminary investigations to be associated with material of apparent molecular mass <50kD which was heat and pH-sensitive (Hollander *et al*, 1986). This material was subsequently isolated from tissue culture medium by a series of size-exclusion chromatography procedures, which resulted in the purification to apparent homogeneity of a lipolytic factor with molecular mass approximately 6kD, as determined by its resolution as a single peak by HPLC-based molecular sieving (Taylor *et al*, 1992). Further analysis confirmed the proteinaceous nature of the factor and that it was heat-stable, protease-resistant and either non-glycosylated or carbohydrate groups if present, were not required for biological

activity. In addition, the factor was reported to be 'highly charged' and 'rich in glutamic acid' though no further information was reported.

2.3.1.3. Mechanism of action of tumour-derived lipolytic factors

Material with lipolytic activity may exert its effects by direct hydrolysis of triglycerides in a manner comparable to that observed by lipoprotein lipase (LPL); alternatively, lipolysis may occur via the induction of intracellular lipase activity through interaction with receptors on the adipocyte cell surface as is observed during catecholamine-induced lipolysis.

Lipase activity was not exhibited by the factor isolated by Kitada and co-workers as determined by measurement of glycerol release during incubation of the material with triolein. Toxohormone-L was also shown to have no hydrolytic effect on neutral fat (Masuno *et al*, 1981; results not shown). In addition, the 6kD lipolytic factor also showed a lack of lipase activity as determined by detection of fatty acids released from free triglycerides (Taylor *et al,* 1992). A four-fold increase in glycerol release was observed however, when material isolated from A375 cell culture medium was incubated with cultured adipocytes. In contrast, no lipolysis was induced by 'lipolytic material' purified by the same methods from normal fibroblast cells. Further experiments by these authors revealed a rapid increase in intracellular lipase activity of adipocytes cultured in the presence of 10μg/ml of purified factor. Maximum activity was observed 8h after the addition of material thus, the authors conclude that lipolysis caused by the 6kD factor must be manifested through an induction in the activity of the intracellular adipocyte lipase, either due to elevated transcription or by increased activation of a pre-synthesised inactive enzyme pool. Lipolytic material isolated from the MAC 16 tumour may also exert its lipid-catabolic effects by induction of the hormone-sensitive lipase. Thus, no triglyceride lipase activity could be measured in MAC 16 lipolytic material as determined by measurement of the release of [^{3}H] oleic acid from [^{3}H] triolein after a 2h incubation

with tumour homogenate (McDevitt *et al*, 1995). Additionally, incubation of isolated adipocytes with partially purified material was observed to cause an elevation of cyclic AMP (cAMP) within 10min that was comparable in magnitude to that induced by 0.165mM of the β-adrenergic agonist salbutamol, and which paralleled the stimulated glycerol release (Tisdale and Beck, 1991). However, unlike the stimulation of cAMP production induced by salbutamol which reached a maximum 1h after exposure and returned to basal levels thereafter, maximum stimulation of cAMP in response to the lipolytic factor was still apparent 2h after exposure; a phenomenon also observed after exposure of intestinal cells to cholera toxin (Sharp and Hynie, 1971). More recently, stimulation of adenylate cyclase has been observed following exposure of isolated adipocyte plasma membranes to partially purified material (Khan *et al*, unpublished results). Lipolysis may therefore be induced by a cascade of events involving activation of adenylate cyclase, elevation of cAMP, activation of a protein kinase and subsequent activation of triglyceride lipase by reversible phosphorylation of serine residue 563. Thus, the biological effect of the MAC 16 factor may be mediated by events analogous to those involved in the rapid stimulation of lipolysis by glucagon and adrenaline. This would be in contrast to the slower induction of lipolysis induced by growth hormone by a mechanism involving adenylate cyclase-independent stimulation of hormone-sensitive lipase; or the induction of lipolysis by dexamethasone involving transcriptional up-regulation of the lipase (Slavin *et al*, 1994). Experiments are currently underway to elucidate the exact molecular mechanism of action of the MAC 16 lipolytic material and in particular its possible interaction with guanine nucleotide-binding proteins (G-proteins) involved in signal transduction across the adipocyte plasma membrane.

2.3.1.4. In vivo effects of tumour-derived lipolytic factors

A toxohormone is defined as a toxic substance produced by cancer cells which causes pathological conditions in normal animals that are similar to those of the tumour-bearing animal (Masuno *et al*, 1981). Thus, in order to determine the importance of a

potential cachectic factor the *in vivo* effects of the material need to be determined. Injection of high molecular weight lipolytic material isolated by Kitada and co-workers (1982) into healthy AKR mice resulted in a 100-fold increase in lipid mobilisation within 2h, as determined by the measurement of [$^{14}CO_2$] from [^{14}C]-labelled adipose tissue transplanted into the peritoneal cavity. However, no data on the effect of this material on total body weight is presented.

Toxohormone-L isolated from ascites fluid of patients with hepatomas was found to be anorexigenic, as determined by injection of the material into the lateral ventricle of healthy adult male rats (Masuno *et al*, 1984). Food and water intake were significantly suppressed for up to three days after treatment, due to a reduced rate of ingestion. A latent period of 5h was observed prior to the anorexic effect and the authors speculate that this may be due to the slow release of a biologically active fragment from the 65.2 - 75kD material. More recently, infusion of toxohormone-L into the third cerebroventricle of healthy rats resulted in a dose-related reduction in food and water intake that was shown to be due to early satiety, as the postprandial inter-meal interval and eating speed were unchanged (Okabe *et al*, 1992). Treated animals in both studies showed no symptoms of toxicity. However, the capacity of the anorexigenic effects of the material to induce the development of 'cachectic' parameters such as total body weight loss, loss of adipose tissue and concomitant elevation in plasma free fatty acids is unknown, as long term administration experiments were not performed in either study. Furthermore, the ability of the material to induce cachexia by systemic administration was not examined.

Administration of the 6kD lipolytic material to BALB/C nude mice by i.v. injection of 50μg at time points 0 and 8h, resulted in loss of total body weight of 3.4% by 24h, with no change in food and water intake (Taylor *et al*, 1992). Adipose tissue was depleted by 85% and serum triglycerides were increased by 43%. Plasma free fatty acids were not measured in this study, but the observed hypertriglyceridaemia caused by administration of this factor suggests a possible inhibitory effect on membrane-bound

LPL in addition to the demonstrated up-regulatory effect on intracellular lipase. Alternatively, the elevation in serum triglycerides could be due to increased hepatic production due to esterification of fatty acids released from the adipose tissue following lipolysis.

Preliminary experiments to investigate the *in vivo* effects of the lipolytic factor isolated from the MAC 16 tumour suggest it may induce the cachectic condition in mice transplanted with the non-cachexigenic MAC 13 tumour (Beck *et al*, 1990) and non tumour-bearing mice (Cariuk *et al*, unpublished results). However, interpretation of these results is complicated by the presence of antigenic 24kD material which was observed to co-purify with the lipolytic factor and was only separable by affinity chromatography (section 2.2.4).

2.3.1.5. Comparison with tumour necrosis factor-α (TNF)

As discussed in sections 1.4.2.1. - 1.4.2.7., cytokines TNFα (TNF), IL-6, IL-1, TGFβ (TGF) and LIF have all been strongly implicated in the development of cancer cachexia, especially with reference to the aberrations in lipid metabolism observed. They are believed to contribute to total body lipid catabolism by their inhibitory action on LPL, thereby preventing the hydrolysis of circulating triglycerides and thus, uptake of non-esterified fatty acids for adipose tissue storage. TNF is the most potent of the cytokines with respect to inhibition of adipose LPL activity thus, a 50ng/ml concentration was observed to reduce enzyme activity in cultured adipocytes by 96% (Greenberg *et al*, 1992). Reduced LPL activity has been associated with malignancy and depletion of lipid stores in human and animal studies. A significant decrease in total heparin-releasable enzyme activity was observed in patients with lung and breast cancer during one study (Vlassara *et al*, 1986). Hepatic LPL and peripheral activities were equally inhibited and the amount of inhibition was correlated to the degree of weight loss rather than to the extent of disease. In addition, a decrease in LPL activity was found to be the first

measurable change in lipid metabolism following innoculation, of mammary carcinoma-bearing rats (Lanza-Jacoby *et al*, 1984). This was followed sequentially by an increase in serum triglycerides, elevation of plasma free fatty acids and reduction in epididymal tissue mass. TNF has been shown to exert its LPL-inhibitory effects primarily through a down-regulation of transcription (Zechner *et al*, 1988; Hauner *et al*, 1995). However, no change in LPL or fatty acid synthase mRNA was detected in cancer patients in which a two-fold increase in circulating triglycerides and free fatty acids were observed (Thompson *et al*, 1993). Increased lipid mobilisation was instead associated with an up-regulation of hormone sensitive lipase transcription, suggesting that fat catabolism in this case may be entirely due to increased lipolysis.

Disruption of lipid metabolism through inhibition of LPL alone is unlikely to induce the massive depletion in total body fat that is a manifestation of the cachectic process. Thus, Type 1 hyperlipidaemia which is characterised by elevated serum chylomicron levels, is caused by an inherited deficiency of LPL; yet patients are not cachectic and have normal fat stores. In contrast to the other cytokines implicated in the pathogenesis of cancer cachexia, TNF has been shown by some studies to have lipolytic properties in addition to those of suppression of LPL activity. However, lipolytic activity has only been observed following long term exposure of cultured adipocytes to the cytokine. Thus, murine TNF at a concentration of 100ng/ml stimulated a two-fold increase in lipolysis of 3T3-L1 cells after 16h exposure (Marshall *et al*, 1994), and a 400-fold increase in glycerol release was observed in cultured human adipocytes incubated with 5nmol/l recombinant human TNF for 6h (Hauner *et al*, 1995). When experimental conditions were the same as those required for the induction of glycerol release by the lipolytic factors however, TNF did not induce lipolysis. Thus, exposure of isolated adipocytes to recombinant human TNF or lipolytic factor for 2h, induced glycerol release only in the latter case, by 100-fold (Taylor *et al*, 1992). Additionally, lipolysis was not induced by incubation of isolated adipocytes for 2h with $4x10^3$-$4x10^5$ units recombinant human TNF; while under the same conditions, a 150-fold increase in free fatty acid

release was induced by MAC 16 tumour extract (Mahoney *et al*, 1988). Hence, TNF may increase activation of intracellular lipase by a different mechanism; perhaps by a process comparable to that employed by growth hormone which also induces lipolysis only after exposure for several hours (Goren *et al*, 1990).

2.3.1.6. Concluding remarks

The lipolytic factors isolated to date appear to satisfy several criteria for consideration as novel cachectic factors. They were isolated from tumours which cause extensive lipid mobilisation in the host, this effect was reproducible with the purified material *in vitro* and, with the exception of Toxohormone-L, *in vivo*. Additionally, preliminary observations suggest that they share few similarities with TNF which to date is the cytokine most frequently cited as causing the aberrations in fat metabolism associated with the cachectic condition.

The four lipolytic factors thus discussed, share several similar characteristics. It is possible that the divergence of some of their properties may be attributed to the different methods of purification; equally, minor differences in amino acid composition could alter post-translational modifications and thus effect the physical and biological properties of the final molecule. Whether they are ultimately shown to be related depends on further analysis of their structures by isolation of their respective genes. However, no further information has been published to date for the factors isolated by Kitada *et al*, Masuno *et al*, and Taylor *et al*. Indeed, Masuno and co-workers subsequently demonstrated that lipolytic activity detected in various human sarcomas and carcinomas was attributable to LPL as determined by hydrolysis of [^{3}H] triolein, inhibition by anti-LPL IgG and detection of 57kD human adipose tissue LPL by western blot (Sakayama *et al*, 1994).

2.3.2. Purification of lipolytic material to homogeneity

2.3.2.1. Structural comparison to Zn-α-2-glycoprotein

Lipolytic activity purified to homogeneity from human and murine tissue sources was found to be associated with a protein which had identity to Zn-α-2-glycoprotein, as determined by SDS-PAGE, non-denaturing PAGE and amino acid sequence analysis. Zn-α-2-glycoprotein has been isolated from the blood plasma (Ohkubo *et al*, 1988) and seminal plasma (Ohkubo *et al*, 1990) of healthy human adults. Protein analysis of the material from both sources revealed identity in 17 N-terminal amino acids and subsequent analysis of the cDNA from liver and prostate revealed both to be identical (Ueyama *et al*, 1991). The proteins differed however, with respect to post-translational modification. Thus, Zn-α-2-glycoprotein from seminal fluid was found to be unglycosylated and lacked an N-terminal pyroglutamic acid residue, properties that were reflected in its lower apparent molecular mass (41 and 42kD for material from blood plasma, 40 and 41kD for material from seminal plasma). Post-translational modification of the circulatory protein probably served to protect the material against N-terminal cleavage with aminopeptidases thus increasing its stability.

Zn-α-2-glycoprotein was thus named on the basis of early observations of its electrophoretic mobility in the α2 region and because it was precipitable by Zn ions (Burgi and Schmid, 1961). Another property of this material is its apparent polymorphism as observed during electrophoretic analysis (Schmid and Takahashi, 1964), and also encountered during our own investigations. Elucidation of the entire protein sequence, as deduced from the cDNA sequence for plasma-derived material, revealed a single protein with apparent molecular mass 38478D and failed to reveal polymorphism in the composition of the 276 amino acids (Araki *et al*, 1988). It has been suggested therefore, that the property may be due to partial sulphation of carbohydrate moieties or that Zn-α-2-glycoprotein may be the product of a small set of highly

homologous genes. The former possibility was further supported by our observations which demonstrated a dose-related loss of biological activity from the lipolytic material following removal of sulphate residues by sulphatase.

In agreement with observations made during analysis of the properties of the lipolytic material, Zn-α-2-glycoprotein has been shown to be a weakly acidic protein. This was determined by its isoelectric focussing properties (pI: 4.7-4.8) and amino acid composition analysis which revealed a predominance of glutamic acid and aspartic acid (Ohkubo *et al*, 1988). The plasma-derived protein is N-glycosylated at three of the four potential asparagine sites, giving rise to a total 18% glycosylation. This property would confer resistance to proteases and may explain the observed resistance of the lipolytic material to trypsin digestion. Thus, Zn-α-2-glycoprotein was only digestible by proteolytic enzymes after desialisation with neuraminidase and reduction and alkylation with iodoacetic acid (Araki *et al*, 1988). Further analysis revealed that residues Asn^{92}, Asn^{108} and Asn^{239} were glycosylated (Ueyama *et al*, 1994), and the carbohydrate moieties comprised N-acetylneuraminic acid, galactose, mannose, fucose and N-acetylglucosamine (Araki *et al*, 1988). There was no evidence of any O-linked glycans. Treatment of lipolytic material to remove N-linked glycan chains and terminal sialyl residues had no effect on the biological activity; however, the presence of carbohydrate moieties was confirmed during recent analyses by digoxigenin glycan detection (Boehringer Mannheim, East Sussex, U.K.) (Todorov *et al*, unpublished results). This suggests that post-translational modification of lipolytic material by glycosylation was not a prerequisite for bioactivity and may instead serve to increase its stability in the circulation. It is possible that material produced by the MAC 13 tumour lacks these properties; which may explain the apparent absence of circulatory lipolytic material detected in MAC 13 tumour-bearing mice, and hence the failure of this tumour to induce cachexia.

Amino acid sequence determination of Zn-α-2-glycoprotein revealed the presence of four cysteine residues and the formation of disulphide bonds between residues 101& 164 and 203&258, comprising 64 and 54 residues respectively. The contribution of disulphide bonds to the secondary structure of the lipolytic factor was also suggested by observations reported in this study. Thus, the material appeared to be susceptible to oxidation as was evidenced by complete loss of biological activity following treatment with periodate; in addition, activity was stabilised by sulphydryl groups.

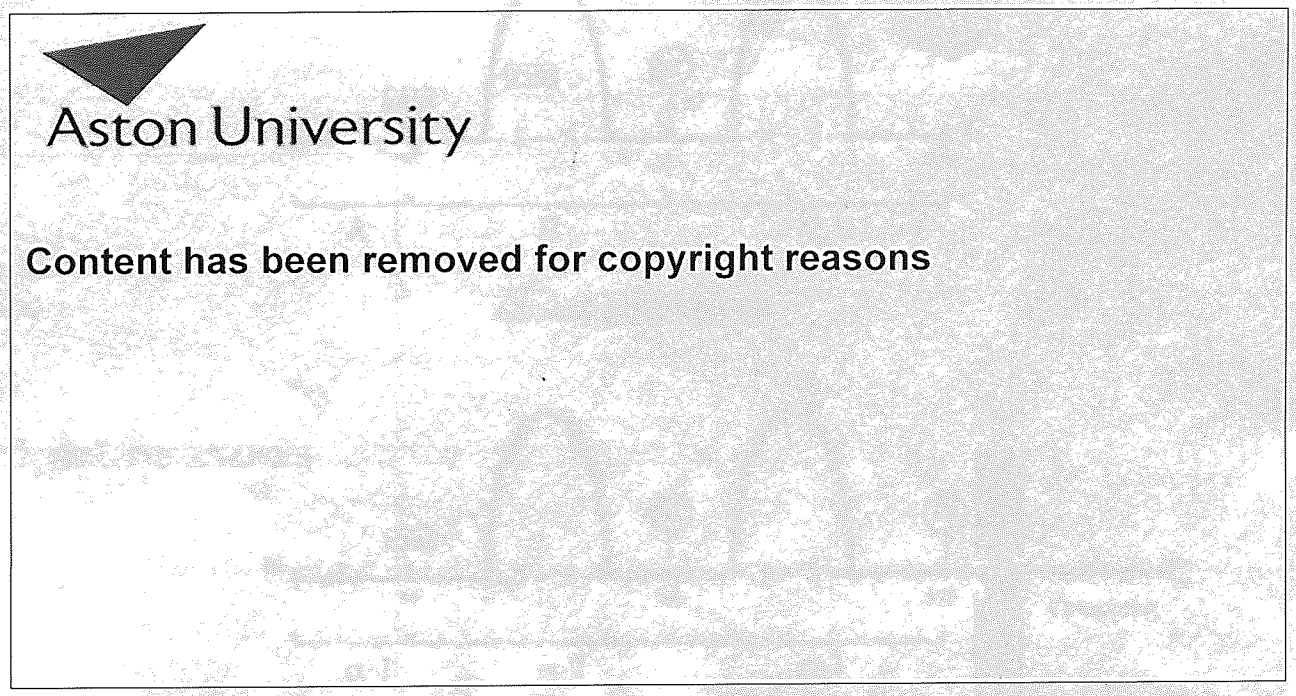
The structure of Zn-α-2-glycoprotein has been further elucidated by the isolation of its cDNA from human liver and prostate (Ueyama *et al*, 1991) which were found to code for 278 and 277 amino acids respectively. Subsequent isolation of cDNA from rat and mouse liver libraries and comparison of the deduced amino acid sequences with that for human revealed that the rodent proteins were larger (279 and 290 amino acids respectively) (Ueyama *et al*, 1994). Approximately 73% homology between human and rodent sequences was observed and all four cysteine residues were found to be conserved. An absence of conservation with respect to glycosylation was apparent however, by the lack of residue Asn^{92} in the rodent protein.

2.3.2.2. Comparison of Zn-α-2-glycoprotein to the MHC antigens

Zn-α-2-glycoprotein was found to share significant homology with the α-chains of HLA (human), H-2 (mouse) and RLA (rat) class 1 major histocompatibility (MHC) antigens with respect to amino acid composition and domain structure (Araki *et al*, 1988). Homology was also detected with the class 11 antigens but this was less significant. Identities of 36-38% for overlaps of 272-279 amino acids were apparent with the class 1 antigens, with a similarity score of 188-141, whereby a score of 80 corresponds to a z-value of 10 which represents statistical significance. The domain structure was found to be very similar to the extracellular domain of the MHC class 1 α-chains, which comprises three domains: α-1, α-2, α-3, each with approximately 90 residues, and each being

Figure 2.3.2.2.1.

Schematic diagram of the domain structures of Zn-α-2-glycoprotein and HMC Class 1 a-chain



Plasma membrane

MHC Class 1 α-chain

AC, acid-cleavage site; Pa, papain-cleavage site; CHO, carbohydrate. The highly homologous region between the two proteins are indicated by bold lines (From Araki *et al*, 1988)

precisely coded for by one exon (figure 2.3.2.2.1). The main differences between this and the composition of the domain for Zn-α-2-glycoprotein was found to be an absence of Asn^{106} and Asn^{237} in the MHC antigen. In addition, glycosylation of residue Asn^{86} is highly conserved in HLA but its counterpart in Zn-α-2-glycoprotein is unglycosylated, probably because it is followed by aspartic acid, which may adversely effect the tripeptide acceptor sequence. Absence of the protease-cleavable transmembrane and cytoplasmic domains of the HLA antigens has led to the suggestion that Zn-α-2-glycoprotein may represent a soluble product that is derived by cleavage with circulatory proteases from a precursor (Araki *et al*, 1988). However, further analysis following isolation of the cDNA revealed the presence of an in-phase stop codon immediately adjacent to the C-terminal $serine^{276}$ residue, thus confirming that Zn-α-2-glycoprotein could not be formed by a protease-mediated mechanism (Freije *et al*, 1991).

The similarities in amino acid composition and domain structure observed between the MHC class 1 antigens and Zn-α-2-glycoprotein has also led to the speculation that the latter protein was possibly a member of the immunoglobulin superfamily and was perhaps descended from the ancestral gene of the immunoglobulin gene family that codes for 90-100 amino acids. However subsequent analyses revealed that Zn-α-2-glycoprotein was coded for by a single gene, with one exon for each domain, and was located on chromosome 7 and not 6 where the genes for MHC reside (Ueyama *et al*, 1991 & 1993).

2.3.2.3. Physiological role of Zn-α-2-glycoprotein

The physiological role of Zn-α-2-glycoprotein is currently unknown. The material has been purified from blood and seminal plasma and has been shown to be present in cerebrospinal fluid, saliva, breast milk, urine, saliva and sweat (Ueyama *et al*, 1991 and references therein). It has also been detected in epithelial cells of prostate, pancreas, exocrine and endocrine sweat glands, and salivary, tracheal, bronchial and oesophageal glands. Its homology to the MHC class 1 antigens has led to speculation that

it may have a role in the expression of the immune response, perhaps as a soluble HLA-like antigen (Freije *et al*, 1991). Thus, it may be similar in function to the truncated transplantation-like antigen identified in mouse serum, which is secreted as the soluble product of a class 1 (H-2) related gene expressed only in liver (Kress *et al*, 1983). This latter protein is thought to be involved in the mediation of active immunological tolerance.

Analysis by *in situ* hybridisation revealed that Zn-α-2-glycoprotein gene expression was localised to specific regions of the rat stomach (Ueyama *et al*, 1994). Thus, mRNA was detected in the isthmus zone where cell multiplication takes place, and in the neck zone in mucous neck cells which are precursors of chief cells. It was suggested therefore, that the protein may play a role in cell proliferation and differentiation respectively in these two areas.

More recently, a role in cell adhesion has been proposed (Takagaki *et al*, 1994). Zn-α-2-glycoprotein contains the amino acid sequence Arg-Gly-Asp-Val (RGDV) which is specifically associated with cell adhesion between cells and extracellular matrices. This sequence is also present in other adhesive proteins such as collagen IV, fibrinogen and fibronectin. In addition, other cell binding regions within Zn-α-2-glycoprotein were identified namely, Arg^{73}-Glu-Asp-Ile^{76} and Ile^{171}-Leu-Asp-Arg^{180} which are also found in fibronectin. The effect of Zn-α-2-glycoprotein on the attachment and spreading characteristics of nine cell lines was therefore examined by these authors, and it was shown that the protein induced these characteristics on cell-lines derived from the kidney. Renal carcinoma cells were induced most strongly, the response was dose-related up to 10μg/ml and spread efficiency was comparable to that observed for fibrinogen and collagen IV. This property was induced by Zn-α-2-glycoprotein from both blood seminal plasma sources and was therefore unaffected by post-translational modification. It may be mediated through β–3 integrins, as a requirement for Mg^{2+} and Mn^{2+} and inhibition by kininogen was observed. In the physiological environment, Zn-α-2-glycoprotein may facilitate the attachment and spread of renal carcinoma cells on the basal membrane.

However, the apparent failure of the protein to induce these properties on other cell lines, in particular, a cell-line derived from prostate tissue, which has been found to produce the highest amount of Zn-α-2-glycoprotein, suggests that the material must have additional properties.

2.3.2.4. Concluding remarks

To date therefore, only a limited number of minor roles have been suggested for Zn-α-2-glycoprotein. The association of this protein with lipolytic material purified from a cachexia-inducing tumour may suggest therefore, the revelation of a novel function for Zn-α-2-glycoprotein. Indeed, electrophoretic analysis of lipolytic material purified to homogeneity revealed the presence of one major protein for which a single sequence for Zn-α-2-glycoprotein was obtained. This material exhibited *in vitro* lipid-mobilising activity which was neutralisable by polyclonal antibody to human Zn-α-2-glycoprotein. Furthermore, preliminary results obtained following *in vivo* administration of 1μg of purified material by four i.v. injections at 2.5 hourly intervals, revealed significant weight loss without a change in food and water intake (Todorov *et al*, unpublished results).

Conclusive identification of Zn-α-2-glycoprotein as a lipolytic factor however, has not yet been achieved, as the latter protein obtained from Dr. I. Ohkubo failed to induce lipid-mobilisation in isolated adipocytes (Todorov *et al*, unpublished results). It is possible that this biological activity was destroyed by long term storage at -20°C, as loss of activity due to freezing has been previously associated with purified lipolytic factor. Future experiments using freshly purified Zn-α-2-glycoprotein should clarify this discrepancy.

Additional observations also serve to cast doubt on the identity of Zn-α-2-glycoprotein as a lipolytic factor. Thus, results obtained during initial isolation of lipolytic material which achieved 207-fold purification from MAC 16 tumour extract,

suggested that activity was associated with material of apparent molecular mass 9.64-47kD with a mean Mr of 22kD as determined by superose gel-exclusion chromatography. SDS-PAGE analysis of this material did not reveal the presence of a major 40kD protein. In addition, Zn-α-2-glycoprotein has been shown to be a highly abundant, ubiquitous constituent of several normal tissue fluids. Thus, measurements of 6.7±1.8mg/100ml (n=233) in blood plasma, 39.1±16mg/100ml (n=38) in seminal plasma and 0.6-5mg/100ml in other fluids have been obtained (Ueyama *et al*, 1988 and 1990). Furthermore, the protein has never been specifically associated with malignancy. Thus, although one study revealed the possibility of a subset of tumours that may synthesise and secrete Zn-α-2-glycoprotein, the highest levels of expression were actually associated with a benign disease condition (Freije *et al*, 1991). Future gene-expression analysis of tumours associated with cachexia using available cDNA clones are required to clarify whether or not an association between cachexia and enhanced expression of this protein exists.

Until evidence is obtained which conclusively identifies the lipolytic factor as Zn-α-2-glycoprotein, the possibility that the latter protein may have a role as a carrier for lipolytic activity should be considered. This role has been previously observed when Zn-α-2-glycoprotein was found to be a carrier protein for nephritogenic renal glycoproteins (Shibata and Miura, 1982).

It is possible that minor modifications in the amino acid composition of Zn-α-2-glycoprotein and subsequent post-translational modification, could result in the production of a protein with very different properties to those of its ubiquitous counterpart. Indeed, up to four glycoforms of the acute phase glycoprotein α1-acid glycoprotein has been shown to be present in serum (Mackiewicz and Mackiewicz, 1995). The relative proportions of the glycoforms was observed to alter with various disease states and thus provided a mechanism whereby disease extent and response to treatment could be assessed.

In the event of the failure of future experiments to demonstrate lipolytic activity for Zn-α-2-glycoprotein, detailed analysis of the protein sequence and carbohydrate side chains of the lipolytic factor may be required to identify any modifications which may give rise to this novel function in Zn-α-2-glycoprotein.

2.3.3. Purification of immunogenic material (P24) from the MAC 16 tumour

2.3.3.1. Significance of the sequence identity of P24 with PA-Ag

Previous studies have provided evidence for the presence of circulatory tumour products in the serum of MAC 16 tumour-bearing mice that are capable of inducing the catabolism of skeletal muscle tissue (Beck and Tisdale, 1987; Smith and Tisdale, 1993a). The present study reports the purification of a 24kD factor (P24) from the tumour which is capable of inducing skeletal muscle protein degradation both *in vitro* and *in vivo* ; and the determination of 18 amino acids of the N-terminus. Purification was aided by the availability of a murine monoclonal antibody (Todorov *et al*, 1996b). Attempts to obtain further sequence information by protease digestion using trysin, chymotrypsin, pronase, pepsin and V-8 protease was unsuccessful. Thus, it appeared that the amino acid sequence obtained may represent the entire proteineous proportion of the molecule. The identity of the sequence of P24 with that of *Streptococcal* pre-absorbing antigen (PA-Ag) for 11/13 of the N-terminal amino acids is 'more than passing' (referee 1; Todorov *et al*, 1996a).

P24 : N- **Y D P E A A S A P G S G N**[13] P S H E A (S) (H)

PA-AG: N- **Y D P E A A S A P G D G D**[13]

Indeed, functional significance has been suggested for regions of sequence similarities observed between prokaryotic and eukaryotic proteins during sequence analysis studies of hormone sensitive lipase; when homologous regions within five bacterial proteins were identified (Langin, 1993). As emphasised by this author, similarities between the proteins of such evolutionary distinct organisms have great structural and functional significance, since they pre-date their divergence between 1.5 and 3.5 billion years ago. The region of similarity in hormone sensitive lipase was found

to be bordered by the lipase consensus catalytic site motif Gly-X-Ser-X-Gly and His-Gly 70-100 amino acids upstream. The functional significance of the similarities has been deduced in two of the cases. Thus in contrast to other eukaryotic lipases, hormone-sensitive lipase has catalytical activity at low temperatures; likewise, lipase 2 from *Moraxella* TA144 is active at 4°C. In addition, similarities in the catalytic processes of *Streptomyces* N-acetyl hydrolase and hormone sensitive lipase were observed.

Pre-absorbing antigen has been isolated from nephritogenic group A *Streptococci* obtained from the pharynx of patients with acute post *Streptococcal* glomerulonephritis (Yoshizawa *et al*, 1992), and was found to be a 43kD protein with an isoelectric point of 4.7. No further information on the structure of this antigen or its role in the pathogenesis of glomerulonephritis was available. Hence, functional comparisons with P24 cannot be made. It is possible that P24 may be a cleavage product of a protein that has diverged from PA-Ag in which the presumed functionally-important sequence has been rendered secretable and systemically stable due to two amino acid substitutions and subsequent acquisition of carbohydrate moieties.

2.3.3.2. Structural analysis of P24

Sequence analysis of P24 revealed the presence of four potential glycosylation sites. There was found to be a possible N-glycosylation site at Asp (N)^{13} within the sequon Asn^{13}-Pro-Ser^{15} which conformed with the consensus sequence: Asn-X-Ser/Thr. Additionally, three potential O-glycosylation sites at Ser^{7}, Ser^{11} and Ser^{15} were apparent, and the position of Ser^{11} within a glycine rich region was suggestive of this residue being a possible site for the binding of a glycosaminoglycan (GAG) chain (Lodish *et al*, 1995). The glycosylation status of P24 was confirmed by further analysis (Todorov *et al*, 1996a). Thus, carbohydrate residues were detected in affinity-purified material by the digoxigenin glycan detection kit (Boehringer Mannheim, East Sussex, U.K.). In addition, complete removal of glycan chains by treatment of iodinated P24 with

anhydrous triflouromethanesulphonic acid resulted in the reduction of its apparent molecular mass to approximately 2kD, in agreement with the size predicted by the amino acid sequence data. Thus, carbohydrate moieties contribute approximately 92% to the final apparent molecular weight. Selective removal of N-glycan chains with peptide-N-glycosidase F, and O-glycan chains with endo-α-N-acetylgalactosaminidase resulted in the size reduction of P24 to 14kD. The use of both enzymes together produced a product of apparent molecular mass 6kD. All treatments involving removal of carbohydrate moieties resulted in complete abolition of the immunogenicity of P24. Similar experiments on material that had been metabolically labelled with [^{35}S] revealed that the glycan chains were sulphated; furthermore, preliminary results obtained following treatment with chondroitinase AC suggested that the O-linked chain may be a GAG (Todorov *et al*, unpublished results). The exact nature of the carbohydrate chains and the amino acid residues involved, await further elucidation.

Fully glycosylated P24 was shown to exist as a free molecule, and in a form bound to albumin. The electrophoretic properties of albumin were unchanged by association with P24, and the complex formed was found to be stable, as it was apparent throughout all stages of the purification process. A similar phenomenon has been observed by other workers. Thus, albumin was identified as being the 70kD protein of the surface of lymphocytes and macrophages which bound peptidoglycan, lipopolysaccharide, lipoteichoic acid, heparin and sulphated heparinoids (Dziarski, 1994). This cell-bound albumin was found to be derived from extracellular sources such as tissue culture medium or serum. The function of this association was not determined, but did not appear to be necessary for cell activation, as cells maintained in standard medium and serum-free medium appeared equally sensitive to stimulation for the production of TNF.

P24 appeared to be specific to the cachexia-inducing MAC 16 tumour, as it was found to be absent from the serum of mice transplanted with the histologically similar, but non-cachexigenic MAC 13 tumour, as determined previously by *in vitro* proteolysis

assays (Beck and Tisdale, 1987; Smith and Tisdale, 1993b). In this report, minimal immunoreactivity was detectable in the tumour and serum of MAC 13 tumour-bearing mice. However, the monoclonal antibody has been shown to detect only glycosylated material; therefore the possibility exists that the peptide core may be synthesised but, as a result of differences in post-translationary processes, may not be secreted. Alternatively, the material may be secreted but biologically inactive due to rapid degradation or a functionally defective structure.

2.3.3.3. Detection of P24 in urine from cachectic cancer patients

Similar material was also detected in the urine of pancreatic cancer patients by immunoblot using murine monoclonal antibody (Todorov *et al*, 1996a). The material appeared to be present in the free form in urine and was not detectable in the urine of normal subjects. Subsequent purification and sequence determination of 14 N-terminal amino acids revealed the material to be identical to that isolated from the MAC 16 tumour. Human studies also confirmed the specificity of this material to the cancer-cachectic condition, as it was found to be absent from the urine of patients with weight loss not associated with malignancy. Furthermore, detection of P24 appeared to be dependent on the degree of weight loss and not on the type of tumour involved. Thus, the protein was detected in the urine of patients with cancers of the lung, breast, ovary and T-cell lymphoma, and weight loss of more than 1.5kg/month; but not was not detectable in the urine of weight-stable patients with breast and lung carcinomas.

2.3.3.4. In vivo effects of P24

Administration of affinity-purified P24 (5 ELISA units/8h, i.v.) and HPLC-purified free P24 (1.26 ELISA units/8h, i.v.) to non-tumour-bearing mice resulted in a rapid weight loss (14% and 8% respectively), in the absence of a change in food and water intake, that was apparent after 24h. Weight loss was accompanied by a 29%

reduction in adipose tissue mass and 14% loss of carcass dry weight. Hypoglycaemia was apparent, but there was no change in circulating triglycerides or total body water content. Thus, P24 appeared to be capable of inducing many of the cachectic parameters apparent in MAC 16 tumour-bearing mice (Bibby *et al*, 1987). Weight loss-inducing properties of P24 were completely abrogated by the administration of 0.8mg of monoclonal antibody prior to treatment (Todorov *et al*, 1996a). Similar effects were also obtained with affinity-purified material from human sources. The presence of carbohydrate moieties was found to be important for mediation of the *in vivo* biological activity of P24, as administration of the synthetic peptide had no effect.

2.3.3.5. In vitro effects of P24

A direct role for P24 in skeletal muscle catabolism has been suggested by results obtained from *in vitro* studies, which revealed enhanced protein degradation, as measured by tyrosine release, from isolated gastrocnemius muscle (Todorov *et al*, 1996a). Affinity-purified P24 was capable of inducing the release of 50μmol of tyrosine/g muscle over 1.5h, and similar effects were also induced by material purified from human sources (Lorite and Tisdale, unpublished results). Tyrosine release was prevented by the inclusion of monoclonal antibody in the incubating medium and by enzymatic removal of carbohydrate groups. In a separate study, induction of tyrosine release by P24 was found to be comparable to that induced by serum from MAC 16 tumour-bearing mice (Todorov *et al*, 1996b). In addition, *in vivo* measurement of protein metabolism by the administration of [4-^{3}H] phenylalanine revealed an increase in muscle degradation of 60% and a 50% decrease in muscle protein synthesis. Prior administration of monoclonal antibody prevented these effects.

Enhanced muscle proteolysis induced by P24 was associated with reduced plasma amino acid concentrations (Beck and Tisdale, 1991; Lorite and Tisdale, unpublished results). In particular, alanine was found to be reduced by approximately 40% in the

latter study by *in vivo* administration of affinity purified P24. Other workers have demonstrated a reduced whole body alanine oxidation rate and reduced uptake of radiolabelled alanine into skeletal muscle (Garcia-Martinez *et al*, 1994b). Thus, it has been suggested that the amino acid may be released as a result of protein degradation and diverted into the gluconeogenesis pathway.

2.3.3.6. Mechanism of action of P24

Skeletal muscle proteolysis induced by serum from MAC 16 tumour-bearing mice (Smith and Tisdale, 1993b) and affinity-purified human P24 (Lorite and Tisdale, unpublished results) has been associated with an increase in muscle prostaglandin E_2 (PGE_2) production. In addition, inclusion of ibuprofen and CV6504 (inhibitors of the cyclooxygenase and lipoxygenase pathways respectively) in the incubation buffers, resulted in the ineffectiveness of material from both sources to induce proteolysis and elevation of PGE_2 in the *in vitro* assay. Similar observations have been made by other workers thus, the non-steroidal anti-inflammatory drug Naproxen prevented muscle proteolysis and the associated rise in PGE_2 when administered to rats transplanted with the Yoshida ascites hepatoma (YAH) (Baracos *et al*, 1995). The functional significance of elevated PGE_2 is unknown. Although prostaglandins have been shown to induce muscle protein degradation through their capacity to activate the lysosomal proteases (Belizario *et al*, 1991), evidence produced by the former study and others (Llovera *et al*, 1995) suggested that protein degradation due to these proteases was minimal. Instead, the most important mechanism involved in protein catabolism in the YAH model of cachexia was attributed to the non-lysosomal, Ca^{2+}-independent, ATP-dependent ubiquitin conjugation pathway. Protein degradation by this mechanism was found to account for 85-100% of the observed total muscle proteolysis in this model (Llovera *et al*, 1995; Baracos *et al*, 1995). Protein degradation by ubiquitin conjugation has also been suggested to be the major mechanism involved during muscle wastage due to fasting and denervation atrophy, glucocorticoid-induced muscle atrophy, acidosis, endotoxin

treatment (Baracos *et al*, 1995, references within) and exercise-induced muscle damage (Thompson and Scordilis, 1994). However, control of this pathway and the extent of its contribution to the entire process of muscle protein breakdown in any of these situations is unknown.

It is possible that the elevation of PGE_2 observed in rats transplanted with the YAH may be due to the increased circulatory levels of TNF associated with the growth of this tumour (Costelli *et al*, 1993), and may not directly be involved in the proteolytic processes. Alternatively involvement of PGE_2 may be through another as yet undetermined mechanism.

Preliminary results obtained as a result of experiments to elucidate the mechanism of protein degradation induced by P24 suggest that the mechanism may be ATP-dependent (Lorite and Tisdale, unpublished results). Experiments to ascertain whether this proteolysis is via ubiquitin conjugation, or via involvement of alternative ATP-dependent proteases such as the multipains (Gottesman and Maurizi, 1992; and references within), are currently underway.

Mechanisms responsible for enhanced proteolysis in the YAH and MAC 16 tumour models of cachexia therefore appear to be similar. However, the observed association of PGE_2 and proteolysis must be significant. The activation of lysosomal proteases cathepsins B and D by P24 through a cascade involving an increase in intracellular Ca2+, activation of phospholipase A2 and subsequent elevation of PGE_2, has yet to be investigated. It is possible that this degradative pathway, in addition to processes involving ubiquitin conjugation and/or the multipains may play a role in the catabolism of muscle protein associated with cachexia induced by the MAC 16 tumour.

2.3.3.7. Significance of the proteoglycan structure of P24

Analysis of the structure of P24 revealed that it may be a small chondroitin sulphate proteoglycan. Thus, it may comprise a small core protein with one or more GAG chains, attached via a trisaccharide linker to a serine residue, and composed of repeating linear polymers of the disaccharide type: [D-glucuronic acid + N-acetyl-D-galactosamine]$_{n=20-60}$. One or more of these sugars would be sulphated thus imparting the highly negatively charged properties that are characteristic of these molecules.

Proteoglycans comprise a diverse family of molecules found in all connective tissues and extracellular matrices and on the surface of many cells (Lodish *et al*, 1995). Aggregan (core protein: 250kD) is the most abundant proteoglycan in cartilage and has been shown to be responsible for the structural properties of this tissue. Other matrix proteoglycans reside in the basal lamina (core proteins: 20-40kD) and play a structural role through the interaction of their GAG chains with type IV collagen. Cell surface proteoglycans are predominantly associated with epithelial cells and are often positioned so that the core protein is divided into three domains namely, a short cytoplasmic domain, a plasma membrane domain and an extracellular domain, to which the GAG chains are attached. Examples of cell-surface proteoglycans include syndecan (core protein: 56kD), serglycin (core protein: 10kD), biglycan and decorin (core proteins: 45kD). Extracellular GAG chains have been shown to bind to fibrous collagens and fibronectin thus anchoring cells to the matrix and complementing integrin-mediated cell adhesion. Proteoglycans have also been shown to mediate many intracellular processes through their capacity to bind growth factors and cytokines. This association appears to serve two main functions firstly, the half-life of the bound molecule may be increased due to protection from degradation by proteases; secondly, binding to cell surface GAG chains may result in the localisation of a high concentration of the molecule close to the target receptor. Release of the active molecule may be by proteolysis of the proteoglycan protein core or partial degradation of the GAG chains. Thus, binding of basic fibroblast growth factor (FGF)

and acidic FGF to heparan sulphate chains of extracellular proteoglycans resulted in increased stability due to increased resistance to protease degradation (Saksela *et al*, 1988 and Damon *et al*, 1989 respectively). In addition, binding of these growth factors to the heparan sulphate chains of cell-bound syndecan was found to be a prerequisite for interaction with their receptors and subsequent biological activity. More recently, insulin-like growth factor binding protein-5 was detectable only as a 23kD proteolytic cleavage product in the tissue culture medium of cultured fibroblast cells. In contrast, the protein was present as an intact molecule bound to heparan sulphate chains of extracellular matrix proteoglycans (Arai *et al*, 1994). Heparin and dermatan sulphate GAG chains also performed the same function, but sulphation of an O-linked chain was essential as sulphation of N-linked chains had no effect.

The core proteins of proteoglycans may also be involved in interactions between macromolecules. Thus, the hyaluronic acid-binding region of the core protein of aggregan interacts with link protein and serves to strengthen the proteoglycan-hyaluronic acid interaction. In addition, binding of decorin to fibronectin and collagen involves core protein interaction. Mediation of the activation of signal transduction pathways by cell-surface proteoglycan protein cores has also been suggested following observations of the anchoring of mouse melanoma heparan sulphate proteoglycan through its protein core to phosphotidylinositol (PI) (Drake *et al*, 1992). These authors suggest that the core protein could laterally associate with other cell surface adhesion molecules following initial binding to fibronectin and thereby fortify the adhesion. Indeed it has been shown that PI-anchored proteins have greater lateral mobility by a factor of ten. In addition, fibronectin-mediated ligation of the proteoglycan could activate specific signal transduction pathways associated with PI turnover as has been observed for Thy-1, a PI-anchored glycoprotein on neurons and thymocytes (Kroczek *et al*, 1986).

In the light of this brief review, P24 appears to be a novel proteoglycan with respect to the size of the core protein, its endocrine mode of action and its systemic

In the light of this brief review, P24 appears to be a novel proteoglycan with respect to the size of the core protein, its endocrine mode of action and its systemic distribution. The antigenic carbohydrate moieties have been shown to be essential for biological activity *in vivo* and *in vitro*; but whether they are involved directly, or indirectly by facilitating secretion and increasing stability of the peptide core, remains to be elucidated.

P24 (9nmol) has been shown to bind strongly to isolated muscle at a concentration of 2.3fmol/g tissue (Todorov *et al*, 1996a). Through interaction with as yet unspecified cell surface receptors, or through its association with cell-bound albumin, the protein may activate various proteolytic systems by causing an influx of extracellular calcium and subsequent enhanced activation of Ca^{2+}-dependent proteases and lysosomal proteases.

2.3.3.8. Additional circulatory proteolytic factors identified to date

A small, glycosylated proteolytic factor has also been detected in the serum of patients with sepsis or trauma in whom accelerated muscle proteolysis was apparent (Clowes *et al*, 1983). This peptide was found to comprise 33 amino acids and had an apparent molecular mass 4274D. The material was subsequently shown to be a stable proteolytic cleavage product of interleukin-1 (IL-1) involved in both enhanced muscle proteolysis and enhanced hepatic protein synthesis in these patients (Dinarello *et al*, 1984). The material appeared to retain many of the properties of the intact molecule but further structural details have not been elucidated, and its involvement in muscle proteolysis associated with other disease states is uncertain. Indeed, anti-IL-1 antibodies only partially abrogated muscle proteolysis in 41% of cancer patients with >10% weight loss in one study (Belizario *et al*, 1991). In contrast, significant inhibition was observed by the inclusion of the Ca^{2+}-chelator quin-2, and indomethacin *in vitro*; thus suggesting that other inducers of proteolysis may play a more important role.

2.3.3.9. Concluding remarks

It is possible that P24 may not cause activation of proteolytic pathways directly, but instead may mediate induction of proteolysis by another as yet unidentified factor produced by macrophages associated with intact muscle tissue. Thus, the protruding GAG chains of P24 attached to the muscle cell surface through the core protein via interaction with a receptor or cell-bound albumin, may bind to specific sites on this factor. This would result in the production of a reservoir of protease-resistant factor in close proximity to its receptor.

The possible role of P24 as a direct activator of muscle protein degradation will be determined by experiments involving measurement of proteolysis induction in cultured muscle cells, which are currently underway.

CHAPTER 3:

ISOLATION OF P24 cDNA

3.1. MATERIALS, SOLUTIONS AND METHODS

3.1.1. Materials

Advanced Biotechnologies Ltd, Surrey, UK

Thermostable DNA polymerase: 5u/μl

dNTP set: dATP, dCTP, dGTP, dTTP

Buffer IV: 200mM $(NH_4)_2SO_4$

750mM Tris HCl pH 9.0

0.1% (w/v) Tween

Buffer II : 500mM KCl

100mM Tris HCl pH 8.3

25mM $MgCl_2$

Amersham International (USB), Bucks., UK

[^{35}S] dATP α S : > 600Ci/mmol

Hybond C nitrocellulose membranes

Sequenase version 2.0 DNA sequencing kit

M13 reverse primer

Bio-Rad Laboratories Ltd, Herts. UK

Acrylamide

N,N'-Methylene-bis-acrylamide (Bis-acrylamide)

Ammonium persulphate

N,N,N',N'-Tetramethylethylenediamine (TEMED)

Sequi-Gen Nucleic acid sequencing cell

Boehringer Mannheim, Lewes, East Sussex, UK.

Molecular weight marker V: pBR322/Hae III

Difco Laboratories, Surrey, UK

Yeast extract

Tryptone

Bacto Agar

Fisons Scientific Equipment, Leics., UK

Ethanol

Chloroform

Glacial acetic acid

Isopropanol

Methanol

Flowgen Instruments Ltd., Kent,UK

Nusieve GTG agarose

SeaPlaque GTG agarose

Gelman Sciences, Northampton, UK

Acrodisc 0.2μm syringe filters

GIBCO BRL Life Technologies Ltd. Scotland, UK

Ultrapure agarose

Enzymes:

SuperScript™ II RT	200u/μl
E. coli RNAse H	1.6u/μl
Xho 1	10u/μl
Pst 1	10u/μl

*Sst*1	10u/μl
T4 kinase	10u/μl
T4 DNA ligase	1u/μl
Large fragment of DNA polymerase 1 (Klenow fragment)	3.3u/μl

Molecular weight markers

1kb DNA ladder

10bp DNA ladder

Lambda/*Hind* III fragments

Hybaid Ltd, Middx, UK

Quigen plasmid miniprep kit

Pharmacia Biotech, Herts., UK

pUC 18/Sma 1/BAP

pUC 18

cDNA Sephacryl S-300 Spin columns

R & D systems, Abingdon, UK.

Mini - Message Maker

Sigma Chemical, Dorset,UK

Ampicillin (sodium salt)

Bovine serum albumin (BSA) (ELISA grade)

5-Bromo-4-chloro-3-indolyl phosphate (BCIP)

5-Bromo-4-chloro-3-indoyl β-D Galactopyranoside (X-Gal)

Bromophenol blue

Calcium chloride

Casein hydrolysate

Diethylpyrocarbonate (DEPC)

Dimethylformamide

Dimethyl sulphoxide (DMSO)

Ethidium bromide

Ethylenediaminetetraacetic acid (EDTA)

Formamide

Formaldehyde

Guanidine isothiocyanate

Glycerol

Isopropyl β-D-Thiogalactopyranoside (IPTG)

Kanomycin

Kodak Processing Chemicals : GBX Developer and Replenisher

Kodak Processing Chemicals : GBX Fixer and Replenisher

Kodak X-Omat AR film 35cm x 43cm

Kodak X-Ray exposure holder 35.6cm x 43.2cm

N-Lauroylsarcosine, Sodium salt (SLS)

Lithium chloride

Magnesium sulphate

Maltose

β-Mercaptoethanol

Mineral oil

3-[N-Morpholino]propanesulphonic acid, (free acid) (MOPS)

Nitroblue tetrazolium

Phenol/Chloroform/isoamyl alcohol (25:24:1)

Polyoxyethylenesorbitan (Tween 20)

Protein A alkaline phophatase conjugate

Proteinase K (from Tritirachium album)

Sodium acetate

Sodium azide

Sodium chloride

Sodium dodecyl sulphate (SDS)

Sodium hydroxide

Tetracycline (hydrochloride crystalline)

Tetramethylammonium chloride (TMAC)

Trizma base

Urea (ultrapure reagent)

Xylene cyanol

Sterilin (Appleton Woods), Birmingham, UK

Bacteria culture plates : 140mm, 84mm

Stratagene Ltd

Escherichia coli XL1-Blue MRF'

Escherichia coli SOLR

Helper phage ExAssist

Whatman Intl., Ltd, Kent, UK

Cellulose nitrate filters (0.2μm, 25mm)

3MM Chr chromatography paper

3.1.2. Solutions and media

All media and solutions were made with ultrapure water (Elgastat Option 4 water purifier).

3.1.2.1. Stock solutions.

Tris.HCl pH 8.0	1M
Tris.HCl pH 7.5	1M
Tris.HCl pH 2.5	1M
Tris.HCl pH 9.5	1M
EDTA pH 8.0	500mM
NaCl	5M
SDS	10% (w/v)
$MgCl_2$	1M
$MgSO_4$	1M
Sodium acetate buffer pH 5.2 (glacial acetic acid)	3M

Stock solutions for RNA work were made with DEPC-treated water and autoclaved on a liquid cycle (15psi) for 15min. Tris stock buffers were made in autoclaved DEPC-treated water and then re-autoclaved. Stock solutions for general molecular biology use were made with purified water and autoclaved. SDS was dissolved in sterile DEPC water by heating to 65°C for 30min.

3.1.2.2. Diethylpyrocarbonate (DEPC)-treatment of water.

Purified water was mixed overnight at 37°C with 0.1% (v/v) DEPC then used directly to make buffers which were subsequently sterilised, or used as a diluent after inactivation by autoclaving.

3.1.2.3. RNA extraction

All solutions for RNA extraction were made with Molecular Biology-grade reagents.

3.1.2.3.1. Lysis buffer.

Guanidine isothiocyanate	5M
Tris.HCl pH 7.5	50mM
EDTA	10mM

These components were dissolved in sterile DEPC-treated water, filter-sterilised through a 0.2μm filter and stored at -20°C in 10ml aliquots.

3.1.2.3.2. Lithium chloride.

6M, 4M and 3M solutions were made with sterile DEPC-treated water, filter-sterilised through 0.2μm filters and stored at 4°C.

3.1.2.3.3.TEN-SDS.

Tris.HCl pH 8.0	10mM
EDTA	1mM
NaCl	150mM
SDS	0.1%

3.1.2.3.4. Proteinase K.

Proteinase K was dissolved in sterile DEPC-treated water (20mg/ml) filter-sterilised through a 0.2μm filter and stored in 50μl aliqouts at -20°C.

3.1.2.3.5. 10 x MOPS buffer.

MOPS	0.4M
Sodium acetate buffer pH 5.2	100mM
EDTA	10mM

3.1.2.4. Poly A RNA extraction.

3.1.2.4.1. Loading buffer (2x)

Tris.HCl pH 7.6	40mM
NaCl	1M
EDTA	2mM
SLS	0.2% (v/v)

A 10% solution of sodium N-Lauroylsarcosine (SLS) was made as described for 10% SDS (2.2.2.1).

3.1.2.4.2. Elution buffer.

Tris.HCl pH 7.6	10mM
EDTA	1mM
SDS	0.05% (v/v)

Loading and elution buffers were made freshly from stocks with sterile DEPC-treated water and re-autoclaved.

3.1.2.5. cDNA library immunoscreening.

3.1.2.5.1. Maltose (20%).

20g maltose was dissolved in sterile water (final volume 100ml), the solution filter-sterilised through a 0.2μm filter and stored at 4°C.

3.1.2.5.2. Tris buffered saline (TBS).

Tris.HCl pH 7.5	20mM
NaCl	150mM

3.1.2.5.3. Tris buffered saline + Tween 20 (TBST).

TBS + 0.05% (v/v) Tween 20

3.1.2.5.4. Antibody diluent.

TBS + 1% BSA

3.1.2.5.5. Colour development solution.

Tris.HCl pH 9.5	100mM
NaCl	100mM
$MgCl_2$	5mM

3.1.2.5.6. Stop solution.

Tris.HCl	20mM
EDTA	1mM

3.1.2.5.7. 5-Bromo-4-chloro-3-indoylphosphate (BCIP).

BCIP was dissolved in dimethylsulphoxide to a final concentration of 50mg/ml, stored at 4°C and used at a concentration of 0.15mg/ml.

3.1.2.5.8. Nitroblue tetrazolium (NBT).

NBT was dissolved in 70% dimethylsulphoxide to a final concentration of 75mg/ml, stored at 4°C and used at a concentration of 0.3mg/ml.

3.1.2.5.9. NZY broth (1 litre).

NZY broth and plates were used for plaque development due to their increased clarity.

NaCl	5g
$MgSO_4$	2g
Yeast extract	5g
Casein hydrolysate	10g
NaOH	to pH 7.5

3.1.2.5.10. NZY agar plates.

Agar (1.5% w/v) was added to NZY broth, the mixture was autoclaved, poured into plates and stored at 4°C.

3.1.2.5.11. NZY top agar

Agarose (0.7%) was added to NZY broth, the mixture was autoclaved and then allowed to set. When required, an aliqout (3ml/82mm plate, 7.5ml/140mm plate) was melted in a microwave oven (3min, full power).

3.1.2.5.12. SM buffer (1 litre).

1M Tris.HCl pH 7.5	50ml
MgSO4	2g
NaCl	5.8g
Gelatin (2%,w/v)	5.0ml

3.1.2.6. General solutions and media.

3.1.2.6.1. Sample loading buffer (6x)

Bromophenol blue	0.25% (w/v)
Xylene cyanol	0.25% (w/v)
Glycerol	30%

Sample loading buffer was made with sterile water and stored at 4°C.

3.1.2.6.2. Tris/EDTA (TE) buffer

Tris.HCl pH 8.0	10mM
EDTA	1mM

3.1.2.6.3. TBE (5x) (1 litre).

Trizma base	54g
Boric acid (sodium salt)	27.5g
EDTA	20ml

3.1.2.6.4. Ethidium bromide.

A stock solution of ethidium bromide solution (10mg/ml) was made by dissolving one tablet (10mg) in 1ml of sterile water. The stock was stored at room temperature wrapped in aluminium foil.

3.1.2.6.5. Ampicillin.

A stock solution (50mg/ml) was made with sterile water, filter-sterilised through a 0.2μm filter and stored in 1.0ml aliquots at -20°C. Ampicillin was used at a final concentration of 50μg/ml.

3.1.2.6.6.Tetracline.

A stock solution (5mg/ml) was made in ethanol and stored in 1.0ml aliquots at -20°C. Tetracycline was used at a final concentration of 12μg/ml).

3.1.2.6.7. Kanomycin.

A stock solution of kanomycin (10mg/ml) was made in sterile water, filter-sterilised and stored in 1.0ml aliquots at -20°C.

3.1.2.6.8. Isopropyl β–D-Thiogalactopyranoside (IPTG).

IPTG was dissolved in sterile water at a concentration of 1.0M. The solution was filter-sterilised through a 0.2μm filter and stored in aliquots of 0.5ml at -20°C. For blue/white colony screening (section 2.2.3.15) the final concentration was 1.3mM. For induction of the *lac Z* gene promoter required for immunoscreening (section 2.3.21.2), the final concentration was 10mM.

3.1.2.6.9. 5-Bromo-4-Chloro-3-Indoyl β–D Galactopyranoside (X-Gal).

One gram of X-Gal was dissolved in 4.0ml of dimethylformamide (613mM) and stored in 0.5ml aliqouts at -20°C. For blue/white screening the final concentration was 0.82mM.

3.1.2.6.10. Acrylamide stock solution.

Acrylamide (38g) and bis-acrylamide (2g) were dissolved in water to a final volume of 100ml and stored in the dark at 4°C.

3.1.2.6.11. Sequencing gel fixing solution.

Methanol	10ml
Glacial acetic acid	10ml
Water	80ml

3.1.2.6.12. Luria broth (LB) (1 litre)

NaCl	10g
Yeast extract	5g
Tryptone	10g
NaOH	to pH 7.0

Luria broth was autoclaved and stored at room temperature in 100ml aliqouts. LB 2x was made by doubling the amount of components.

3.12.6.13. LB-agar plates.

Agar (1.5%) was added to Luria broth, the mixture was autoclaved, cooled to 45°C and antibiotic added if required. The agar was then poured into plates (25ml), allowed to cool and stored at 4°C. Antibiotic plates were stored for a maximum of two weeks.

3.1.2.6.14. LB- top agar.

Agar (1.2%) was added to Luria broth, the mixture autoclaved and allowed to set for storage at room temperature. For use, the top agar (3.0ml/plate) was melted in a microwave oven (3min full power).

3.1.3. Methods

3.1.3.1. Extraction of total RNA from tumour tissue.

In order to minimise RNAse contamination, all solutions used for RNA extraction were made with water treated with diethylpyrocarbonate (DEPC, section 3.1.2.2) and autoclaved for 15min on a liquid cycle (15psi). Glassware was soaked in DEPC (0.1% w/v) overnight then autoclaved prior to use, and gloves were worn throughout all procedures.

Total RNA was extracted by a modification of the method of Cathala *et al*, (1989). MAC 16 tumours were excised from cachectic NMRI mice, rinsed briefly in ice-cold PBS and homogenised immediately in guanidine isothiocyanate lysis buffer (3.1.2.3.1.), (10ml/tumour)+mercaptoethanol (0.8ml/10ml lysis buffer). Tumour homogenates could be stored at -70°C until required. Ice-cold lithium chloride (6M) was added to a final concentration of 4M and the mixture homogenised further by drawing five times through a 25-guage needle and 10ml syringe. Lithium chloride (4M) was added (final ratio of lithium chloride : guanidinium thiocyanate: 5.8 : 1) and RNA was precipitated overnight at 4°C. Total RNA was pelleted by centrifugation (10000g, 90min, 4°C), resuspended in 35ml of 3M lithium chloride and homogenised again through a 25-guage needle. Following re-centrifugation (10000g, 30min, 4°C), the pellets were resuspended in 5.0ml TEN-SDS (3.1.2.3.3.), proteinase K was added (final concentration 400μg/ml) and the samples were incubated at 37°C for 30min. Proteinaceous material was removed by phenol/chloroform extractions (3.1.3.5), RNA was precipitated as described (3.1.3.6) and resuspended in 50μl DEPC-treated, sterilised water. Total RNA isolated was quantified by measurement of 5μl in a total volume of 1.0ml water at 260nm (1.0OD unit = 40μg). Quality was assessed by the ratio of absorbance readings at 260nm and 280nm (1.7/2.0 : 1) and by formaldehyde gel electrophoresis (3.1.3.2).

3.1.3.2. Formaldehyde gel electrophoresis.

An aliquot of total RNA from each tumour was analysed by electrophoresis under denaturing conditions for the presence of two distinct bands representing the 28S and 18S subunits of ribosomal RNA. The presense of these bands in a ratio of intensity of ethidium bromide staining 2:1 indicated that undegraded RNA had been obtained. The samples were electrophoresed through a formaldehyde agarose gel in a horizontal mini-gel apparatus (Pharmacia Biotech). The gel (50ml) was prepared by melting 0.6g ultrapure agarose (final cancentration 1.2%) in 30ml DEPC-treated water (3.1.2.2.). Formaldehyde (8.95ml) and 5.0ml of 10x MOPS buffer (3.1.2.3.5) were added followed by water to 50ml and 3μl ethidium bromide (10mg/ml). The gel was cast and allowed to set for at least 30min at room temperature. RNA samples were prepared as follows: To approximately 5μg of RNA (2.0μl), 16.35μl formamide, 5.72μl formaldehyde, and 3.27μl 10x MOPS buffer were added. The samples were heated at 65°C for 10min, cooled on ice and 5.0μl 6x loading buffer was added; they were then loaded and electrophoresed at 50V (10 mAmp) in 1x MOPS buffer. The gel was viewed on a UV transilluminator and photographed.

3.1.3.3. Extraction of Poly A RNA.

3.1.3.3.1. Extraction of Poly A RNA from total RNA.

Messenger RNA was separated from ribosomal and transfer RNA by passage through an oligo dT column (Sambrook *et al*, 1989). A sterile, siliconised pasteur pipette was plugged with glass wool and glass beads (also siliconised and autoclaved) and packed with 0.5g Oligo-dT cellulose (stored in suspension at 4°C in 0.1M NaOH). The column was washed with 3x volumes DEPC-treated sterile water (3.1.2.2) and 3x volumes of loading buffer (3.1.2.4.1). Prior to loading, the RNA sample was denatured by incubation at 65°C for 5min and cooled quickly on ice. An equal volume of 2x loading

buffer was added to 0.5mg total RNA, the sample was loaded and the eluate was collected immediately. The column was washed with one volume of loading buffer and all eluate was pooled, denatured by heating at 65°C for 10min and cooled on ice. The eluate was then reloaded and the column washed with 5x volumes of loading buffer. Poly A RNA was eluted with 2-3x volumes of elution buffer (3.1.2.4.2) and 1ml fractions were collected. Fractions containing RNA as determined by absorbance at 260nm were pooled, denatured and subjected to a second passage through the column. Finally, fractions containing RNA were pooled and the RNA precipitated as described (3.1.3.6). Pellets were resuspended in DEPC-treated water and an aliquot removed for quantitation by absorbance at 260nm. For storage, the mRNA was aliquoted into 5μg amounts and stored under 2.5x volumes of ethanol at -70°C to be re-precipitated with one tenth of the original volume of sodium acetate buffer when required.

3.1.3.3.2. One-step extraction of Poly A RNA from tissue culture cells

MAC 16 and MAC 13 cells were grown in RPMI medium supplemented with 20mM glutamine and 25mM HEPES buffer at 37°C in a humid atmosphere of 95% air/5% CO_2. Approximately 6 x 10^6 MAC 13 cells were obtained from an 800ml flask when the cells were grown to confluency and 8 x 10^6 MAC 16 cells were obtained from the same size flask when grown in 250ml medium to confluency. The cells were harvested by centrifugation (1500rpm 5min) in a bench-top centrifuge. They were washed three times in ice-cold PBS, transferred to microfuge tubes and pelleted by centrifugation at 13000rpm for 5min. Poly A RNA was isolated directly by the Mini-Message Maker kit (R&D Systems) according to the manufacturers instructions. Briefly, the cell pellets were resuspended in 1.0ml of lysis buffer and homogenised by drawing through a 25-guage needle and 5.0ml syringe ten times until the viscosity was reduced. Cell debris was removed by centrifugation (13000rpm, 30 sec) and the supernatant was transferred to a fresh tube. Lysis buffer was added to 1.5ml followed by 50μl Oligo dT latex beads; after mixing by ten tube inversions, the suspension was incubated at room

temperature for 10min. The beads + poly A RNA were recovered by centrifugation (13000rpm, 5min) and resuspended in 350μl lysis buffer. The suspension was transferred to a spin column unit and the beads were washed by two centrifugation (13000rpm, 30sec) and resuspension steps in wash buffer. Poly A RNA was finally eluted from the beads by the same procedure using 2 x 50μl hot elution buffer (70°C). Further enrichment of mRNA was achieved by the addition of 1.0ml of lysis buffer + 50μl of beads to the eluate and repetition of the described steps.

3.1.3.4. First strand cDNA synthesis

Complementary DNA was prepared from mRNA using Moloney Murine Leukaemia Virus (MoMuLV) reverse transcriptase (SUPERSCRIPT™ II) according to the manufacturers instructions. Briefly, 1μg random hexamers, 1μg (0.5nM) of primer based on two amino acids at the carboxy terminus of the N-terminal peptide sequence for the cachectic factor, or 1.0μl (0.5μg) Oligo $(dT)_{12\text{-}18}$ were added to approximately 5.0μg of mRNA, and DEPC water was added to 8.0μl. The RNA and primer mix was denatured by heating at 70°C for 10min and quick-chilled on ice. The sample was briefly spun and the following components added: 5x enzyme buffer (4.0μl), 0.1M DTT (2μl), and 1μl mixed dNTP stock (10mM each dATP, dGTP, dCTP, dTTP at neutral pH). The contents were mixed by gently vortexing, enzyme was added (200u/μg RNA) mixed gently with a pipette tip and incubated 1h for 37°C or 42°C. The reaction was terminated by the addition of 4.0μl 0.5M EDTA. If the cDNA was to be used for the amplification of products greater than 1kb, the RNA template was degraded by the addition of 3.0units of *E. coli* RNAse H followed by incubation at 37°C for 20min. First strand cDNA was prepared for analysis by PCR by phenol/chloroform extraction, ethanol precipitation (3.1.3.5 and 3.1.3.6) and centrifugation through a spun-column according to the manufacturers instructions (Pharmacia Biotech).

3.1.3.4.1. Analysis by alkaline agarose electrophoresis

[α^{32}P] dCTP (approximately 1μCi) was added to a pilot cDNA synthesis reaction and the mix was electrophoresed through a 1.9% agarose gel under alkaline conditions. The agarose gel was made with 50mM NaOH, 1mM EDTA (final concentration) and run in the same buffer. Alkaline loading buffer (0.2x volume) was added to the samples (300mM NaOH, 6mM EDTA, 18% ficoll type 400, 0.15% bromocresol green and 0.25% xylene cyanol FF) and they were electrophoresed at 0.25V/cm. The gel was soaked in 7% trichloroacetic acid for 30min, mounted on a glass plate, dried for 8h under weighted paper towels, then exposed to autoradiography film overnight

3.1.3.5. Phenol/chloroform extraction of nucleic acid.

Protein was removed from DNA and RNA following enzyme-modification reactions by two extractions with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and one extraction with chloroform/isoamyl alcohol (24:1). At each step, the mixture was vortexed, centrifuged (13000rpm, 2min) and the supernatant transferred to a fresh tube without disturbing the interface.

3.1.3.6. Ethanol precipitation of nucleic acid.

DNA was precipitated by the addition of 0.1x volume of sodium acetate buffer (3.1.2.1) and 2x volumes of ice-cold ethanol (100%). The mixture was mixed, incubated at -20°C for 1h and DNA recovered by centrifugation (13000rpm, 10min) in a microfuge. The supernatant was disgarded, the pellets were washed with 0.3ml ice-cold 70% ethanol, re-centrifuged and the supernatant carefully removed using a Gilson pipette (200μl). The pellets were allowed to air-dry at room temperature and dissolved in an appropriate buffer. RNA was precipitated as described above with 2.5x volumes ethanol (100%) and incubation at -70°C prior to centrifugation.

3.1.3.7. Polymerase Chain Reaction (PCR).

All plastic-ware, solutions and pipettes to be used for PCR were kept specifically for this purpose and reactions were assembled at a separate area of the bench to minimise the risk of contamination.

PCR components were assembled in 0.5μl microfuge tubes as 50μl or 25μl reaction volumes. For a final volume of 50μl, the following constituents were added: 20 pmoles forward and reverse oligonucleotides, 5.0ng cDNA, 5.0μl 10x reaction buffer IV, 1.5mM $MgCl_2$, 8.0μl mixed dNTP stock (200μM final concentration each dATP, dCTP, dGTP, dTTP), 5.0mM TMAC, 1.25units Taq polymerase and water to 50μl. The amounts were reduced by 50% for a reaction volume of 25μl.

The components of the reaction mixture were assembled as a master mix, mixed gently and then aliquoted to tubes containing oligonucleotides and template DNA. The samples were spun briefly, one drop of mineral oil was added and the tubes were placed in an Omnigene thermal cycling system (Hybaid Ltd) set previously at 94°C. Cycle programs involved a preliminary denaturation step at 94°C for 4min, followed by 30 cycles consisting of a denaturation step at 94°C for 1min, an anealling step at temperatures ranging from 48°C to 60°C for 1-2min, and an extension step at a temperature of 72°C for 20-60 seconds. On completion of the 30 cycles, there was a further 2min extension step at 72°C followed by a return to room temperature.

Samples were then placed on ice, transferred to fresh tubes by pipetting through the mineral oil, and analysed by agarose gel electrophoresis (3.1.3.8). A control reaction comprising MAC16 cDNA template and oligonucleotides based on the human GSTT1 gene sequence (figure 3.2.1.1.9.) designed to produce a 53bp amplified product (kindly donated by Dr. J. Taylor, London) was included in every experiment. Preliminary reactions were run with additional controls involving oligonucleotides in the absence of template, and template with a single oligonucleotide.

3.1.3.7.1. PCR analysis of tissue culture whole cells

Cells were washed twice in PBS and finally resuspended in PBS at a density of 100 cells/μl. The suspension was placed in a boiling water bath for 5min and cell debris removed by centrifugation in a microfuge (13000rpm, 5min). An aliquot from the supernatant (1μl/reaction) was then analysed by PCR.

3.1.3.8. Analysis of PCR products by agarose gel electrophoresis.

PCR products were analysed on a 3% Nusieve + 1% agarose gel for gel elution or 2-3% agarose for analysis using a midi-gel rig (Gibco BRL) with either two 20-well combs (3mm x 2mm) for 36 samples or two 14-well combs (4mm x 2mm) for 24 samples. The gel was made by melting 3g Nusieve + 1g agarose (or 2-3g agarose) in 80ml water, 10ml of 5x TBE buffer (3.1.2.6.3) was then added followed by water to 100ml. Ethidium bromide was added (5.0μl of 10mg/ml stock) and the mixture allowed to cool to approximately 50°C before casting. PCR samples were prepared for electrophoresis by transferring 20μl to a fresh tube containing 3.0μl 6x loading buffer (3.1.2.6.1). They were then loaded and electrophoresed alongside markers (1kb or 10bp, Gibco BRL) at 100V (20 mAmp) until the bromophenol blue had reached the bottom of the gel. Products were viewed under a UV transilluminator and photographed.

3.1.3.9. Analysis of PCR products by polyacrylamide gel electrophresis.

PCR products were analysed by electrophoresis through a non-denaturing polyacrylamide gel alongside the control 53bp product in order to obtain a better resolution and size assessment of bands. A polyacrylamide gel (7.5%) of 1.3mm thickness was cast in a vertical sequencing gel apparatus 21cm x 50cm, (Bio-Rad Laboratories Ltd) using a comb with well dimensions 15mm x 5.5mm x 1mm . The gel components were 7.5g acrylamide, 0.255g bis-acrylamide, 96ml 1 x TBE (3.1.2.6.3),

4.0ml 1.6% ammonium persulphate and 60µl TEMED in a total volume of 100ml. The gel was poured and allowed to set for 1h before use.

Samples to be analysed (50µl) were prepared for loading by the addition of 8.0µl of 6x loading buffer (3.1.2.6.1). The gel was run at 600V (43 mAmp) in 1x TBE. Once the bromophenol blue in the loading buffer had reached the bottom of the gel, the apparatus was switched off and the gel allowed to cool before dismantling. The gel was then transferred to a shallow tray, stained in 1x TBE + 0.5µg/ml ethidium bromide for 30min and destained in 1x TBE for 15min. The gel was then viewed under the transilluminator and photographed.

3.1.3.10. Gel elution of PCR products.

3.1.3.10.1. Elution from polyacrylamide.

After electrophoresis through polyacryamide, the position of the control 53bp band was used to select an area of gel approximately 2cm x 7mm from each lane (in which products often could not be seen). These areas were excised from the gel, chopped into small pieces with a sterile scalpel blade, immersed in 30µl TE buffer (3.1.2.6.2) and eluted overnight at 4°C. The mixture was vortexed, pelleted (13,000rpm, 5min) and 1.0µl was used as a template for re-amplification. The re-amplification reactions were set up as described (3.1.3.7) except that 10x reaction buffer II was used in place of buffer IV. The absence of ammonium sulphate in the latter allowed blunt-ending of PCR products to be performed immediately after completion of amplification without the need for purification by gel elution.

3.1.3.10.2. Elution from agarose gel.

PCR products to be eluted were resolved on either 3% Nusieve + 1% agarose (<200bp) or 2% SeaPlaque (>200bp) as described (3.1.3.8). A preparative gel was made

for large-scale elution (using a preparative comb) while an analytical comb was used for elution of products from a single lane. Two strips of NA45 membrane were cut to the same width as the band to be eluted and equilibrated in TE buffer (3.1.2.6.2)+ 2.5M sodium chloride for 1h at room temperature. The membranes were rinsed in water and soaked in 1x TBE (3.1.2.6.3) prior to use. Membranes were inserted immediately infront of and behind the bands to be eluted, these were then electrophoresed in the reverse direction until they became immobilised on the membrane behind them as determined by viewing under a UV transilluminator. Membranes were removed, rinsed in water, cut into 2mm x 2mm squares and eluted in TE + 1.5M sodium chloride (300μl for large-scale elution, 100μl for single-lane elution) at 70°C for 1h. The samples were vortexed during the incubation to aid elution. Finally, the eluates were removed, extracted with phenol/chloroform/isoamyl alcohol and precipitated as described (3.1.3.5 and 3.1.3.6). Due to the high salt concentration in the samples, they were precipitated at room temperature. Samples thus prepared could then be cloned as described (3.1.3.11-3.1.3.14)

3.1.3.11. Blunt-ending PCR products.

Total eluate from a single lane was used in the blunt-end reaction, one-tenth of the eluate from a preparative gel (3.1.3.10.2) was used and if PCR gave rise to a single product ie after elution from polyacrylamide gel and re-amplification in PCR buffer II, (3.1.3.10.1) the components listed below were added directly to the PCR tube.

PCR products were blunt-ended in preparation for ligation into pUC18 by the addition of 2.0μl mixed dNTP (50μM final concentration of each dNTP), 6.0μl ATP (from 20μm stock), 3.0μl $MgCl_2$, (from 25mM stock), 2.0μl Klenow fragment (5.88u/μl), and 1.0μ T4 polynucleotide kinase (10u/μl) in a reaction volume of 50μl. The mixture was gently tapped to mix, spun briefly and incubated at 37°C for 30min. The sample was then extracted twice with an equal volume of of phenol/chloroform/isoamyl alcohol and once with an equal volume of chloroform/isoamyl alcohol, and precipitated as

described (3.1.3.5 and 3.1.3.6) dissolved in 10μl TE buffer (3.1.2.6.2), 2-5μl was then used in the ligation reaction.

3.1.3.12. Ligation of blunt-ended PCR products into pUC 18 plasmid.

A 20μl ligation mix was set up with the following components: 2-5μl of blunt-ended PCR product, 1.0μl pUC18 (100ng) cut with *Sma*1 and treated with alkaline phosphatase (Pharmacia Biotech), 2.0μl 5x ligase buffer and 1 unit of T4 DNA ligase. The mixture was tapped gently to mix, briefly spun and incubated overnight at 15°C

3.1.3.13. Preparation of competant E.coli JM83 cells.

An innoculum from glycerol stock of *E.coli* JM83 at -70°C was streaked onto an LB-agar plate (3.1.2.6.13) and grown overnight at 37°C. During the same overnight incubation period as the ligation reaction (3.1.2.13), one colony from the plate was grown in 10ml LB-broth (3.1.2.6.12) at at 37°C in a shaking incubator. An aliquot (0.5ml) from the cell suspension was added to 50ml LB-broth and the cells grown to mid-log phase (OD_{600nm} 0.3-0.5). After incubation on ice for 10min, the cells were collected by centrifugation (2000g, 10min, 4°C), and resuspended gently in 20ml of 50mM calcium chloride. The cells were incubated on ice for 20min then harvested by centrifugation (2000g, 10min, 4°C), resuspended in 1.6ml 50mM calcium chloride and after furthur incubation on ice for 30min they were ready to use.

3.1.3.14. Transformation of competant E.coli JM83.

Freshly prepared *E.coli* cells (300μl, section 2.2.3.14) were incubated with the ligation mix (3.1.3.12) on ice for 1h. The cells were heat-shocked by incubation at 46°C for 5min, placed on ice for 10min and then incubated at 37°C in 0.8ml of 2x LB broth (3.1.2.6.12) for 30min. The cells were harvested by centrifugation on the lower speed

setting of a microfuge (6000rpm) for 3min. All but 200μl of the supernatant was removed in which the cells were gently resuspended and spread on an LB-ampicillin (50μg/ml) agar plate (3.1.2.6.13) overlaid with top-agar (3.1.2.6.14) containing ampicillin (50μg/ml), isopropyl β-D-Thiogalactopyranoside (IPTG, 1.3μM, 3.1.2.6.8) and 5-Bromo-4-chloro-3-indolyl β-D galactopyranoside (X-GAL, 0.82mM, 3.1.2.6.9). The plates were then incubated overnight at 37°C in an inverted position.

3.1.3.15. PCR analysis of plasmid DNA in bacterial cells.

A single white colony was transferred to 1.0ml LB broth (3.1.2.6.12) + ampicillin (50μg/ml) and incubated at 37°C for 2h. An aliquot (1.0μl) from this innoculum was used as a template for PCR as described (3.1.3.7). PCR products were analysed by electrophoresis as described (3.1.3.8) and those bacterial cell suspensions giving rise to a PCR product of the correct size were selected for plasmid DNA purification.

3.1.3.16. Extraction of plasmid DNA for sequence analysis.

Plasmid DNA was purified from bacterial cell suspensions selected by PCR analysis (3.1.2.16) using the Quigen plasmid miniprep kit according to the manufacturers instructions. A volume of 100μl from a 1.0ml bacterial cell suspension (3.1.2.15) was added to 10ml LB broth (3.1.2.6.12) + ampicillin (50μg/ml) and incubated overnight at 37°C in a shaking water bath. Cells from 3ml of this innoculum were harvested and the plasmid extracted. Briefly, the pellet was resuspended in 0.3ml buffer P1 containing RNAase A, 0.3ml of buffer P2 was added and the sample mixed by inversion 4-6 times. After incubation at room temperature for 5min, 0.3ml of chilled buffer P3 was added, mixed by inversion and incubated on ice for 10min. The precipitate was pelleted by centrifugation (13,000rpm 10min) and the supernatant loaded onto a Quigen column. After several washes with wash buffer during which RNA, proteins and other cellular contaminants were removed, approximately 20μl of DNA was eluted by 0.8ml elution

buffer. The DNA was precipitated by the addition of 0.7x volume isopropanol followed by incubation at room temperature for 20min, and the pellet was collected by centrifugation (13,000rpm, 10min), washed with 0.3ml 70% ethanol and resuspended in 20μl TE buffer.

3.1.3.17. Analysis of plasmid DNA by restriction enzyme digest.

Restriction sites flanking the *Sma* 1 insertion site were used for excision of the insert for size analysis. Restriction enzyme digests were set up by adding the following components to 5μl plasmid in TE buffer (3.1.2.6.2): 2.0μl 10x enzyme reaction buffer (React 2, Gibco BRL), 10units *Sst* 1, 10units *Pst* 1 and water to 20μl. After incubation at 37°C for 30min, 3.0μl 6x loading buffer was added and the samples were electrophoresed through 1.5% agarose in 0.5x TBE buffer (3.1.2.6.3) adjacent to the 1kb marker as described (3.1.3.9.).

3.1.3.18. Sequence analysis of PCR products cloned into plasmid DNA.

3.1.3.18.1. Polyacrylamide gel assembly.

The gel apparatus for a gel of dimensions 21cm x 80cm x 0.4mm was prepared as follows: Surfaces to be in contact with the gel were cleaned thoroughly with a powder-based cream cleanser and then with a mild detergent rinsing extensively with purified water between both stages. After drying, the surfaces were cleaned with 70% ethanol and the surface of the integral plate/chambers was siliconised with Sigmacote to prevent the gel from sticking to both plates. The gel apparatus was assembled according to the manufacturers instructions (Bio-Rad Laboratories Ltd) using 0.4mm spacers and a 24-well shark-tooth comb. A 6% gel was then made with the following components: 36g urea, 10.8ml acrylamide solution (38% acrylamide, 2% Bis-acrylamide), 14.4ml 5x TBE (3.1.2.6.3), and 20ml H_2O. The mixture was warmed to dissolve the urea, then 440μl

ammonium persulphate (22.8%) was added and the gel solution filtered through a 0.2μm cellulose nitrtate filter. An aliquot of this solution (approximately 15ml) was used to seal the gel plates after the addition of 50μl TEMED. To the remainder of the gel mix, 15μl TEMED was added and the gel poured immediately using a 20ml syringe. The gel was allowed to set for 1-2 h and prior to loading it was pre-run at 50W until the temperature was 45°C-50°C.

3.1.3.18.2. Preparation of plasmid DNA for sequence analysis.

Quigen minipreps of plasmid DNA (3.1.3.16) were sequenced using the chain-termination method in the form of the Sequenase version 2.0 DNA sequencing kit. The protocol used was based on that provided with the kit. All reagents involved in the annealling, extension, chain -termination and final stop reaction were provided in the kit : To 5.0μl plasmid prep (2-4μg), 5.0μl water and 50μl of a freshly prepared solution of 0.2M NaOH + 1.0mM EDTA were added and the sample incubated at 37°C for 30min to denature the DNA. The sample was then precipitated with 0.1x volume 3M sodium acetate buffer (3.1.2.1) and 2x volume of ethanol at -70°C for 25min as described (3.1.3.6). The pellet was resuspended in 7.0μl water and to ensure complete dissolution of the DNA, the sample was incubated at 65°C for 5min.

The sequencing reaction proceeded as follows: To the sample dissolved in 7.0μl of water, 2.0μl of 5x sequenase buffer and either 1.0μl (0.5 pmoles) of M13 -40 primer (antisense strand), or M13 reverse primer (sense strand) was added. Primer and plasmid DNA were allowed to aneal at 37°C for 20min, and DNA synthesis from the primer was started by the addition of 1.0μl 0.1uM DTT, 2.0μl labelling mix (0.2x dilution of the kit stock), 1.0μl [^{35}S] dATP (10mCi/ml) and 2.0μl sequenase (the enzyme was diluted prior to use: 1.0μl enzyme + 8.0μl enzyme dilution buffer). The mixture was lightly tapped to mix and extension was allowed to run for 5min at room temperature. During this time, four 0.5ml microfuge tubes were labelled A,G,C,T and 2.5μl of the appropriate termination mixture dispensed.

The synthesis reaction was terminated by the addition of 3.5μl of the mixture to each of the four tubes followed by incubation at 37°C for 5min. After this time, 4.0μl of stop solution containing formamide was added and the sample stored at -20°C until required. Prior to loading, the sample was denatured by heating in a boiling water bath for 3min, then placed on ice and 4.0μl was loaded immediately. The remainder of the sample was stored at -20°C. Loaded samples were electrophoresed at 50W until the bromophenol blue in the stop solution had reached the bottom of the gel. The gel was then removed and allowed to cool to room temperature, after which it was dismantled and fixed in a solution of 10% methanol + 10% glacial acetic acid for 20min. It was then dried at 80°C for 2h and exposed to Kodak X-omat AR film overnight at room temperature in a light-proof cassette. The film was developed for 3 min in Kodak developer and replenisher solution and fixed for 5min in Kodak fixer and replenisher.

3.1.3.19. Analysis of nucleotide sequence.

Nucleotide sequences obtained were entered into the FASTA program in the GCG package (University of Wisconsin Genetics Computer Group) on the SERC seqnet facility at Daresbury, UK. This was used to compare nucleic acid sequences obtained with other sequences in the Genbank and EMBL databases.

3.1.3.20. cDNA library immunoscreening.

A MAC16 cDNA library was contructed by Stratagene Limited from 20μg mRNA isolated as described (3.1.3.1. and 3.1.3.2). The cDNA was synthesised using both Oligo $(dT)_{12\text{-}18}$ and random primers, size-fractionated to select cDNA greater than 400bp and inserted into lambda Uni-ZAP XR unidirectionally in the sense orientation between the *Eco* R1 and *Xho* 1 restriction sites. During quality control tests run by the company, the library was assessed by actin screening, blue/white colour assessment and excision to check insert size. A primary library of 1.4 x 10^6 plaque forming units and an amplfied

library of 3.7 x 10^{11} were made and on arrival they were stored at -70°C as 250μl and 1.0ml aliquots after the addition of dimethylsulphoxide (DMSO) (7% final concentration).

The library was screened with polyclonal antisera raised in rabbits against a synthetic peptide based on the first fourteen amino acid residues of the cachectic factor protein sequence. The peptide was synthesised on a Synergy 4320 A Biosynthesiser (Applied Biosystems Ltd.) by Dr. D Poyner. In order to induce an immune reponse, the peptide was linked to keyhole limpet haemocyanin and rabbits were innoculated and sera monitored for the presence of antibodies by Dr. P. Cariuk and collegues.

3.1.3.20.1. Preparation of competant E.coli XL1-Blue MRF' cells

The method of screening followed was a modification of the protocol provided with the Picoblue™ immunoscreening kit. An innoculum from a glycerol stock of *E. coli* XL1-Blue MRF' was streaked onto an LB-agar plate (3.1.2.6.13) containing 12.5μg/ml tetracycline, and incubated overnight at 37°C. This stock plate was sealed with parafilm and stored at 4°C for one week after which a fresh plate was streaked. A single colony was picked and grown overnight in LB-broth supplemented with 10mM $MgSO_4$ and 0.2% maltose (3.1.2.5.1) overnight at 37°C in a shaking incubator. An aliquot (0.5ml) from this cell suspension was added to 50ml supplemented LB-broth and the cells grown to mid-log phase (OD_{600nm} 0.3-0.5) They were harvested by centrifugation (2000g, 10min), and resuspended in 10mM $MgSO_4$ (OD_{600nm}0.5) the cells were stored of ice and remained viable for two days.

3.1.3.20.2. Immunoscreening Lambda UNI-ZAP™ plaques.

Approximately 4 x 10^4 plaque-forming units from the cDNA library were incubated with 600μl *E. coli* XL1-blue MRF' cells (OD_{600nm}0.5) prepared as described in section 3.1.3.20.1, for 15min to allow phage to attach to the cells. Molten NZY top-agar (7.5ml, 48°C) (3.1.2.5.11) was then added and the mixture poured onto pre-warmed

and dried 140mm NZY agar plates (3.1.2.5.10)). Plates were incubated at 42°C until plaques were just visible (3.0-3.5h), circular nitrocellulose membranes (132mm) pre-soaked in 10mM isopropyl β-D-thiogalactopyranoside (IPTG, 3.1.2.6.8) were placed onto the surface of the agar and the plates incubated for 3.5h at 37°C. The membranes were orientated by piercing with a sterile needle at appropriate points, then they were removed and washed in four changes of TBST (3.1.2.5.3) for 15min each. Non-specific binding sites were blocked by incubation in TBS (3.1.2.5.2) + 1% BSA (w/v) overnight at 4°C. The membranes were incubated in TBS + 1% BSA (w/v) + polyclonal antiserum (1:1000) for 2h at room temperature. The serum had previously been titred to obtain a suitable dilution, and treated to reduce background binding caused by the presense of antibodies against *E.coli* and phage proteins (as described in the Picoblue™ immunoscreening kit protocol, Stratagene Ltd.). The diluted serum solution was stored at 4°C after the addition of sodium azide (0.05%, w/v); the solution was used four times without loss of signal, with significant reduction of background. After three washes with TBST, 15min each, the membranes were incubated in TBS + 1% BSA + 0.25mg/ml Protein A conjugated with alkaline phosphatase for 1h at room temperature. The membranes were washed with TBST and incubated in colour development solution (3.1.2.5.5) containing substrates 0.3mg/ml of nitroblue tetrazolium (NBT, 3.1.2.5.8) and 0.15mg/ml of 5-bromo-4-chloro-3-indolylphosphate (BCIP, 3.1.2.5.7) in the dark for 30-60min. The reaction was then terminated by incubation of the membranes in stop solution (3.1.2.5.6).

3.1.3.20.3. Isolation of positive phages.

Positive plaques were removed in plugs of agar using the wide end of a yellow pipette tip and allowed to elute in 500μl SM buffer (3.1.2.5.12) + 20μl chloroform, overnight at 4°C. The phage suspensions were vortexed and plated at three dilutions in SM buffer: 1:200, 1:2000, 1:20,000 on 82mm NZY-agar plates with 200μl *E.coli* XL1-blue MRF' (OD_{600nm} 0.5) (3.1.3.17.1) in 3.0ml NZY top agar. Plaque development,

IPTG-protein induction and antibody screening were as described (3.1.3.21.2.). This procedure was repeated until a single homogenous phage had been isolated this was then stored in 500μl SM buffer + 20μl chloroform for further analysis.

3.1.3.20.4. Excision of pBluescript phagemid

This procedure allowed analysis of the cloned insert in a plasmid system. The excision protocol was as described in the Uni-ZAP™ XR library instruction manual (Stratagene Ltd). Briefly, an innoculum from a glycerol stock of *E. coli* SOLR was streaked onto an LB-agar plate (3.1.2.6.13) containing 50μg/ml kanomycin, and incubated overnight at 37°C. This stock plate was sealed with parafilm and stored at 4°C for one week after which a fresh plate was streaked. A single colony was picked and grown overnight in 10ml of LB-broth (3.1.2.6.12) overnight at 37°C in a shaking incubator. An aliquot (0.5ml) from this cell suspension was added to 50ml of LB-broth and the cells grown to a cell density of 1.0 (OD_{600nm}).

An aliquot (0.5ml) from an overnight 10ml culture of *E.coli* XL1-blue MRF' (3.1.3.20.1) was grown in 50ml LB broth until the cell density was 0.5 (OD_{600nm}). *E.coli* XL1-blue MRF' cells were harvested by centrifugation (2000g, 10min) and resuspended in 10mM $MgSO_4$ to a density of 1.0 (OD_{600nm}) and 200μl was incubated with 250μl of phage suspension and 1.0μl of ExAssist helper phage for 15min at 37°C. The excision reaction was allowed to proceed overnight at 37°C after the addition of 3.0ml of LB broth after this time, cloudy growth could be seen. The cells were removed by centrifugation (2000g, 15min) and the supernatant containing excised phagemid pBluescript packaged as filamentous phage particles was transferred to a fresh tube and could be stored at 4°C for 1-2 months. pBluescript plasmid was obtained by incubating 10μl of this phage stock with 200μl *E.coli* SOLR cells (OD_{600nm}1.0) for 15min at 37°C and streaking 10μl onto a LB agar plate + ampicillin (50μg/ml). Colonies obtained after overnight incubation contained double-stranded pBluescript plasmid with the cloned

cDNA insert and were analysed by PCR, purified and sequenced as described. (3.1.3.15-3.1.3.19).

3.2. RESULTS

3.2.1. Methods employing the polymerase chain reaction (PCR)

3.2.1.1. General introduction

N-terminal amino acid sequence analysis of 24kD immunogenic material isolated from MAC 16 tumour extract yielded a homogeneous sequence of 18 amino acids (figure 3.2.1.1.1). No further sequence information was obtained by protease digestion thus suggesting that the sequence obtained may represent the total protein content of the material. This was confirmed by more recent experiments which showed that the material was composed of a 2kD core protein and that its apparent molecular mass of 24kD was due to the presence of N- and O-glycan chains, some of which were of the glycosaminoglycan type containing sulphate residues (Todorov *et al,* in press). Attempts to isolate the cDNA from MAC 16 tumour total cDNA therefore involved the use of PCR with degenerate oligonucleotides based on the 18-amino acid sequence. A modification of the method employed to isolate the cDNA for mitochondrial NADH-ubiquinone reductase (Pilkington *et al,* 1991) was used. Briefly, four forward oligonucleotides based on the first six amino acids of the 24kD material were designed to include all amino acid codon possibilities and to have minimum degeneracy at the 3' end (figure 3.2.1.1.2). Two reverse oligonucleotides based on the last six amino acids of the 24kD material were designed to cover all six options for serine with minimum degeneracy (figure 3.2.1.1.3). The oligonucleotides were used in all combinations with a MAC 16 DNA template in order to obtain a 53bp PCR product containing the internal 18bp sequence. Sequence determination of such a product would allow synthesis of a non-degenerate oligonucleotide which could be used as a probe for isolation of the cDNA. The main modification of the published method involved the addition of tetramethylammonium chloride (TMAC) to the PCR mix in order to eliminate the preferential melting of A-T versus G-C base pairs (Wood *et al,* 1985). Oligonucleotides based on the human GSTT1

gene sequence (figure 3.2.1.1.9) were included in all experiments in order to test the quality of the cDNA and the efficiency of the PCR. Other controls included incubation of oligonucleotides without template and incubation of template with single oligonucleotides. PCR components were assembled and products analysed as described (3.1.3.7 and 3.1.3.8-3.1.3.19 respectively).

Figure 3.2.1.1.1. **N-Terminal amino acid sequence of P24**

Y D P E A A S A P G S G N P S H E A

Figure 3.2.1.1.2. **Forward oligonucleotides: Based on YDPEAA**

	Y	D	P	E	A	A		
5'	TAC T	GAC T	CCA G C T	GAA G	GCA	GC	3'	**(A)**
5'	TAC T	GAC T	CCA G C T	GAA G	GCG	GC	3'	**(B)**
5'	TAC T	GAC T	CCA G C T	GAA G	GCC	GC	3'	**(C)**
5'	TAC T	GAC T	CCA G C T	GAA G	GCT	GC	3'	**(D)**

Properties of oligonucleotides A-D:

Degeneracy		32
Melting temperature (°C)		48-58
MW		5610
OD_{260nm}	**A** **B** **C** **D**	18.2 18.2 17.2 21.6

Figure 3.2.1.1.3. **Reverse oligonucleotides: Based on NPSHEA**

	N	P	S	H	E	A		
3'	TTA	GGA	AGA	GTA	CTT	CG	5'	(E)
	G	G	G	G	C			
		C	C					
		T	T					
3'	TTA	GGA	TCA	GTA	CTT	CG	5'	(F)
	G	G	G	G	C			
		C						
		T						

Properties of reverse oligonucleotides E & F:

Degeneracy: E	128
F	64
Melting temperature (°C)	48-58
MW	5610
OD_{260nm} : E	24.1
F	20.5

Figure 3.2.1.1.4. **P24 internal DNA sequence as predicted from the six internal amino acids of the N-terminal sequence**

YDPEAASAPGSGNPSHEA

	S	A	P	G	S	G	
5'	TCA	GCA	CCA	GGA	TCA	GGA	3'
	AGG	G	G	G	AGG	G	
	C	C	C	C	C	C	
	T	T	T	T	T	T	

Figure 3.2.1.1.5. **Nested reverse oligonucleotides: Based on GNPSHE**

	G	N	P	S	H	E		
3'	CCA G C T	TTA G	GGG C	AGA G C T	GTA G	CT	5'	**(G)**
3'	CCA G C T	TTA G	GGG C	TCA G	GTA G	CT	5'	**(H)**

Properties of nested oligonucleotides G & H:

Degeneracy	128
Melting temperature (°C)	50-58
MW	5610
OD_{260nm} : G	14.9
H	21.3

Figure 3.2.1.1.6. **Gene-specific primer used for cDNA synthesis:** Based on the last two amino acids of the protein sequence (antisense format for binding to mRNA)

YDPEAASAPGSGNPSHEA

E **A**

3' 5'

CTT CGA

C G

C

T

Figure 3.2.1.1.7. **Universal dT oligonucleotide (dT):**

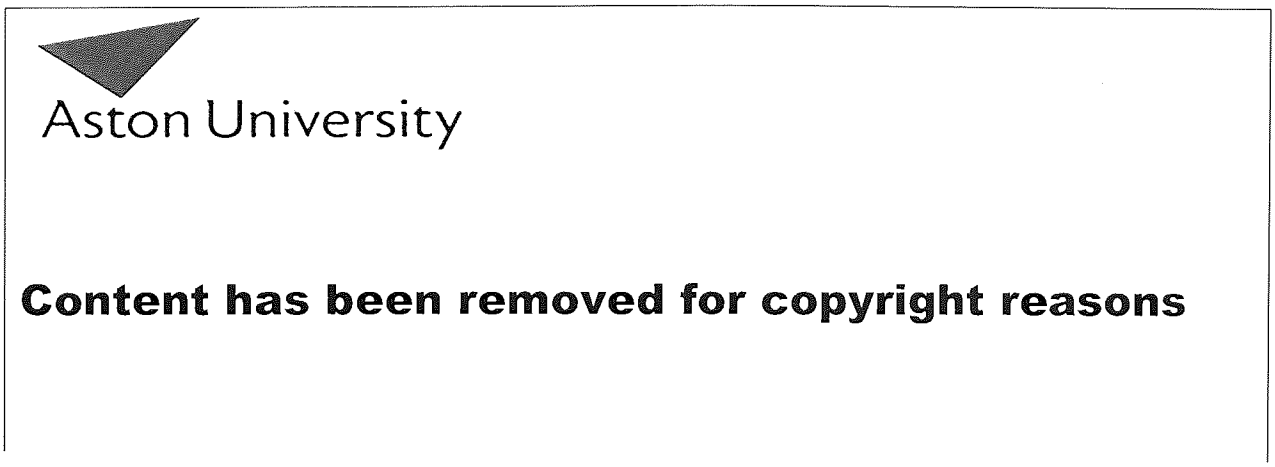
5' GCGGCCGCTTTTTTTTTTTTTT 3'

Figure 3.2.1.1.8. **Sequencing oligonucleotides:** M13 -40 and reverse oligos

5' GTTTTCCCAGTCACGAC 3' **(M13,-40)**

5' TTCACACAGGAAACAG 3' **(M13, R)**

Figure 3.2.1.1.9. **Oligonucleotides based on the human GSTT1 gene sequence (1002bp) (Pemble *et al*, 1994):**



Oligonucleotide based on the rat GST su 12 gene sequence (reverse):

$^{5'}$TCAGGGAATCCTGGCAATTC$^{3'}$ **(298)**

Products obtained by PCR analysis of human GSTT1 cDNA using oligonucleotides to the GST gene sequence:

Oligos (F+R)	297+299	297+481	297+198	297+827	297+298
PCR product (bp)	53	87	100	657	750

*3.2.1.2. **PCR method (1):** Random primed cDNA + oligonucleotides A, B, C, D,E, F*

All combinations of forward and reverse oligonucleotides were incubated with cDNA synthsised from MAC 16 tumour mRNA by random priming (3.1.3.4). Total RNA was isolated from MAC 16 tumours excised from mice with a mean weight loss of 4.0g. The integrity of total RNA was tested by formaldehyde gel electrophoresis prior to fractionation for isolation mRNA. The presence of the 28S and 18S subunits of ribosomal RNA with a quantity ratio of approximately 2:1 (figure 3.2.1.2.1) confirmed that minimum degradation had occurred during the isolation process. Total RNA was quantified by measurement of its absorbance at 260nm and purity assessed by comparison of the absorbance at 260nm and 280nm. A 260nm : 280nm ratio of two was an indication of minimum contamination of the sample with protein (table 3.2.1.2.1).

The efficiency of the reverse transcriptase reaction of cDNA synthesis was tested by PCR with oligonucleotides based on the human GSTT1 cDNA sequence. PCR products of the correct size were obtained (figures 3.2.1.2.2 and 3.2.1.2.3) indicating that the 5' region of the mRNA representing the 18 N-terminal amino acids was represented in the pool of cDNA synthesised.

The initial PCR involved eight reactions to include all oligonucleotide combinations and these were performed at anealling temperatures 50°C-56°C in order to cover the possible melting temperatures for the degenerate oligonucleotides (figures 3.2.1.1.2 and 3.2.1.1.3). Most of the PCR reactions produced non-specific, high molecular weight material (figure 3.2.1.2.4). The samples were electrophoresed by non-denaturing PAGE (3.1.3.9); a strip of gel corresponding to the migration of 53bp was then cut from all the lanes and the eluted cDNA was amplified further using all oligonucleotide combinations (figure 3.2.1.2.5). Re-amplification resulted in two PCR products of approximately 53bp with oligonucleotides B+E and B+F (figure 3.2.1.2.5 lanes 3&4 respectively). These products were eluted and cloned into pUC 18 plasmid. The insert size of purified plasmid preps were tested by restriction enzyme digestion (figure 3.2.1.2.6) and sequenced. Inserts of 17-28bp were obtained and found to

comprise seven different sequences (table 3.2.1.2.2), the most frequently occurring of which was for a 21bp insert. Though two of the PCR products had an insert of the correct size of 19bp, none of the sequences matched that predicted for the six internal amino acids (figure 3.2.1.1.4). A database search (EMBL and Genbank) failed to yield a match for the most frequent sequence obtained.

Figure 3.2.1.2.1.

Formaldehyde gel electrophoresis of total RNA (5μg) extracted from the MAC 16 tumour:

Analysis on a 1.2% agarose gel

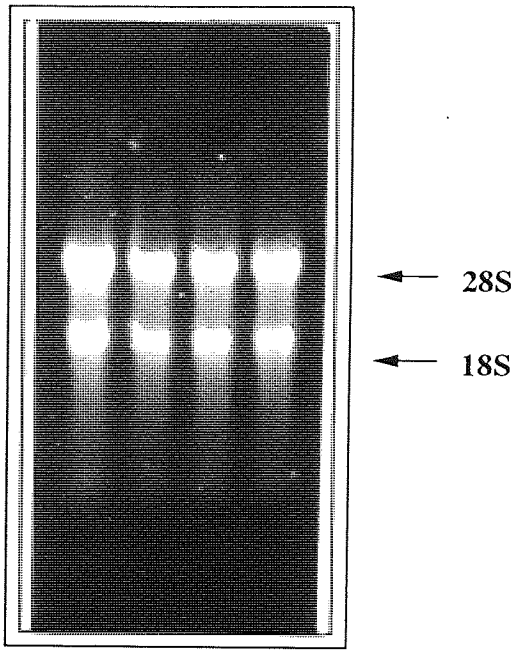


Table 3.2.1.2.1.

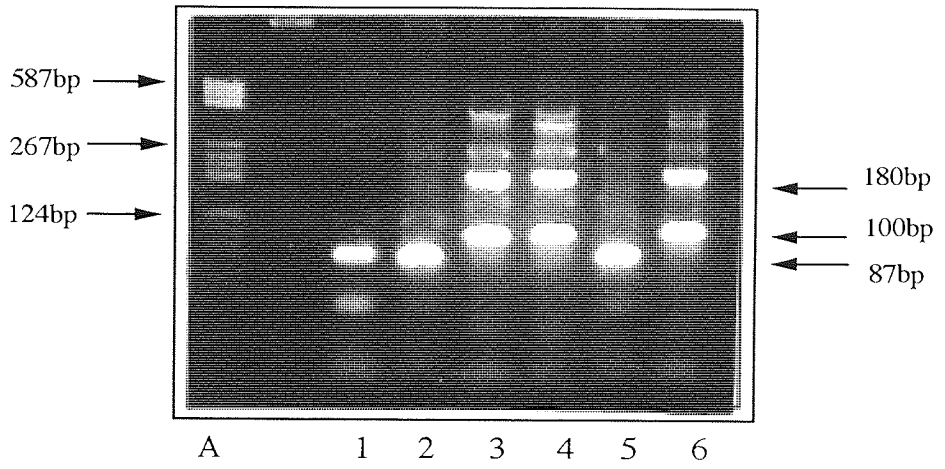
Extraction of total RNA from four MAC 16 tumours:

Tumour	Weight loss (g)	Tumour size (g)	Total RNA (mg)	260:280nm	mRNA (μg)
1	5.3	0.28	1.3	1.9	35.5
2	4	0.33	0.9	1.9	
3	3.1	0.31	0.98	2	
4	3.3	0.35	0.88	2.1	

Figure 3.2.1.2.2.

PCR analysis of cDNA from MAC 16 tumour using oligonucleotides to the human GSTT1 cDNA sequence:

Analysis of products on a 4% agarose gel



1: MAC 16 cDNA (1) + oligos 297+481

2: MAC 16 cDNA (2) + oligos 297+481

3: MAC 16 cDNA (1) + oligos 200+198

4: MAC 16 cDNA (2) + oligos 200+198

5: MAC 16 cDNA (3) + oligos 297+481

6: MAC 16 cDNA (3) + oligos 200+198

A: pBR322/*Hae* 111

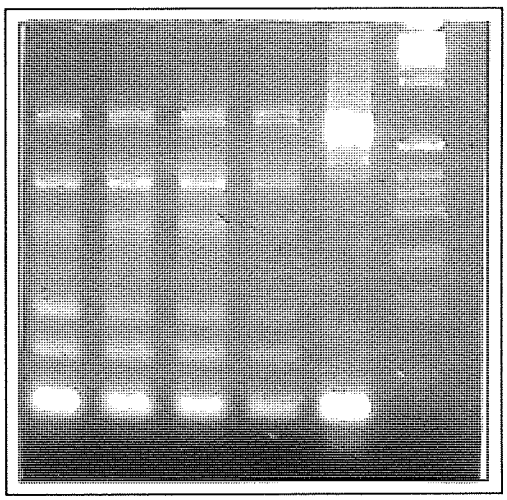
cDNA (1-3) represent cDNA prepared from three separate mRNA preps

PCR temperatures (°C): Denaturation: 94 (1min)
Anealling: 55 (1min)
Extension: 72 (1min)
] 30 cycles

Figure 3.2.1.2.3.

PCR analysis of MAC16 tumour cDNA using oligonucleotides to the human GSTT1 cDNA sequence designed to yield a 53bp product:

Analysis of products on a 4% agarose gel



1: 50ng MAC 16 cDNA + oligos 297+299

2: 5ng MAC 16 cDNA + oligos 297+299

3: 0.5ng MAC 16 cDNA + oligos 297+299

4: 0.05ng MAC 16 cDNA + oligos 297+299

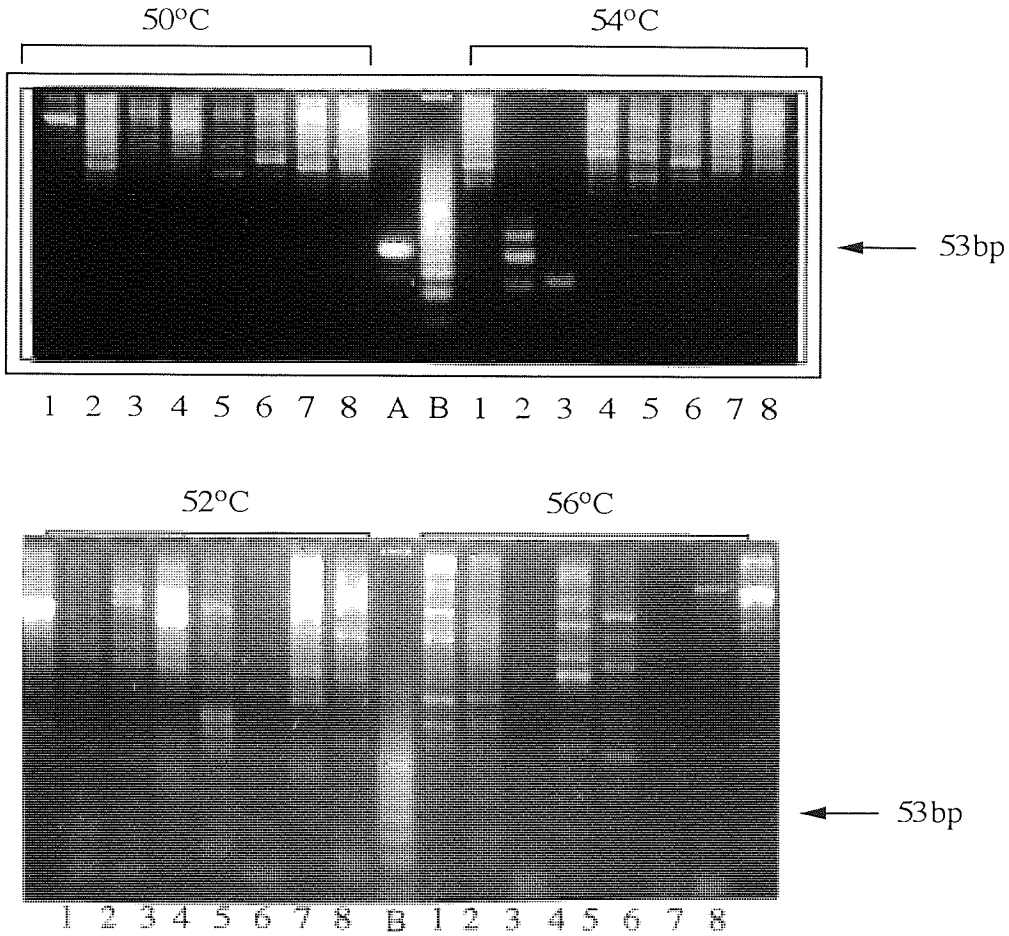
5: 5ng GSTT1 cDNA + oligos 297+299

A: 1kb ladder

PCR temperatures (°C): Denaturation: 94 (1min)
Anealling : 55 (1min)
Extension: 72 (1min)
] 30 cycles

Figure 3.2.1.2.4.

PCR analysis of MAC 16 tumour cDNA (5ng) using oligonucleotides A,B,C,D (forward) & E,F (reverse):
Analysis of products on a 4% agarose gel



1: 5ng MAC 16 cDNA + oligos A+E
2: 5ng MAC 16 cDNA + oligos A+F

3: 5ng MAC 16 cDNA + oligos B+E
4: 5ng MAC 16 cDNA + oligos B+F

5: 5ng MAC 16 cDNA + oligos C+E
6: 5ng MAC 16 cDNA + oligos C+F

7: 5ng MAC 16 cDNA + oligos D+E
8: 5ng MAC 16 cDNA + oligos D+F

A: 5ng MAC 16 cDNA + oligos 297+299
B: 10bp ladder

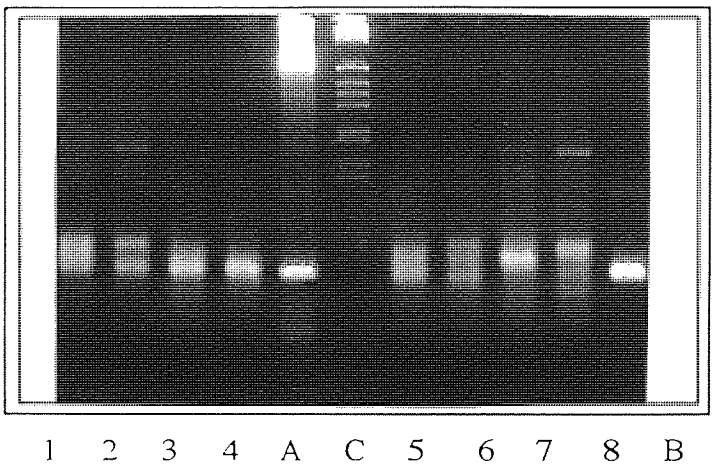
PCR temperatures (°C):

Denaturation: 94 (1min) Anealling: as indicated (2min) Extension: 72 (20sec)	30 cycles

Figure 3.2.1.2.5

Amplification of PAGE-eluted PCR products by PCR using oligonucleotides A,B,C,D (forward) & E,F (reverse):

Analysis on a 4% agarose gel



1: Eluted product (A+E) + oligos A+E
2: Eluted product (A+F) + oligos A+F

3: Eluted product (B+E) + oligos B+E
4: Eluted product (B+F) + oligos B+F

5: Eluted product (C+E) + oligos C+E
6: Eluted product (C+F) + oligos C+F

7: Eluted product (D+E) + oligos D+E
8: Eluted product (D+F) + oligos D+F

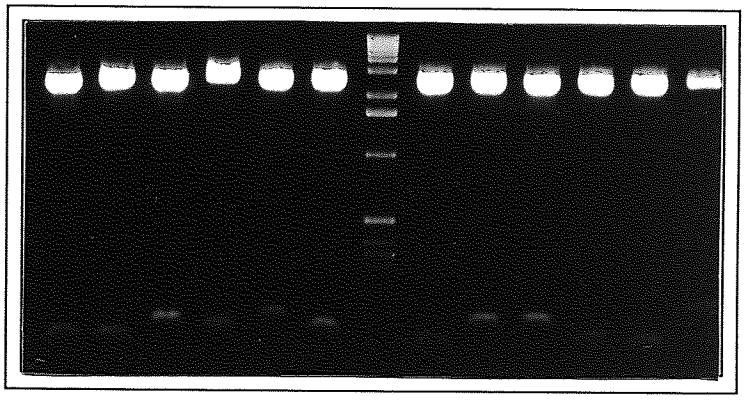
A: 5ng MAC16 cDNA + oligos 297+299
B: eluted product (297+299) + oligos 297+299
C: 1kb ladder

PCR temperatures (°C): Denaturation: 94 (1min)
Annealing: 55 (1min)
Extension: 72 (20sec)
] 30 cycles

Figure 3.2.1.2.6.

Restriction enzyme digest (*Pst*1 & *Sst*1) of plasmid DNA isolated from 12 transformed *E.coli* JM83 colonies:

Analysis on a 1.5% agarose gel



A

A: 1kb ladder

Table 3.2.1.2.2.

PCR method 1: ***Sequence analysis of the 53bp products obtained by PCR using MAC 16 tumour cDNA and oligonucleotides B+E and B+F***

Sequence (sense orientation, 5'-3')	Insert size (bp)	Frequency (per 20 PCR products analysed)
CTTCAAAGAACGATGAACTAT*	21	10
CCCACCGACAAGCCTTTCTCC	21	3
TCATAGATGCTGTTGTAGG	19	1
TGCTGAGCGATGGGTTGGG*	19	1
CCTACAACAGCATCTATA	18	1
GGCCGGTTCCCCCTGGA	17	1
AAACTAACCAACCATATAATTAACCTCC	28	2

* Identical sequence obtained with oligo combinations B+E, B+F and D+F (table 3.2.1.3.1)

3.2.1.3. ***PCR method 2:*** *Specific primer-primed cDNA + oligonucleotides A, B, C, D, E, F.*

A degenerate hexamer based on the last two amino acids of the N-terminal sequence (figure 3.2.1.1.6) was used to prime cDNA synthesis in an attempt to increase representation of the correct gene in the total cDNA pool. PCR was then performed with all oligonucleotides at four anealling temperatures (figure 3.2.1.3.1), PCR products in the area of 53bp were eluted and re-amplified as described (3.2.1.2). Several products of approximately 53bp were obtained by this method (figure 3.2.1.3.2) and these were tested further by PCR using forward oligonucleotides A, B, C, D and nested oligonucleotides G,H (figure 3.2.1.1.5). A 53bp product with the correct sequence would be expected to be a template for the nested oligonucleotides resulting in the formation of a 50bp PCR product. Two 53bp products using oligonucleotides A+E and D+F were identified by this method (figure 3.2.1.3.3, lanes 1&2 and 19&20) and were tested further by PCR at an anealling temperature of 60°C. The presence of two G-C bonds involved in the binding of the 3' end of the nested oligonucleotide to the correct template (figure 3.2.1.1.5) would confer a high degree of stability to the 50bp product and ensure its formation even at high anealling temperature. The 53bp PCR product formed with oligonucleotides A+E failed to be an efficient template for binding of the nested oligonucleotide at 60°C (figure 3.2.1.3.4, lanes 1&2) in contrast, a 50bp band was observed when the 53bp PCR product formed with oligonucleotides D+F was used as a template (figure 3.2.1.3.4, lanes 3&4). This 53bp product was therefore cloned into pUC 18 plasmid and sequenced. Seven sequences were obtained for the seventeen plasmid inserts analysed (table 3.2.1.3.1) two of which were identical to sequences obtained observed previously (table 3.2.1.2.2). Four of the sequences revealed an insert of the correct size of 19bp but none of these matched the predicted internal sequence (figure 3.2.1.1.4).

A nested PCR product was observed to be stable at anealling temperatures of 62°C and 64°C as shown in figure 3.2.1.3.5. in which the 50bp product was electrophoresed

further and loaded adjacent to a 10bp ladder in order to obtain a more accurate assessment of its size. This product was cloned into pUC 18 plasmid and plasmid DNA isolated from transformed *E.coli* JM83 transformed colonies was screened by PCR at anealling temperatures 60°C and 62°C (figure 3.2.1.3.6 a & b respectively). Three inserts giving rise to stable nested products were analysed by sequencing.

Sequence analysis revealed inserts of 15bp, 16bp and 13bp for the 50bp PCR products (table 3.2.1.3.2). Although an insert of the correct size had been obtained (16bp), none of the sequences matched that predicted for the correct insert (figure 3.2.1.1.4). The nested insert sequences obtained were observed to match those for three of the 53bp products previously analysed (3.2.1.3.1). The explanation for the stability of these products which allowed their formation at high anealling temperatures was revealed by sequence analysis which confirmed the presence of G-C bonds at the site of initiation of DNA synthesis by Taq polymerase (figure 3.2.1.3.7).

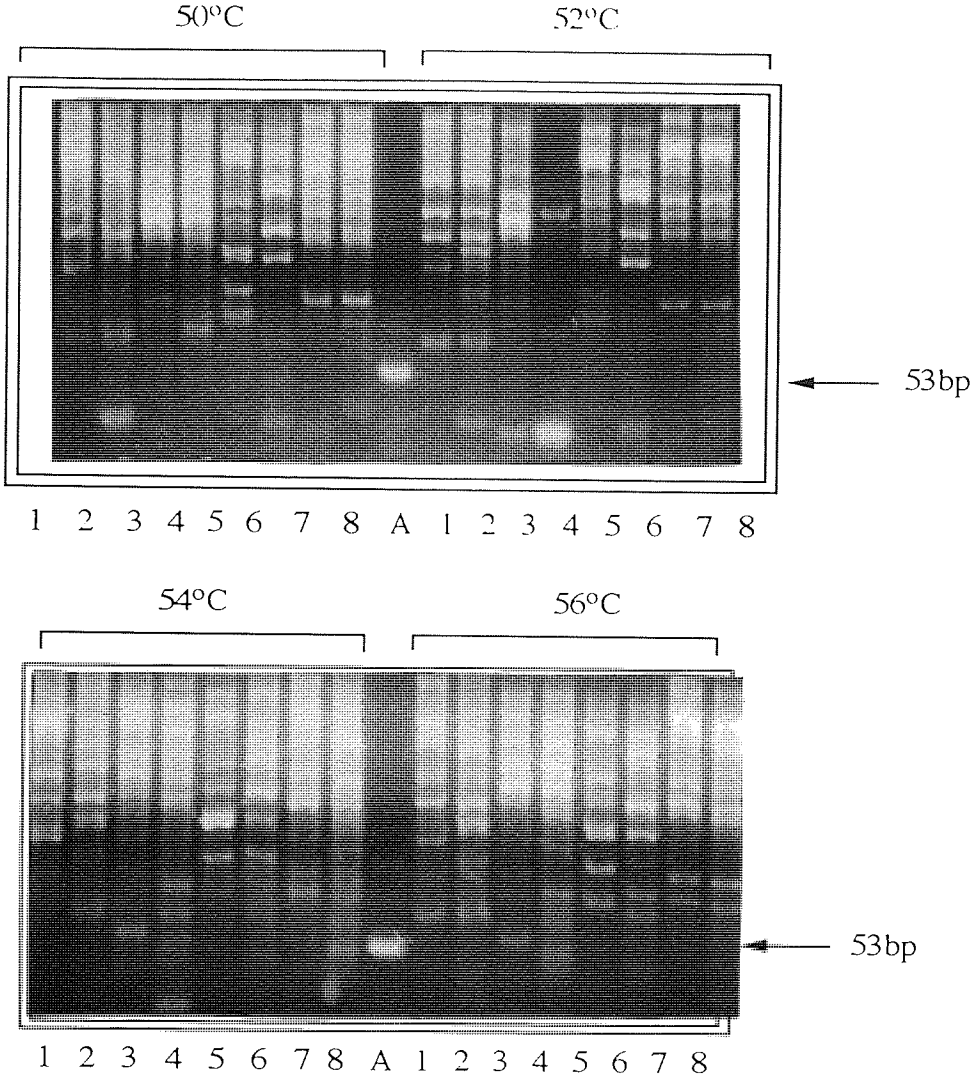
3.2.1.4. Conclusion

Efforts to isolate the cDNA for the 24kD material using oligonucleotides based on the six N-terminal and six carboxy amino acids of the 18-amino acid protein sequence were unsuccessful. PCR products obtained that were apparently homogeneous and of the correct size, as determined by agarose gel electrophoresis were revealed by cloning and sequence analysis to consist of several products. In total, 53 inserts were analysed and although five were of the correct size (19bp) a match with the DNA sequence predicted for the internal six amino acids was not observed.

Figure 3.2.1.3.1.

PCR analysis of MAC 16 tumour cDNA (5ng) using oligonucleotides A,B,C,D (forward) & E,F (reverse):

Analysis on a 4% agarose gel



1: 5ng MAC 16 cDNA + oligos A+E
2: 5ng MAC 16 cDNA + oligos A+F

3: 5ng MAC 16 cDNA + oligos B+E
4: 5ng MAC 16 cDNA + oligos B+F

5: 5ng MAC 16 cDNA + oligos C+E
6: 5ng MAC 16 cDNA + oligos C+F

7: 5ng MAC 16 cDNA + oligos D+E
8: 5ng MAC 16 cDNA + oligos D+F

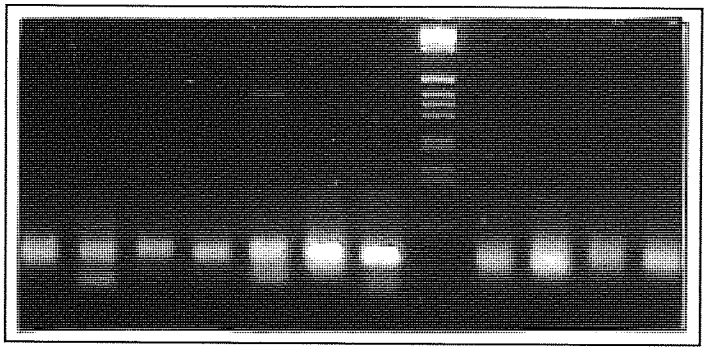
A: 5ng MAC 16 cDNA + oligos 297+299

PCR temperatures (°C):
Denaturation: 94 (1min)
Annealing: as indicated (2min)
Extension: 72 (20sec)
30 cycles

Figure 3.2.1.3.2.

Amplification of PAGE-eluted PCR products by PCR using oligonucleotides A,B,C,D (forward) & E,F (reverse):

Analysis on a 4% agarose gel



1: Eluted product (A+E) + oligos A+E
2: Eluted product (A+F) + oligos A+F

3: Eluted product (B+E) + oligos B+E
4: Eluted product (B+F) + oligos B+F

5: Eluted product (B+E) + oligos B+E
6: Eluted product (B+F) + oligos B+F

Product from PCR method 1 (figures 3.2.1.2.5., lanes 3&4)

7: Eluted product (C+E) + oligos C+E
8: Eluted product (C+F) + oligos C+F

9: Eluted product (D+E) + oligos D+E
10: Eluted product (D+F) + oligos D+F

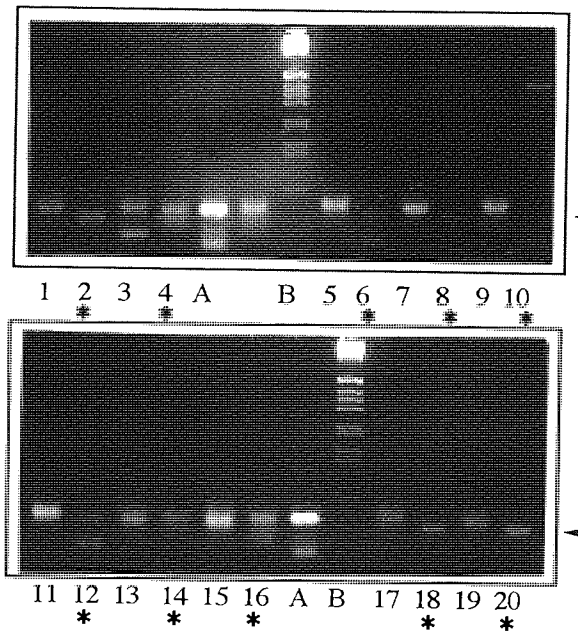
A: MAC 16 cDNA + oligos 297+299
B: 1kb ladder

PCR temperatures (°C): Denaturation: 94 (1min)
Anealling: 55 (1min)
Extension: 72 (20sec)

30 cycles

Figure 3.2.1.3.3.

PCR analysis of PAGE-eluted PCR products using oligonucleotides A, B, C, D (forward) and G, H (nested):
Analysis on a 4% agarose gel

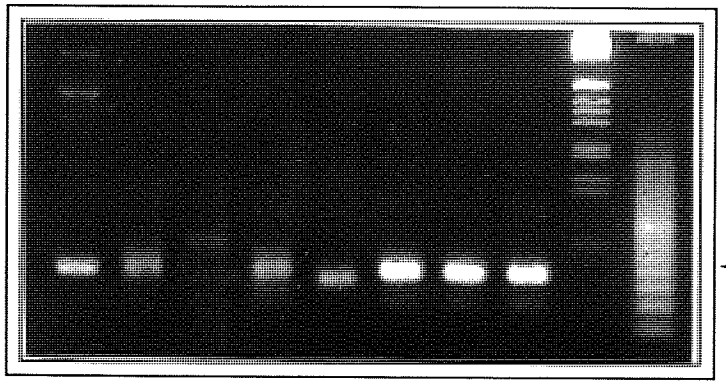


Lane	PAGE-eluted 53bp PCR product	Oligos	Expected PCR product (bp)
1	(A+E)	A, E	53
*2	(A+E)	A, G	50
3	(A+F)	A, F	53
*4	(A+F)	A, H	50
5	(B+E)	B, E	53
*6	(B+E)	B, G	50
7	(B+F)	B, F	53
*8	(B+F)	B, H	50
9	(B+E)	B, E	53
*10	(B+E)	B, G	50
11	(B+F)	B, F	53
*12	(B+F)	B, H	50
13	(C+E)	C, E	53
*14	(C+E)	C, G	50
15	(C+F)	C, F	53
*16	(C+F)	C, H	50
17	(D+E)	D, E	53
*18	(D+E)	D, G	50
19	(D+F)	D, F	53
*20	(D+F)	D, H	50

Figure 3.2.1.3.4.

Verification of PAGE-eluted PCR products by PCR using oligonucleotides A, D (forward) and G, H (reverse, nested) at an elevated anealling temperature:

Analysis on a 4% agarose gel



1: Eluted PCR product (A+E) + oligos A and E

* **2**: Eluted PCR product (A+E) + oligos A and G

3: Eluted PCR product (D+F) + oligos D and F

* **4**: Eluted PCR product (D+F) + oligos D and H

A: Eluted PCR product (297 + 299) + oligos 297 and 299
B: 1kb ladder
C: 10 bp ladder

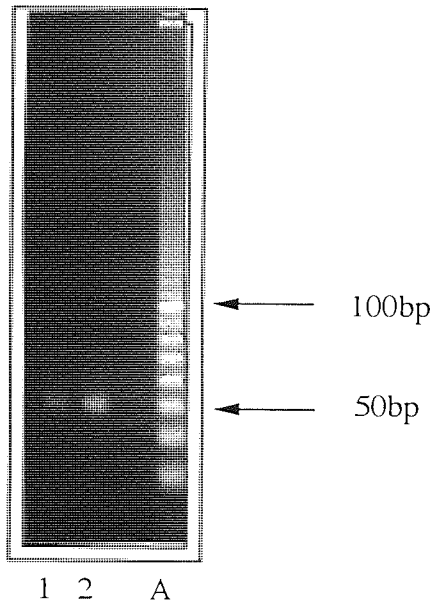
* Nested PCR

PCR temperatures (°**C**): Denaturation 94 (1min)
Anealling 60 (1min)
Extension 72 (20sec)

Figure 3.2.1.3.5.

Verification of PAGE-eluted 53bp PCR product (D+F) by nested PCR with oligos D and H at anealling temperatures 62 and 64°C

Analysis on a 4% agarose gel



1: Eluted PCR product (D+F) + oligos D and H :**Anealling temperature: 64°C**

2: Eluted PCR product (D+F) + oligos D and H :**Anealling temperature : 62°C**

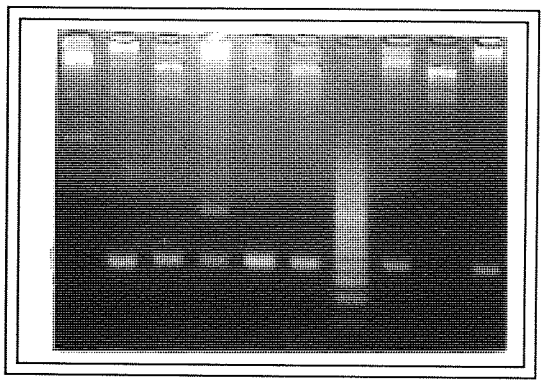
A: 10bp ladder

Figure 3.2.1.3.6.

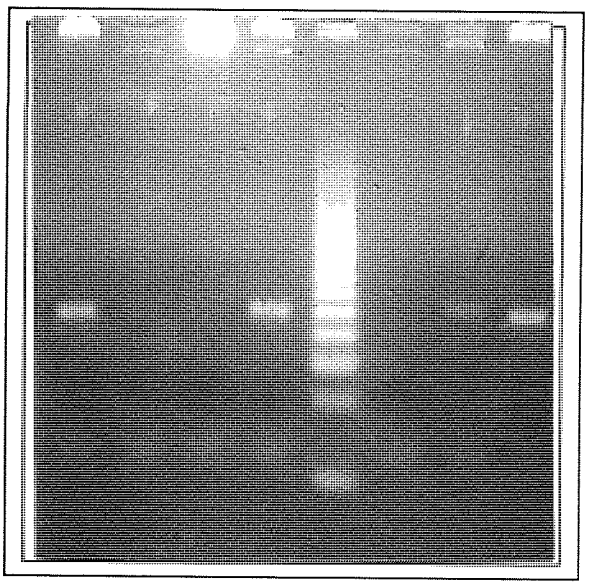
PCR analysis of plasmid DNA isolated from 12 transformed *E.coli* JM83 colonies using oligonucleotides D (forward), and H (reverse, nested):

Analysis on a 4% agarose gel

(A): Anealling temperature: 60°C



(B): Anealling temperature: 62°C



A: 10kb ladder

PCR temperatures (°C): Denaturation: 94 (1min)
Anealling: as indicated (1min)
Extension: 72 (20sec)
] 30 cycles

* Plasmid inserts analysed by sequencing

Table 3.2.1.3.1.

PCR method 2: ***Sequence analysis of the 53bp products obtained by PCR of MAC 16 tumour cDNA and oligonucleotides D+F***

Sequence (sense orientation, 5'-3')	Insert size (bp)	Frequency (per 17 PCR products analysed)
CTTCAAAGAACGATGAACTAT*	21	2
TGCTGAGCGATGGGTTGGG*	19	1
TCATTGAGAATCTGCGAG	18	8
ACTGTGAAACCCGGGC	16	2
TACGATCCTGAGGCTACTG	19	1
CTGCTATTATCCAACTCCC	19	1
ACAGATAGCACATGCCTTT	19	1
CCAGCGGTTCACATTTTACCTT	22	1

* Identical sequence obtained with oligo combinations B+E, B+F and D+F (table 3.2.1.2.2)

Table 3.2.1.3.2.

PCR method 2: ***Sequence analysis of the 53bp and nested 50bp PCR products that were stable at an anealling temperature of 62°C***

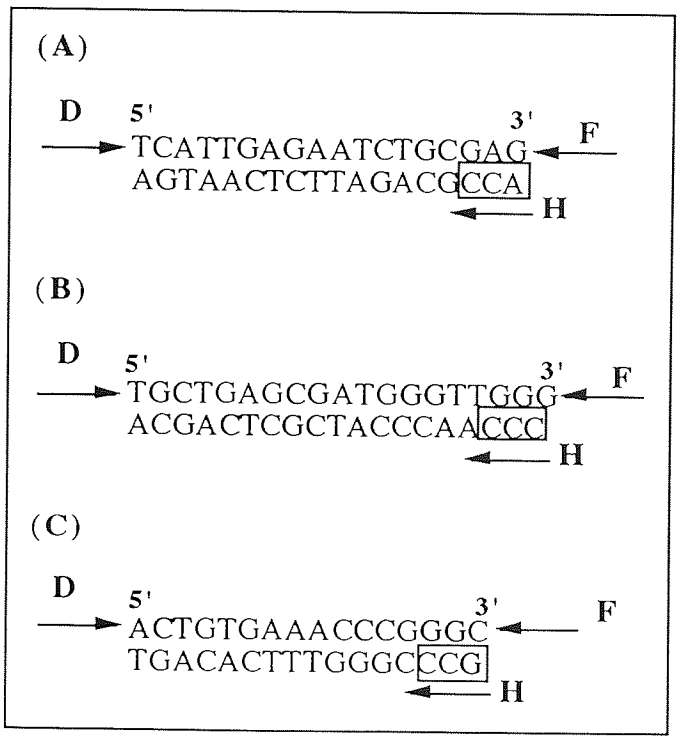
Full insert sequence (Sense format, 5'-3')	Insert size (bp)	Nested insert sequence (Sense format, 5'-3')	Nested insert size(bp)
(A) * TCATTGAGAATCTGCGAG	18	TCATTGAGAATCTGC	15
(B) * TGCTGAGCGATGGGTTGGG	19	TGCTGAGCGATGGGTT	16
(C) * ACTGTGAAACCCGGGC	16	ACTGTGAAACCCG	13

(* From table 3.2.1.2.2.)

Figure 3.2.1.3.7.

Sequence homology of the 3' base triplet of the nested oligo (H) with the 5' base triplet region of the 53bp insert

(A): Plasmid 2 (figure 3.2.1.3.6.)
(B): Plasmid 5 (")
(C): Plasmid 9 (")



*3.2.1.5. **PCR method 3:** Oligo dT primed cDNA + oligonucleotides A, B, C, D and universal oligonucleotide dT (dT)*

MAC 16 cDNA was synthesised by priming with the Oligo $(dT)_{12-18}$ primer (3.1.3.4). This binds to the poly A tail of mRNA, and its inclusion in the reverse transcriptase reaction should therefore result in the synthesis of large cDNA species representing the entire length of the gene. cDNA synthesised by this method was tested prior to use by electrophoresis of radioactively labelled cDNA through an alkaline agarose gel (3.1.3.4.1). Efficient synthesis would be observed as a band of radioactivity between 500-5000 bases, with the bulk of the label migrating at approximately 1500-2000bases (Sambrook *et al*, 1989). An additional test involved PCR with oligonucleotides based on the human GSTT1 gene sequence that were designed to result in large PCR products (figure 3.2.1.1.9). The cDNA was then analysed by PCR using forward oligonucleotides A, B, C, D and reverse oligonucleotide dT (figure 3.2.1.1.7) in a total of six reactions. Products that were reproducibly obtained in high yield were analysed further.

Analysis of the cDNA synthesised by oligo $(dT)_{12-18}$ priming by alkaline agarose gel electrophoresis confirmed that large cDNA species had been produced (figure 3.2.1.5.1). In addition, PCR analysis with oligonucleotides based on the GSTT1 gene sequence resulted in products of 548bp and 740bp when oligo $(dT)_{12-18}$ -primed cDNA was the template (figure 3.2.1.5.2); in contrast, amplification of the 740bp product was greatly reduced when random-primed cDNA was the template. These latter results suggested that the entire GSTT1 cDNA (1002bp) had been synthesised and served as a indicator of the efficient synthesis of all cDNA species. PCR analysis with oligonucleotides A, B, C, D and dT at temperatures 52°C, 55°C and 58°C resulted in one specific product of 398bp with oligonucleotides A+dT (figure 3.2.1.5.2, lane 1). This product was formed maximally at an anealling temperature of 55°C but was also present after PCR at 58°C. Other products observed were non-specific as determined by control PCR results using the oligonucleotides singly (results not shown).

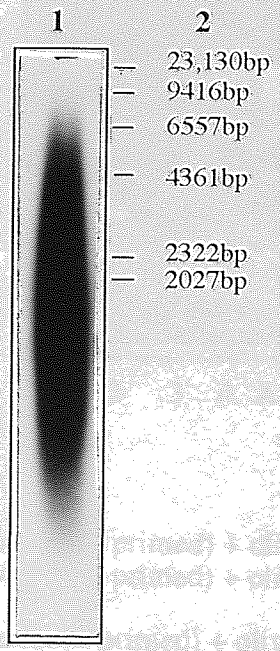
A sequence of 171bases was obtained following analysis of the cloned 398bp PCR product (figure 3.2.1.5.3) and a database search for similar sequences revealed a match with the gene for rat ribosomal protein L23 in 94.771% of the bases. The formation of this product was made possible by the identity of the 3' region of oligonucleotide A with bases 171-187 of the L23 gene sequence.

3.3.1.6. Conclusion:

Efforts to isolate the cDNA for the 24kD material by PCR with oligo $(dT)_{12-18}$ -primed template and oligonucleotides A, D, E, F, and dT with the aim of amplifying the entire gene, were unsuccessful. One product was obtained as a result of non-specific binding of oligonucleotide A, but no other products were observed. The failure to obtain full length PCR products may be due to limitations of the method as was evidenced by an attempt to test its potential by PCR using oligonucleotides 297 and dT in order to obtain a 1002bp product representing GSTT1 cDNA. No product was obtained by PCR using a range of anealling temperatures 60°C-66°C, a phenomenon also observed by other workers (Pemble and Taylor, personal communication).

Figure 3.2.1.5.1.

Analysis of the efficiency of oligo dT-primed cDNA synthesis by alkaline agarose gel electrophoresis



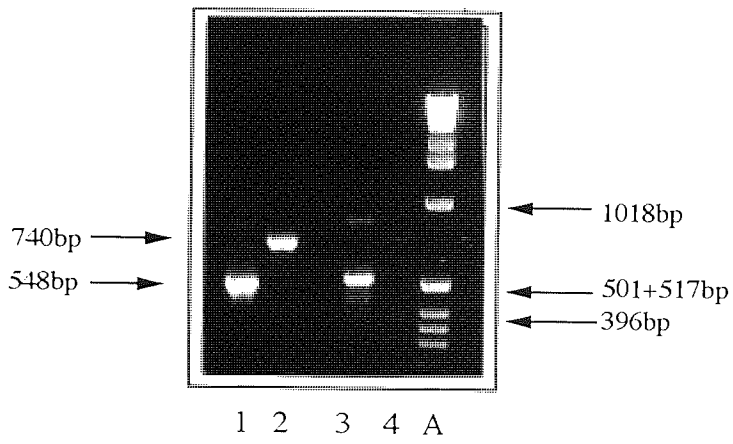
1: cDNA labelled with ^{32}P dCTP during the synthesis reaction and electrophoresed as described (2.2.3.5.1.)

2: Molecular weight markers: Lambda DNA/*Hind* III fragments

Figure 3.2.1.5.2.

PCR analysis of MAC 16 tumour cDNA using oligonucleotides to the human GSTT1 cDNA sequence to confirm the synthesis of full length cDNA by the oligo dT-priming method:

Analysis on a 1.5% agarose gel



1: MAC 16 cDNA (oligo dT-primed) + oligos 297 and 827
2: MAC 16 cDNA (oligo dT-primed) + oligos 297 and 295

3: MAC 16 cDNA (random-primed) + oligos 297 and 827
4: MAC 16 cDNA (random-primed) + oligos 297 and 295

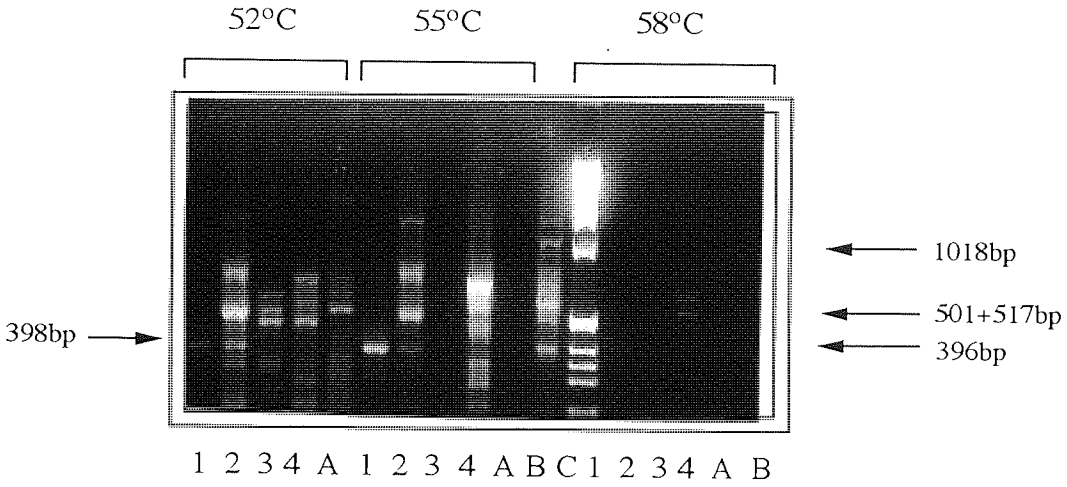
A: 1kb ladder

PCR temperatures (°C): Denaturation: 94 (1min)
Anealling: 55 (1min)
Extension: 72 (1min)
] 30 cycles

Figure 3.2.1.5.3.

PCR analysis of MAC 16 tumour cDNA using oligonucleotides A,B,C,D (forward) and universal dT primer (reverse):

Analysis on a 1.5% agarose gel



1: MAC 16 cDNA + oligos A+dT

2: MAC 16 cDNA + oligos B+dT

3: MAC 16 cDNA + oligos C+dT

4: MAC 16 cDNA + oligos D+dT

A: MAC 16 cDNA + oligos 297+dT

B: MAC 16 cDNA + oligos 299+dT

C: 1kb ladder

PCR temperatures (°C)**:** Denaturation: 94 (1min)
Anealling: As indicated (2min)
Extension: 72 (1min)
] 30 cycles

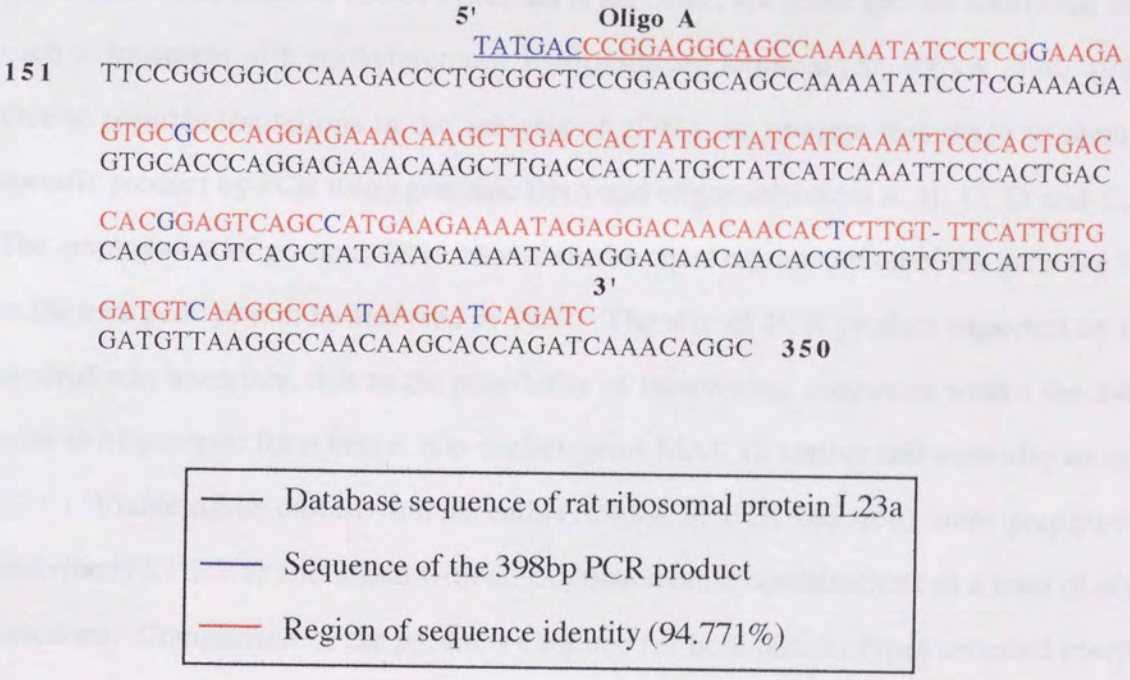
Figure 3.2.1.5.4

PCR method 3: ***Sequence analysis of the 398bp PCR product obtained with MAC 16 cDNA and oligos A (forward) and dT (reverse)***

Database sequence identified: Rat ribosomal protein L23a

Accession number : X65228

Reference **:** Suzuki, K (unpublished)



3.2.1.7. PCR method 4: MAC 16 whole cells and oligonucleotides A, B, C, D ,E, F

The failure to isolate cDNA for the 24kD material by PCR analysis of total MAC 16 cDNA may in part be due to the low abundance of its mRNA. Protein purification data revealed a recovery of 40ppb (table 2.2.4.2.1) which may be indicative of a low rate of transcription or instability of the mRNA. Either of these possibilities would impede the isolation of adequate material for cDNA synthesis. In addition, efficient transcription requires the removal of RNA secondary structure and while for most mRNA species the denaturation step prior to cDNA synthesis is adequate, for some species additional steps such as treatment with methylmercuric hydroxide are required (Sambrook *et al*, 1989). Due to possible limitations in the analysis of cDNA, an attempt was made to obtain a specific product by PCR using genomic DNA and oligonucleotides A, B, C, D and E, F. The employment of genomic DNA should ensure equal representation of the gene for P24 in the total gene pool to be analysed by PCR. The size of PCR product expected by this method was uncertain, due to the possibility of intervening sequences within the 24kD gene in its genomic form hence, non-cachexigenic MAC 13 tumour cell were also tested.

Viable tissue culture tumour cells (1000/25μl PCR reaction) were prepared as described (3.1.2.7.1) and tested with all oligonucleotide combinations as a total of eight reactions. Comparison of the products obtained for both tumour types revealed specific PCR products with oligonucleotides B+E (452bp, figure 3.2.1.7.1, lane 6) and C+E (455bp, figure 3.2.1.7.1, lane 8). These products were specific as determined by comparison with MAC 13 cells (figure 3.2.1.7.1 lane 6* and 8* respectively) and by control reactions using the oligonucleotides singly (figure 3.2.1.7.2 lanes 5, 9 and 10). Additional PCR analysis revealed the presence of a further two products of 612bp and 241bp for oligonucleotides B+E which also appeared to specific (figure 3.2.1.7.2). All four products were cloned into pUC 18 plasmid and the insert size was determined by PCR with forward and reverse M13 oligonucleotides (figure 3.2.1.1.8). The PCR product size was determined by PCR with oligonucleotides B+E and C+E (figure

3.2.1.7.3) and a total of sixteen plasmid inserts were sequenced using M13 forward and reverse oligonucleotides.

Analysis of the DNA sequences obtained revealed no match with that predicted for the six internal amino acids of the 24kD material (figure 3.2.1.1.4). A database search for similar sequences revealed a match with a mouse mitochondrial DNA hydrophobic protein for one of the products obtained with oligonucleotides B+E and C+E which occurred with a frequency of 3/16 (figure 3.2.1.7.4). A match with mouse retrovirus-like intracistronic type A particle element was also observed for 1/16 of the products (figure 3.2.1.7.5).

Sequence analysis revealed that the formation of these non-specific products was made possible by the occurrence of suitable anealling sites within the template sequence, particularly with respect the 3' region of the oligonucleotides.

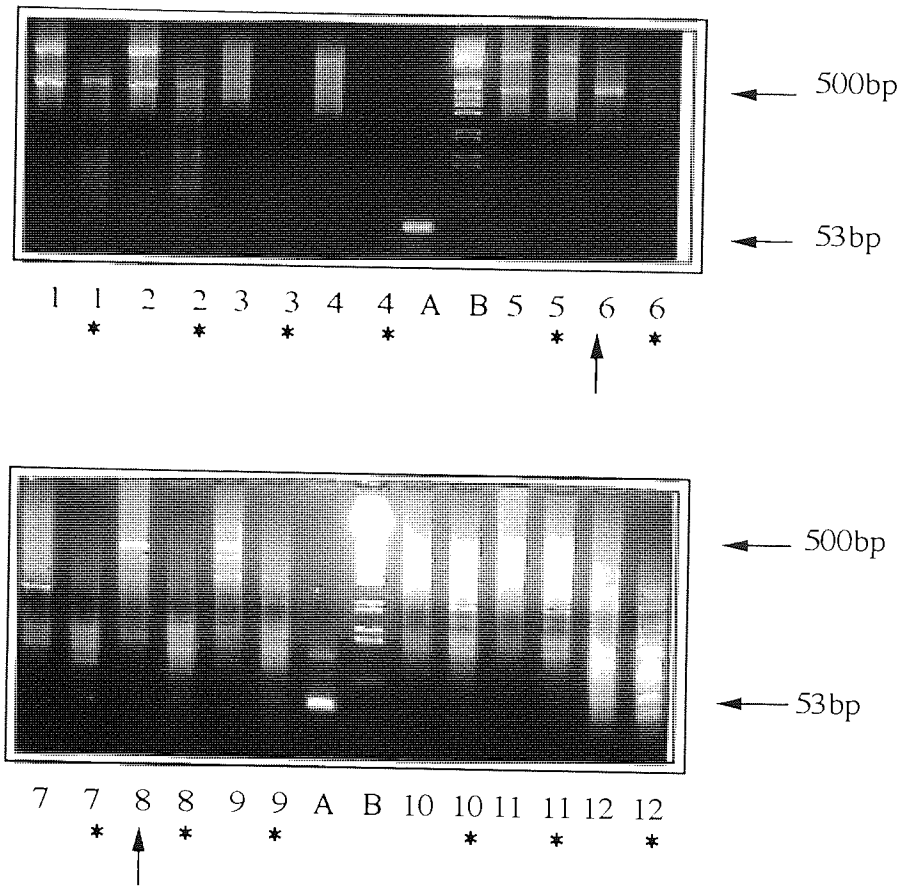
3.2.1.8. Conclusion:

An attempt to obtain a specific PCR product from genomic DNA was unsuccessful. As with PCR analyses 3.2.1.2, 3.2.1.3, and 3.2.1.5, only non-specific PCR products were obtained as a result of sequence similarities between the 3' regions of the oligonucleotides and non-specific gene templates. The reason for the apparent failure of oligonucleotides A, B, C, D and E, F to aneal to the cDNA for the 24kD material and result in a specific product is unknown.

Figure 3.2.1.7.1.

PCR analysis of MAC 16 and MAC 13 whole cells using oligonucleotides A,B,C,D (forward) and E,F (reverse):

Analysis on a 4% agarose gel



1: Cells + oligos A+E
2: Cells + oligo A
3: Cells + oligos E
4: Cells + oligos F
5: Cells + oligos A+F
6: Cells + oligos B+E
7: Cells + oligos B+F
8: Cells + oligos C+E
9: Cells + oligos C+F
10: Cells + oligos D+E
11: Cells + oligos D+F
12: Cells + oligos 297+299

*** MAC 13 cells**

PCR temperatures (°C):
Denaturation: 94 (1min)
Anealling: as indicated (2min)
Extension: 72 (1min)
] 30 cycles

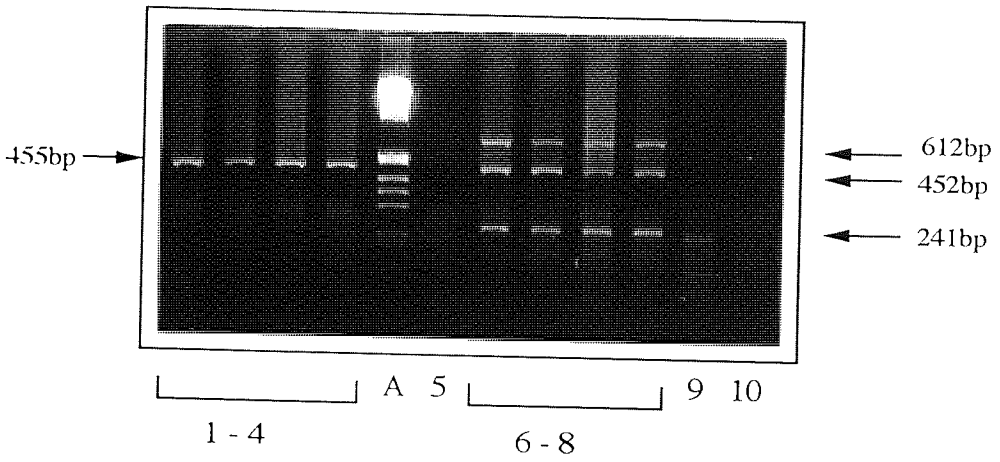
A: MAC 16 cDNA + oligos 297+299
B: 1kb ladder

Arrows indicate products that were analysed further

Figure 3.2.1.7.2.

PCR analysis of MAC 16 whole cells with oligonucleotides B,C (forward) and E (reverse):

Analysis on a 1.5% agarose gel



1 - 4 : MAC 16 whole cells + oligos C+E

5: MAC 16 whole cells + oligo C

6 - 8 : MAC 16 whole cells + oligos B+E

9: MAC 16 whole cells + oligo B

10: MAC 16 whole cells + oligo E

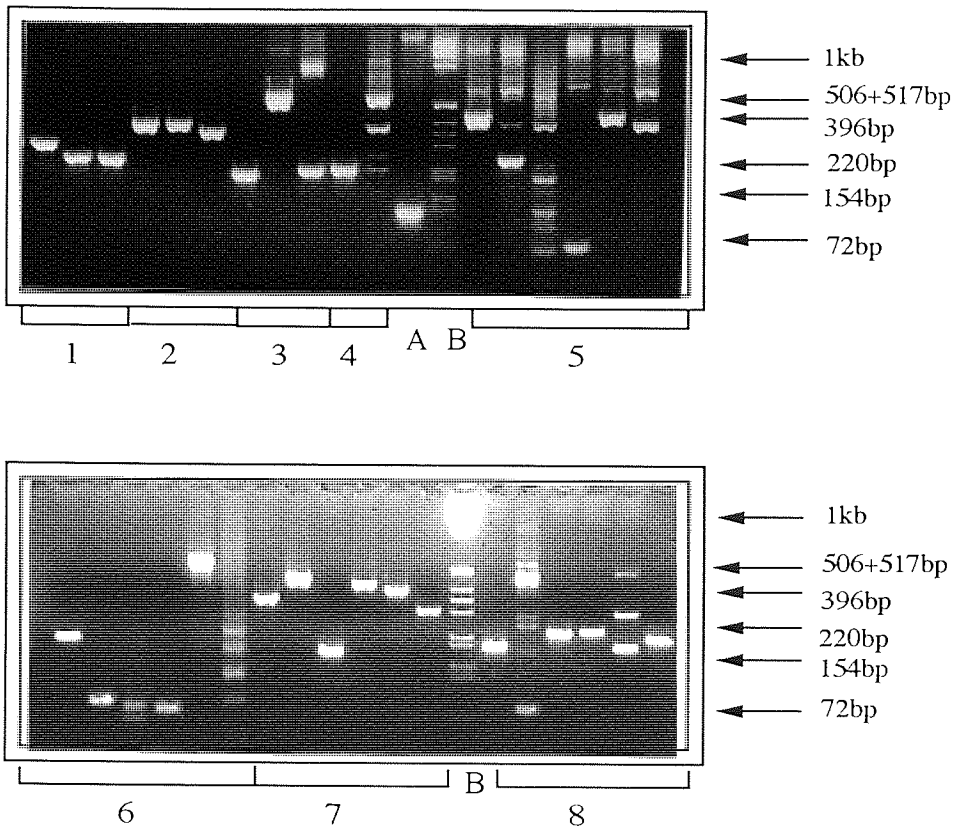
A: 1kb ladder

PCR temperatures (°C): Denaturation: 94 (1min)
Anealling: 55 (2min)
Extension: 72 (1min)
] 30 cycles

Figure 3.2.1.7.3.

PCR analysis of plasmid DNA isolated from transformed *E.coli* JM 83 colonies:

Analysis on a 1.5% agarose gel



Analysis of plasmid insert size:

1: Product (C+E) +M13 -40 and reverse oligos
2: Product $(B+E)_1$+ "
3: Product $(B+E)_2$ + "
4: Product $(B+E)_3$ + "
A: pUC 18 plasmid (no insert)

Analysis of plasmid insert PCR product size:

5: Product (C+E) + oligos C and E
6: Product $(B+E)_1$ + oligos B and E
7: Product $(B+E)_2$ + "
8: Product $(B+E)_3$ + "
B: 1kb ladder

PCR temperatures (°C):
Denaturation: 94 (1min)
Anealling: 55 (1min)
Extension: 72 (1min)
30 cycles

Figure 3.2.1.7.4.1.

PCR method 4: ***Sequence analysis of one of the 450bp products obtained with MAC 16 whole cells and oligos B,C (forward) and E (reverse)***

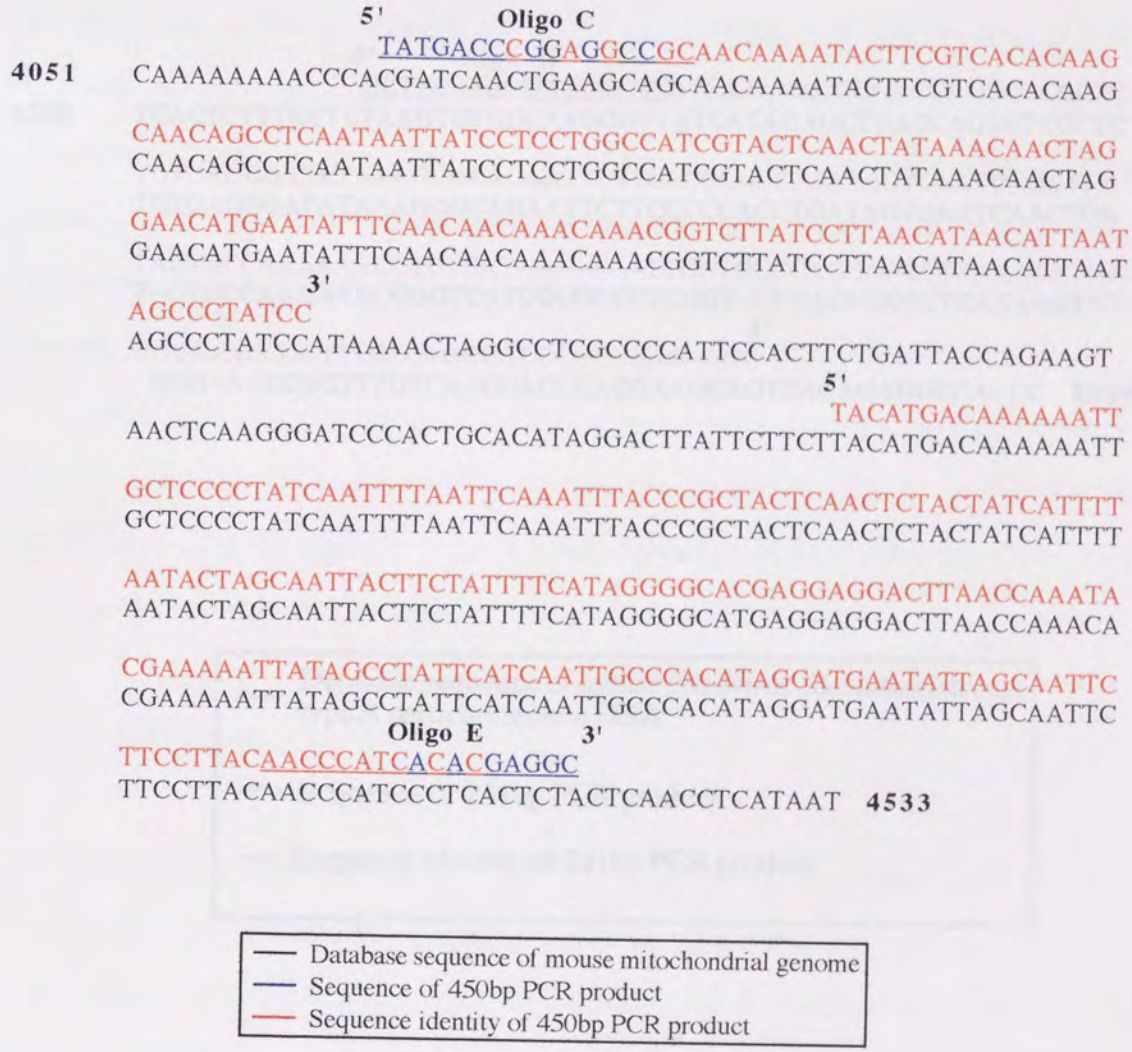
Frequency per 16 products sequenced : 3

Database sequence identified : Mouse mitochondrial genome hydrophobic protein (16295bp)

Accession number : V00711

Identity : 92.4% in 197bp overlap

Reference : Bibb *et al* (1981)



— Database sequence of mouse mitochondrial genome
— Sequence of 450bp PCR product
— Sequence identity of 450bp PCR product

Figure 3.2.1.7.4.2.

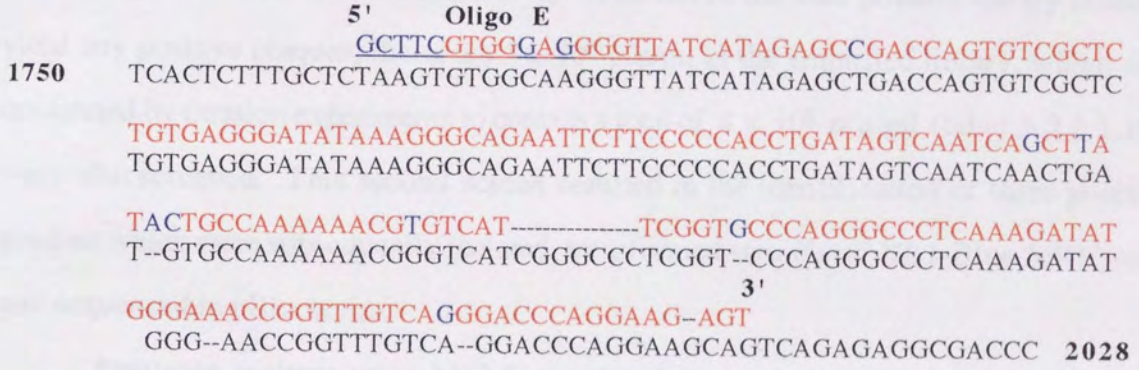
PCR method 4: ***Sequence analysis of one of the 241bp products obtained with MAC 16 whole cells and oligos B (forward) and E (reverse)***

Frequency per 16 products sequenced : 1

Database sequence identified : Mouse retrovirus-like intracistronic type A particle element DNA (3889bp)

Accession number : M18252

Sequence identity : 90.2% in 163bp overlap



___	Database sequence of mouse retrovirus-like intracistronic type A particle element DNA
___	Sequence of 241bp PCR product
___	Sequence identity of 241bp PCR product

3.2.2. Expression screening of a cDNA library using a polyclonal antibody the 24kD protein sequence

3.2.2.1. Results.

A cDNA library was constructed from 20μg mRNA in lambda Uni-ZAP XR by Stratagene Limited. Library screening and analysis of positive plaques were as described (3.1.3.20-3.1.3.21).

A titration of the primary library constructed by Stratagene revealed that it contained only 1 x 10^4 plaque forming units (pfu)/ml as opposed to 1 x 10^6 pfu/ml claimed by the company (table 3.2.2.1.1a). A screen of the total primary library failed to yield any positive plaques, therefore 5 x 10^6 pfu/ml in the amplified library, which was confirmed by titration experiments to contain a total of 4 x 10^8 pfu/ml (table 3.2.2.1.1b), were also screened. This second screen resulted in the identification of three positive plaques which were subsequently isolated, transformed into *E.coli* XL1-Blue MRF' cells and sequenced in pBluescript.

Sequence analysis using M13 forward and reverse primers resulted in sequence data from both the 5' and 3' regions of the gene (figure 3.2.2.1.1). The sequences obtained for all three positive plaques were identical and a database search revealed a match with the mouse NK10 gene.

3.2.2.2. Conclusion.

An attempt to isolate the cDNA for the 24kD material by screening a cDNA library with a polyclonal antibody to a synthetic peptide with the amino acid sequence of the 24kD material was unsuccessful. The cDNA library constructed by Stratagene was found to contain 100-fold less pfu than was claimed by the company for both the primary and amplified libraries. At least 1x10^6 pfu/primary library are required to ensure representation of most genes (Sambrook et al, 1989); therefore, the probability of

isolating the cDNA for the 24kD material shown by protein purification to be approximately $4x10^{-3}$% of total tumour proteins (table 2.2.4.2.1) from such a library is low.

Table 3.2.2.1.1.

Titration of the MAC 16 tumour cDNA Llambda Uni-ZAP™ library

(A) Primary library

Expected titre: 1×10^6 pfu/ml

Actual titre : 1×10^4 pfu/ml

Library dilution	Volume tested (μl)	Expected pfu	Actual pfu
1×10^{-3}	15	15	0
5×10^{-2}	15	30	0
1×10^{-2}	15	150	4
5×10^{-1}	15	300	12
No dilution	15	15000	120

(B) Amplified library

Expected titre: 4×10^{10}

Actual titre: 4×10^7

Library dilution	Volume tested (μl)	Expected pfu	Actual pfu
1×10^{-6}	2.5	100	4
1×10^{-6}	25	1000	36
1×10^{-4}	2.5	10000	116
2×10^{-2}	1	200000	2000

Figure 3.2.2.1.1.

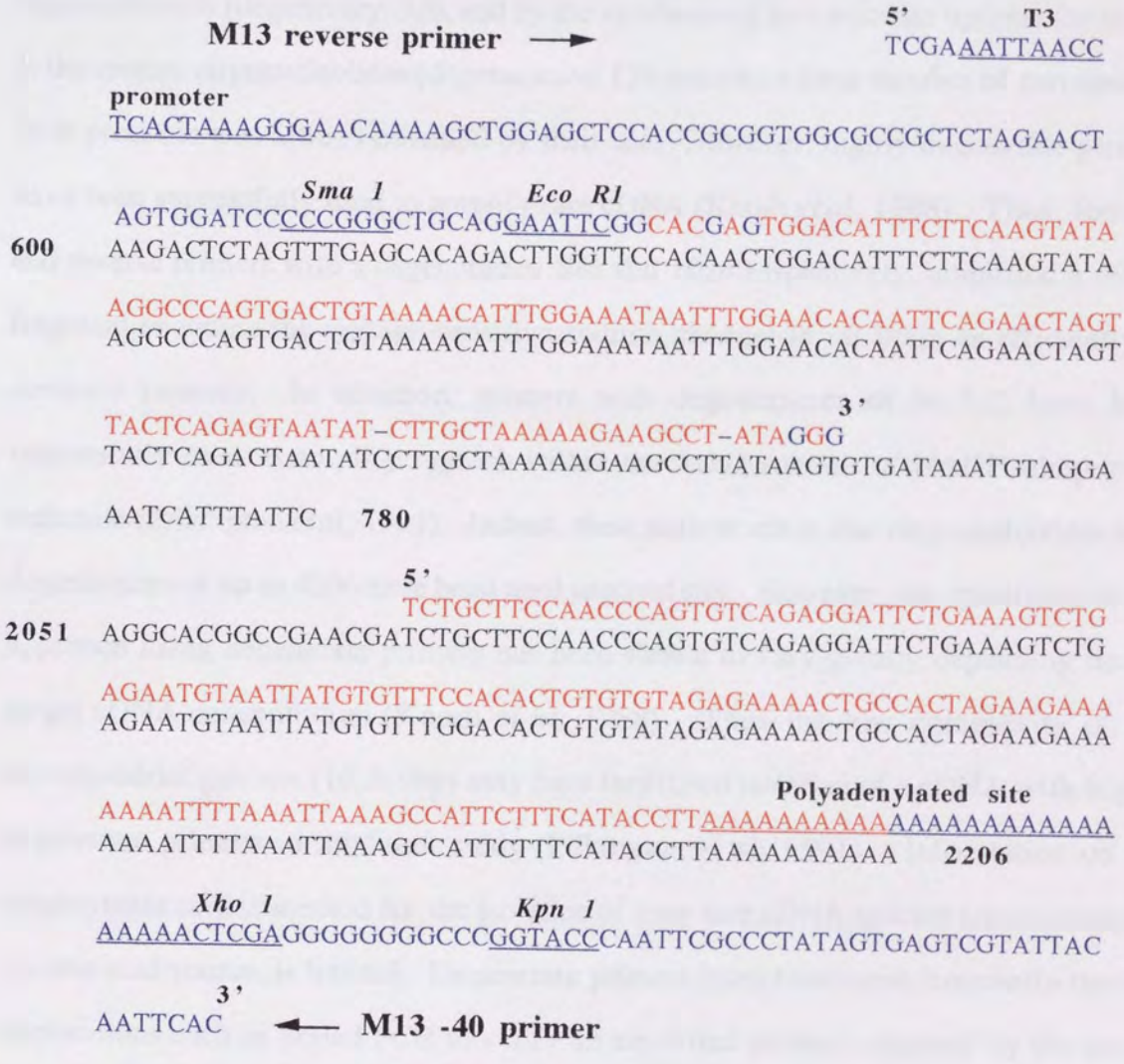
Expression library screen: ***Sequence analysis of the three positive plaques obtained***

Database sequence identified : Mus musculus NK10 gene

Accession number : X79828

Sequence identity : 97.2% in 108bp overlap

Reference : Lange, R. *et al* (unpublished)



— Database sequence of Mus musculus NK10 gene (5450bp)
— Sequence of positive plaque cloned into pBluescript
— Sequence identity of positive plaque

3.3. DISCUSSION

Methods employed to isolate the gene for P24 have so far been unsuccessful. Limited protein sequence information was available, therefore the design of oligonucleotides with high degeneracy was unavoidable. This problem was most apparent for the construction of the reverse oligonucleotide which required the use of $Ser^{1,5}$ and subsequent incorporation of six codon possibilities into its design. Thus, although attempts were made to limit the degeneracy at the 3' end of the forward oligonucleotide (degeneracy: 32), and by the synthesis of two separate options for serine in the reverse oligonucleotides (degeneracies: 128 and 64); a large number of non-specific PCR products was always obtained by their use. However, highly degenerate primers have been successfully used to amplify rare cDNA (Knoth *et al*, 1988). Thus, forward and reverse primers with a degeneracies 576 and 1024 respectively, amplified a 698bp fragment encoding the voltage-sensitive sodium channel in rat brain as efficiently as accurate primers. In addition, primers with degeneracies of 64-512 have been successfully used to amplify regions within the 51kD subunit of NADH-ubiquinone reductase (Pilkington *et al*, 1991). Indeed, these authors claim that oligonucleotides with degeneracies of up to 4096 have been used successfully. However, the specificity of the approach using degenerate primers has been shown to vary greatly depending on the target cDNA concentration (Knoth *et al*, 1988). Thus the low complexity of the mitochondrial genome (16,569bp) may have facilitated isolation of a cDNA with highly degenerate primers in the latter study (Pilkington *et al*, 1991). Information on the employment of this method for the isolation of very rare cDNA species from a complex nucleic acid source, is limited. Degenerate primers have been most frequently used in applications such as nested PCR to verify an amplified product obtained by the use of accurate primers (Chen and Suttle, 1995). In this case the first-step PCR would provide million-fold increases in the designated regions and also would reduce the nucleic acid complexity exponentially. In another application, degenerate primers based on highly conserved regions of the known DNA sequence of GTP-cyclohydrolase 1 from several

unrelated species', were used for the isolation of the gene from other unrelated species by homology cloning (Maier *et al*, 1995). Thus, although the nucleic acid complexity was not reduced in this case, the extensive sequence information available for the design of primers enabled four pairs of degenerate oligonucleotides based on different highly conserved regions of the gene to be constructed.

Information obtained as a result of the purification of P24 suggested that it may represent as little as 40ppb of the total tumour proteins (Todorov *et al*, 1996) and may therefore be in too low an abundance for detection by degenerate oligonucleotides. The heterogenous nature of the non-cloned MAC 16 cell-line and tumour may be the main contributing factor towards this low abundance. Thus, it has been shown that the proportion of MAC 16 tissue culture cells capable of inducing cachexia *in vivo* decreases with increased passage number (Tisdale, unpublished results). In addition, excision of a 0.5g weight loss-inducing, non-necrotic tumour and subsequent subcutaneous inoculation of $1mm^3$ portions into thirty mice, has been shown to result in variable induction of the cachectic condition. Thus, weight loss may occur in 50-80% of the mice with varying degrees of potency (Wynter, personal communication). In a similar situation, cloning of the cachexia-inducing colon 26 tumour resulted in the identification and subsequent isolation of eight clones with very different capacities to induce the syndrome (Fujimoto-Ouchi *et al*, 1995).

The presence of P24 in such low abundance would therefore hamper efforts to isolate the gene by PCR using degenerate primers which often requires low initial anealling temperatures (<40°C) (Knoth *et al*, 1988; Pilkington *et al*, 1991). In addition, low abundance would also render the probability of its representation in a cDNA library unlikely. Hence, it may be necessary to add an enrichment step prior to these procedures.

Enrichment was not necessary for the isolation of the cDNA for the core proteins of other proteoglycans, as they have been found to occur in high abundance. Thus, the

cDNA for serglycin core protein was isolated from rat yolk sac tumour cells by library screening using three, 17-mer oligonucleotide probes based on 19 amino acids of the N-terminus (Bourdon et al, 1985). By this method, the detection rate for positive clones was 6/35000. In addition, the cDNA for biglycan core protein was obtained by two-fold expression screening of a bone-derived cell line cDNA library, using polyclonal antisera both to the whole proteoglycan and to a synthetic peptide comprising 15 N-terminal amino acid residues (Fisher *et al*, 1989). This procedure was able to detect 12 positive clones in the primary library. In contrast, interleukin-1 (IL-1) mRNA has been estimated to be present as $5x10^{-6}\%$ of the total mRNA of the $P388D_1$ macrophage cell line (Lomedico *et al*, 1984). Therefore, isolation of its cDNA involved a super-induction step prior to the construction of a cDNA library. This procedure involved exposure of cell-line macrophage cells to 10μg/ml phorbol myristic acetate in the presence of 2mm sodium butyrate and 10μg/ml cyclohexamide; and resulted in an induction of IL-1 production of 1393-fold (Mizel and Mizel, 1981). mRNA was further enriched by fractionation on a superose density gradient and selection of the active fraction, as determined by analysis of translation products. This fraction of mRNA was then used for the construction of a cDNA library (Lomedico *et al*, 1984). The enrichment procedure employed by this group resulted in a detection rate of 1/2000 clones.

Future attempts to isolate P24 cDNA from the MAC 16 tumour may employ a similar procedure. Thus, P24 mRNA may be enriched by selection of polyclonal antibody-reactive translation products, and subsequently analysed by reverse transcriptase-PCR and expression library screening.

CHAPTER 4:

GENERAL DISCUSSION

The significance of the lipolytic factor and P24 with respect to the aetiology of cancer cachexia and the effectiveness of amelioratory therapies

As has already been discussed in this report (1.4), evidence exists to suggest the involvement of three physiological processes in the aetiology of cancer cachexia. These are the host neuroendocrine system involving glucocorticoid hormones, catecholamines and insulin-glucagon homeostasis; the host immune response and subsequent cytokine-mediated inflammatory response; and circulatory tumour-derived catabolic products. Many studies have attempted to elucidate the relative contribution of these processes to the mediation of cachexia in various animal models and in patients. It is now generally believed that the condition in humans arises as result of the interplay of all these events to cause anorexia, catabolism of host tissues, elevated hepatic synthesis of acute phase proteins and subsequent elevation in resting energy expenditure (Giacosa *et al*, 1996).

Most recent research into the condition in humans has focussed on the prostaglandin-mediated effects of cytokines produced by activated macrophages in response to the presence of a tumour. Continued secretion of cytokines is thought to result in secondary effects of anorexia, metabolic abnormalities and wasting due in part to the induction of an inflammatory response and the production of acute phase reactants. Indeed, recent observations from studies involving cancer patients with advanced disease revealed that the resting energy expenditure of patients in whom an acute phase response was apparent, as measured by serum levels of C-reactive protein, was elevated by approximately 25% compared to cancer patients with normal C-reactive protein levels (Falconer *et al*, 1994a). Furthermore, the presence of an acute phase response in these patients was associated with a markedly reduced survival (Falconer *et al*, 1995). The hepatic acute phase response is thought to be mediated mainly by IL-6 and partly by cortisol, as determined by experiments with isolated human hepatocytes (Castell *et al*,

1990). Human studies further suggested that the cytokine-induced response may be mediated locally by tissue-associated and circulatory activated macrophages rather than systemically (Falconer *et al*, 1994a).

Cytokines are believed to mediate their effects through interactions with receptors on the plasma membrane and subsequent activation of signal transduction pathways. In addition, the production of arachidonate-derived eicosanoids following release of the n-6 fatty acid from the membrane phospholipid bilayer by activated phospholipase A_2, is thought to be important for the inflammatory and tissue-damaging effects of the cytokines.

The contributory role of the cytokines in the mediation of cachexia in MAC 16-tumour-bearing mice, or in non tumour-bearing mice treated with purified cachectic factors is unclear, as parameters such as anorexia and elevated plasma triglycerides usually associated with their increased production, have never been observed during the development of the condition in this model. There is evidence to suggest that arachidonic acid metabolites may play a role in the mediation of cachexia by P24, as inhibition of muscle PGE_2 production with indomethacin, and inhibition of leukotriene B_4 production by the lipoxygenase inhibitor CV6504, resulted in almost complete inhibition of P24-induced *in vitro* muscle proteolysis (Lorite and Tisdale, unpublished results). Furthermore, *in vivo* administration of both of these compounds to MAC 16 tumour-bearing mice at doses of 5mg/kg/12h for indomethacin (Hussey and Tisdale, unpublished results), and 10-25mg/kg/day for CV6504 (Hussey and Tisdale, in press), resulted in the attenuation of weight loss, reduction in tumour growth, and a consequential increased survival. Other workers have also reported improved cachectic parameters following administration of non-steroidal anti-inflammatory drugs (NSAIDS). Thus, an increased survival was observed in MCG 101-tumour-bearing mice treated with indomethacin (1mg/kg/d) (Sandstrom *et al*, 1990). Food intake was significantly improved and 40% of the mice were complete responders. In another study with the same cachexia model it

was noted that mice treated with indomethacin no longer died due to the cachectic condition, but were able to survive with a much larger tumour burden than untreated controls (Gelin *et al*, 1991). The cachexia-specific effects of indomethacin were further illustrated by a study in which the drug had a beneficial effect on survival in mice transplanted with a cachexia-inducing tumour associated with elevated tumour, plasma, liver and muscle PGE_2 levels (Lonnroth *et al*, 1995). In contrast, no effect on survival was observed following administration of indomethacin to mice translanted with a non-cachexigenic melanoma-derived tumour which did not induce an elevated production of PGE_2. Improved cachectic parameters due to treatment with NSAIDS were also apparent in a human study. Thus, attenuation of both accelerated whole body protein kinetics and acute phase response was observed in cachectic patients with advanced colonic adenocarcinoma treated with ibuprofen (3x400mg/d) (Preston *et al*, 1995). Serum levels of TNF, IL-6, IL-1 and cortisol remained elevated during this treatment thus, indomethacin had no effect on mechanisms controlling the enhanced production of these molecules but instead, may have acted to reduce their effect. Observations from epidemiological studies have also led to suggestions that NSAIDS may have beneficial anti-tumour effects. Thus, the risk of breast cancer was found to be cut by one third by the intake of aspirin three times weekly in one study. In another, Clinoril administration was associated with a reduction in the incidence of potentially cancerous polyp formation by 50% in patients with a rare inherited susceptibility to colon cancer. Furthermore, it has also been shown that NSAIDS users could reduce colon cancer-induced morbidity by approximately 50% (Sternberg, 1996).

Long-term administration of NSAIDS is limited however, by the requirement for frequent administration in order to maintain adequate serum levels of drug due to the short half-life of many of them ($t_{1/2}$ for ibubrofen: 2h). In addition, the increased risk of haemorrhage and gastric irritation associated with their long term use; and toxicity observed with the administration of doses >5mg/kg/12 in mice (Hussey and Tisdale, unpublished results) precludes their consideration as effective anti-cachectic agents.

The polyunsaturated fatty acid (PUFA) EPA has been shown to be the most effective anti-cachectic agent tested to date. Initial animal studies with EPA as a component of fish oil revealed that it had potent anti-cachectic (Tisdale and Dhesi, 1990) and anti-tumour properties (Tisdale and Dhesi, 1990; Dagnelie *et al*, 1994). In a human study, administration of 12g/d fish oil as MaxEPA capsules to patients with unresectable pancreatic cancer, over a period of three months resulted in the establishment of weight stability in most patients and weight gain in some (Wigmore *et al*, 1996). EPA was revealed to be the active component of the fish oil, as a result of further animal studies and anti-cachectic and anti-tumour activity was observed with doses of 1.25-2.5g/kg/d (Tisdale and Beck, 1990; Beck *et al*, 1991). Preliminary results from a human study suggest that administration of 6g/d EPA may reduce weight loss in patients with pancreatic cancer (Fearon *et al*, unpublished results). In addition, a trend towards reduced acute phase response, as measured by C-reactive protein was apparent, as was a slight reduction in resting energy expenditure. Reduced spontaneous and stimulated production of IL-6 and TNF from isolated peripheral blood mononuclear cells (PBMC) was also observed.

It has been shown that cytokine production by macrophages can be modulated by dietary fat. Administration of fish oil in the form of 18g/d MaxEPA comprising 2.7g EPA and 1.85g DHA to healthy volunteers over six weeks, resulted in the reduced production of IL-1β, IL-1α and TNF from activated (PBMC) *in vitro* (Endres *et al*, 1989). There is evidence to suggest that cytokine gene expression may be regulated by products of lipid metabolism, perhaps via modulation of inducible transcription factors such as AP-1 and Nuclear Factor-Kappa B (NF-κB) whose activation is in turn modulated by the reduction-oxidation status (redox) of the cell (Meyer *et al*, 1994). NF-κB is activated by a pro-oxidant state and is potentially inhibited by antioxidants, while AP-1 is strongly activated by antioxidants and shows reduced activity in the presence of oxidants. AP-1 activation depends on transcription of *c-fos* and *c-jun* mRNA and production of proteins Fos and Jun which form a complex which binds to the DNA

regulatory element: AP-1 binding site (Abate *et al*, 1990). This binding is determined by the oxidation status of two cysteine residues within the binding domains of the complex thus, reduction of the cysteine residues results in DNA binding and subsequent production of AP-1.

NF-κB is an inducible transcription factor believed to be involved in the induction of multiple genes during mediation of the inflammatory process. Activation involves removal of the inhibitory subunits: I Kappa B-alpha and beta from a latent cytoplasmic complex in a process that involves phosphorylation (Link *et al*, 1992) and proteolysis (Traenckner *et al*, 1994). Activation results in nuclear translocation and is controlled by the redox of the cell. The factor is ubiquitous; however, its properties have most often been associated with cells of the immune system. Thus in monocytes, NF-κB has been suggested to be an important factor involved in the expression of cytokine genes and activation has been observed in response to many pathogenic signals including eicosanoids, and products of lipid peroxidation (Baeuerle and Henkel, 1994). Interference with its activation or activity may therefore be beneficial in the suppression of acute inflammatory reactions and the acute phase response. Indeed, inhibitory effects of physiological concentrations of vitamin E on cellular TNF production, perhaps through reduced cleavage of the inhibitory NF-κB subunits, has been observed (Giacosa *et al*, 1996, and references therein).

EPA has been shown to inhibit glycerol release induced by the lipolytic factor produced by the MAC 16 tumour in an *in vitro* assay (Tisdale and Beck, 1990). In addition, an inhibitory effect on cAMP production was also observed, which has been subsequently shown to be a consequence of reduced adenylate cyclase activity (Adamson and Tisdale, unpublished results), perhaps as a result of activation of the inhibitory G-protein. EPA has also been shown to inhibit PGE_2 production and muscle proteolysis induced by serum from MAC 16 tumour-bearing mice (Smith and Tisdale, 1993b) and affinity-purified P24 (Lorite and Tisdale, unpublished results).

Dietary supplementation with a high dose of EPA has been shown to alter the PUFA composition of the membrane phospholipid bilayer resulting in increased representation of the administered fatty acid (Wigmore *et al*, 1996). Thus the ability of EPA to antagonise the catabolic effects of P24 may be due to a reduction in the production of reactive arachidonic acid metabolites and an increase in less potent EPA-derived PGE_3 and leukotrienes B5. In addition, the alteration in membrane fluidity caused by changes in the PUFA composition may affect receptor recognition, signal transduction and subsequent gene expression thus further antagonising P24-mediated effects.

EPA has also been shown to have a potent anti-tumour effect ,as observed during *in vivo* studies with MAC 16 tumour-bearing mice (Tisdale and Beck, 1990; Beck *et al*, 1991). Evidence that this was indeed a direct effect of the fatty acid rather than an indirect effect through the inhibition of catabolic factors, was provided by a study to compare the relative anti-tumour properties of various PUFAs on the cell growth of five pancreatic cell lines (Falconer *et al*, 1994b). Results from this investigation revealed that EPA had the most potent anti-tumour activity and caused almost complete inhibition of cell growth at a concentration of 10μM. The anti-tumour effect of EPA was found to be dose-related and due to cytostasis rather than cytotoxicity. The mechanism by which EPA exerts its cell-growth-inhibitory effects are unknown, but may be mediated by lipid peroxidation products, as suggested by dose-related increase in these products and the abolition of the effect in the presence of vitamin E. However, a specific EPA-derived peroxide product may be required, as a reduction in anti-tumour effect was observed when EPA and oleic acid were incubated together in concentrations that gave rise to the same level of lipid peroxidation. Animal studies revealed an initial increase in the rate of tumour cell loss during treatment with EPA that was followed by a reduction in the doubling time (Hudson *et al*, 1993).

Thus, amelioration of cancer cachectic parameters by EPA may involve inhibition of cytokine production and induction of tumour cell-stasis due to increases in intracellular

lipid peroxidation products. In addition effects of catabolic tumour-derived products may be antagonised by alteration of the membrane composition and by competition of EPA for phospholipase A2 with subsequent dilution of prostaglandin-mediated processes.

The relative importance of the three processes implicated in the development of cancer cachexia and the mechanism of their interaction is unknown. The importance of the cytokine-mediated acute phase response in the mediation of cachexia in human pancreatic cancer patients has been suggested, and has been shown to have significant prognostic implications (Falconer *et al*, 1995). The importance of tumour-derived catabolic factors for the development of the cachectic condition in mice has been demonstrated. Indeed, administration of P24 alone has been shown to cause catabolism of both muscle and adipose tissue thus, suggesting that this factor may be more important than the lipolytic factor for manifestation of cachectic parameters. The requirement of arachidonic acid metabolites for the mediation of the muscle proteolytic effects of P24 has been demonstrated therefore, the catabolic effects of this factor may be exacerbated by the additional presence of the lipolytic factor. Preliminary studies suggest that both of these factors may be involved in the mediation of the cachectic condition in humans (Groundwater *et al*, 1990; Todorov *et al*, 1996; Todorov *et al*, unpublished results). The cachectic condition in humans and that induced by the MAC 16 tumour in mice were shown to be inhibitable by the administration of EPA thus, suggesting that they may be mediated by similar mechanisms. Future studies need to be undertaken to determine the relationship between the host acute phase response and tumour-derived circulatory factors with reference to the development of cancer cachexia. It may be possible that tumour-derived factors represent an earlier event in the process and that the acute phase reponse develops due to increasing host tissue damage. Measurement of the factors in the serum or urine of may therefore provide a useful tool for the prediction of the development of cachexia; thus, allowing earlier intervention to prevent the condition which drastically shortens survival in cancer patients. Although measurement of P24 in the urine of patients with weight loss > 1.5kg/month has been successfully achieved using murine

monoclonal antibody (Todorov *et al*, 1996), identification of the gene for this factor would facilitate even earlier detection of changes which may predict the development of cachexia. Thus, up-regulation of P24 gene-expression by some tumours may be one of the first steps in the process. Isolation of the gene would also enable information on the regulation of its expression to be obtained and hence, the identification of possible new targets for therapeutic intervention. In addition, production of recombinant material would facilitate the development of human monoclonal antibodies thus providing an additional effective therapy, as has been demonstrated in the murine model of cachexia (Todorov *et al*, 1996)

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APPENDICES.

Appendix A: Amino acids.

Amino acid	Abbreviation	Code letter	Mass	Properties (hydrophilicity)
Alanine	Ala	A	89.09	Neutral (-0.5)
Arginine	Arg	R	174.2	Basic (3.0)
Asparagine	Asn	N	132.1	Neutral (0.2)
Aspartic acid	Asp	D	133.1	Acidic (3.0)
Cysteine	Cys	C	121.12	Neutral (-1.0)
Glutamic acid	Glu	E	147.13	Acidic (3.0)
Glutamine	Gln	Q	146.15	Neutral (0.2)
Glycine	Gly	G	75.05	Neutral (0.0)
Histidine	His	H	155.16	Basic (-0.5)
Isoleucine	Ile	I	131.17	Neutral (-1.8)
Leucine	Leu	L	131.17	Neutral (-1.8)
Lysine	Lys	K	146.19	Basic (3.0)
Methionine	Met	M	149.21	Neutral (-1.3)
Phenylalanine	Phe	F	165.19	Neutral (-2.5)
Proline	Pro	P	115.13	Neutral (0.0)
Serine	Ser	S	105.09	Neutral (0.3)
Threonine	Thr	T	119.12	Neutral (-0.4)
Tryptophan	Trp	W	204.22	Neutral (-3.4)
Tyrosine	Tyr	Y	181.19	Neutral (-2.3)
Valine	Val	V	117.15	Neutral (-1.5)

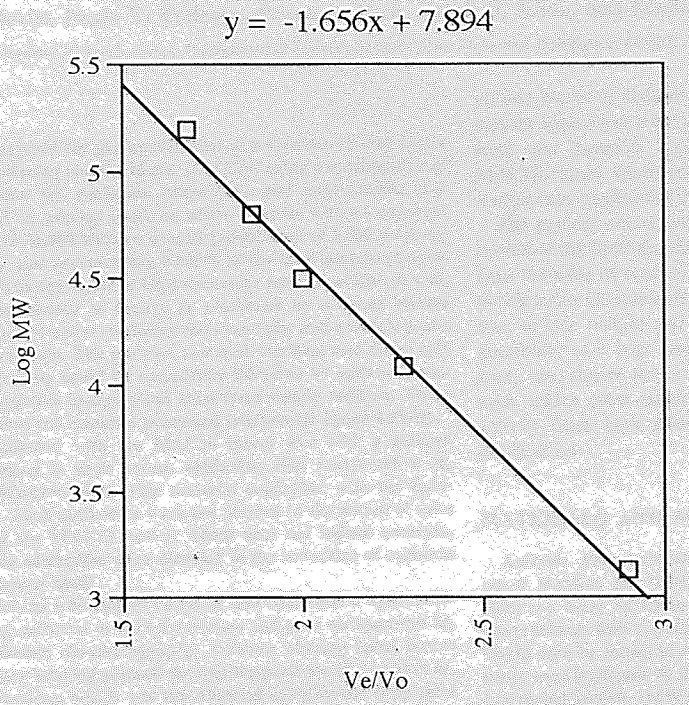
Hydrophilicity value according to Hopp and Woods (1981)

Appendix B. The genetic code (DNA to amino acids)

TTT	Phe	TCT	Ser	TAT	Tyr	TGT	Cys
TTC	Phe	TCC	Ser	TAC	Tyr	TGC	Cys
TTA	Leu	TCA	Ser	TAA	Stop	TGA	Stop
TTG	Leu	TCG	Ser	TAG	Stop	TGG	Trp
CTT	Leu	CCT	Pro	CAT	His	CGT	Arg
CTC	Leu	CCC	Pro	CAC	His	CGC	Arg
CTA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
CTG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
ATT	Ile	ACT	Thr	AAT	Asn	AGT	Ser
ATC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
ATA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
ATG	Met	ACG	Thr	AAG	Lys	AGG	Arg
GTT	Val	GCT	Ala	GAT	Asp	GGT	Gly
GTC	Val	GCC	Ala	GAC	Asp	GGC	Gly
GTA	Val	GCA	Ala	GAA	Glu	GGA	Gly
GTG	Val	GCG	Ala	GAG	Glu	GGG	Gly

(Modified from Sambrook *et al*, 1989)

Appendix C: Superose calibration graph



Fractionation of molecular weight markers on Superose 12 pre-packed 10/30

MW Standard	Elution volume (ml)	MW
IgG	12.1	160000
HSA	13.4	67000
B-Lactalbumin	14.4	35000
Cytochrome C	16.4	12400
B12	20.9	1355
Cytidine	22.6	246

Column bed volume: 24ml
Column void volume: 8ml

[CANCER RESEARCH 55, 1458–1463, April 1, 1995]

Purification and Characterization of a Lipid-mobilizing Factor Associated with Cachexia-inducing Tumors in Mice and Humans[1]

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Characterization of a cancer cachectic factor

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Induction of Muscle Protein Degradation and Weight Loss by a Tumor Product[1]

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