Studies on alpha 2 macroglobulin and cytokine interactions

Presented by

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PhD
The University of Edinburgh
1995

Acknowledgements

I would like to acknowledge the support, assistance and friendship of people who collectively have made the study and completion of my thesis possible.

First and foremost, I acknowledge the excellent leadership of Professor Keith James. He has been with me through the highs and lows of scientific research. In addition, he has made me believe in myself. For all this, I thank him.

I would like to thank the staff of the Wilkie Laboratories. I specifically mention Ian Milne and Jim Black for their technical support, and Andrew Cunningham who was my co-conspirator in the zinc studies and a sounding board for my ideas.

I would like to thank the remaining staff of the Wilkie Laboratories, with special mention of Melany Jackson and Petra Hoffmann for their continuous encouragement and friendship. I thank Mary Guckin and Ali El-Ghorr for the proof reading of my manuscript and for helpful comment.

I thank Graham Pettigrew and Ian Nimmo of the Dept. of Biochemistry of Edinburgh University for their helpful discussion of the binding studies.

I would like to acknowledge the help of my family and friends. They have all given me so much love, support and encouragement during the time of my thesis. I specifically mention my sisters, brother and spouses, Sharon, Steven, Dawn, Gordon and Alison, and my friends Margaret Anderson, Tracey Carberry, Julie Coldwell, Noranne Ellis, Chris Oswald and Carol Press.

Finally, I wish to dedicate this thesis to my parents, Jean and Frank Elliott, who have supported me every step of the way.

Declaration

I wish to declare that the work presented herein has been carried out and written up by myself. I collaborated with A.Cunningham in the zinc affinity studies (Sec.2.4.3 and 3.6), and in part with I.Milne in the immunoassay investigations (Sec.2.5.2 and 3.7).

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Abstract

Alpha2 macroglobulin (α2M) is a major blood protein (2-4mg/ml in adult blood) with a molecular weight of 725kD. Recent reports have indicated that this molecule may bind a variety of cytokines. Such interactions could have important implications with respect to the activity and quantitation of cytokines *in vivo* and *in vitro*. The aim of the present study was to establish if α2M bound a number of previously unstudied cytokines of immunological importance, and if so, did this influence their immuno- and bio-reactivity. The cytokines in question were recombinant derived human IL2, TNFα and IFNγ. The initial studies were qualitative in nature employing G200 Sephadex, FPLC on Superose 6B, zinc affinity chromatography on Sepharose, and native and SDS PAGE. The aim of these studies was to separate preincubated α2M and cytokine, and thus to identify cytokine that had bound to the serum protein. From this work it was apparent that IL2, TNFα and IFNγ bound to α2M. For all 3 cytokines the binding was stronger with α2M-methylamine (α2Mm), a chemically altered form of α2M that has a physiological equivalent. Subsequent biochemical studies were carried out using α2Mm. To analyse the quantitative interaction of cytokines with α2Mm, a novel radioimmunoassay system was developed using anti-human α2M polyclonal reagent as the capture antibody. In addition, the zinc affinity Sepharose system that had been used in earlier qualitative studies was adapted to a tube system to permit batch analysis of α2Mm-cytokine interactions. These techniques revealed a binding affinity for IL2 with α2Mm of K_d=2.1-2.5x10^{-6}M, while that of TNFα with α2Mm was K_d=0.96-1.36x10^{-6}M. Furthermore, IL2 bound non-specifically whereas TNFα showed a specific interaction with the serum protein. The relevance of such interactions was studied by determining the effect of α2M on commercial cytokine immunoassays. It was found that α2M and its derivatives may mask or enhance the detection of a number of cytokines, the effect being dependent on the cytokine and on the source of the kit. The influence of α2Mm on the bioactivity of IL2 and TNFα was examined in lymphoproliferative and antiproliferative assays respectively. These studies revealed that the complexing of TNFα with α2Mm ablated its bioactivity, whilst that of IL2 was maintained when complexed with the protein. In conclusion, the results presented corroborate previous work in the literature and extend our knowledge of α2M-cytokine interactions and their effects.

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Abbreviations

The following list of abbreviations appear in the main body of this thesis.

AD - Alzheimer's Disease

αM - alpha macroglobulin

α2M - alpha 2 macroglobulin

ARDS - adult respiratory distress syndrome

bFGF - basic fibroblast growth factor

BAC - bovine adrenocortical cells

BSA - bovine serum albumin

CF - cystic fibrosis

cisDDP - cisdichlorodiamineplatinum

CNS - central nervous system

EDTA - ethylenediaminetetraacetic acid

EGF - epidermal growth factor

5HT - 5 hydoxytestosterone

IFNγ - interferon gamma

IL1β - interleukin 1beta

IL2 - interleukin 2

IL4 - interleukin 4

IL6 - interleukin 6

IL8 - interleukin 8

K_d - dissociation constant

LDL - low density lipoprotein

LRP - LDL-related protein

LMW - low molecule weight

MAF - macrophage activating factor

MIF - macrophage inhibitory factor

NGF - nerve growth factor

PZP - pregnancy zone protein

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PDGF - platelet derived growth factor

RA - rheumatoid arthritis

RIA - radioimmunoassay

SDS - sodium dodecyl sulphate

TEPP - thiol ester plasma proteins

TGFβ - tissue growth factor beta

TNFα - tumour necrosis factor alpha

Section 1: Introduction

1.1: Alpha 2 macroglobulin

1.1.1: An introduction to α2M

Alpha 2 macroglobulin (α2M) is a 725kDa protein found at a concentration of 2-4mg/ml in human plasma (Ganrot and Schersten 1967; Tunstall, Merriman, Milne and James 1975). It is also present in extravascular fluids such as in synovial fluid and in neurological tissue (Teodorescu, Gaspar, Spear, Skoskey and Ganea 1984; Strauss, Bauer, Ganter, Jonas, Berger and Volk 1992). α2M's activity as a proteinase inhibitor has been extensively researched (Barrett and Starkey 1973; Barrett, Brown and Sayers 1979, see Sec.1.6 and 1.7). It appears in the circulation as an open cylinder that interacts with proteinases resulting in a conformational change of the α2M molecule (Fig.1.1.1, see Sec.1.6 and 1.7). The conformational change results in a shift in mobility under the conditions of polyacrylamide gel electrophoresis (PAGE) giving rise to the designations of slow (native) and fast (converted) α2M. The conformational change entraps the proteinase and reveals a binding site for the α2M receptor. Under normal conditions, the α2M-proteinase complex is rapidly removed from the circulation via cells bearing the α2M receptor.

It has become apparent that α2M may have a role within the immune response (James 1980; Borth 1992, see Sec.1.8.). This may be implemented by its inhibitory effect on proteinases. In the literature, a novel function has been proposed: namely that α2M may bind cytokines and thus modify their activity *in vivo* (reviewed in James 1990, see Sec.1.9). Herein this proposition is examined.

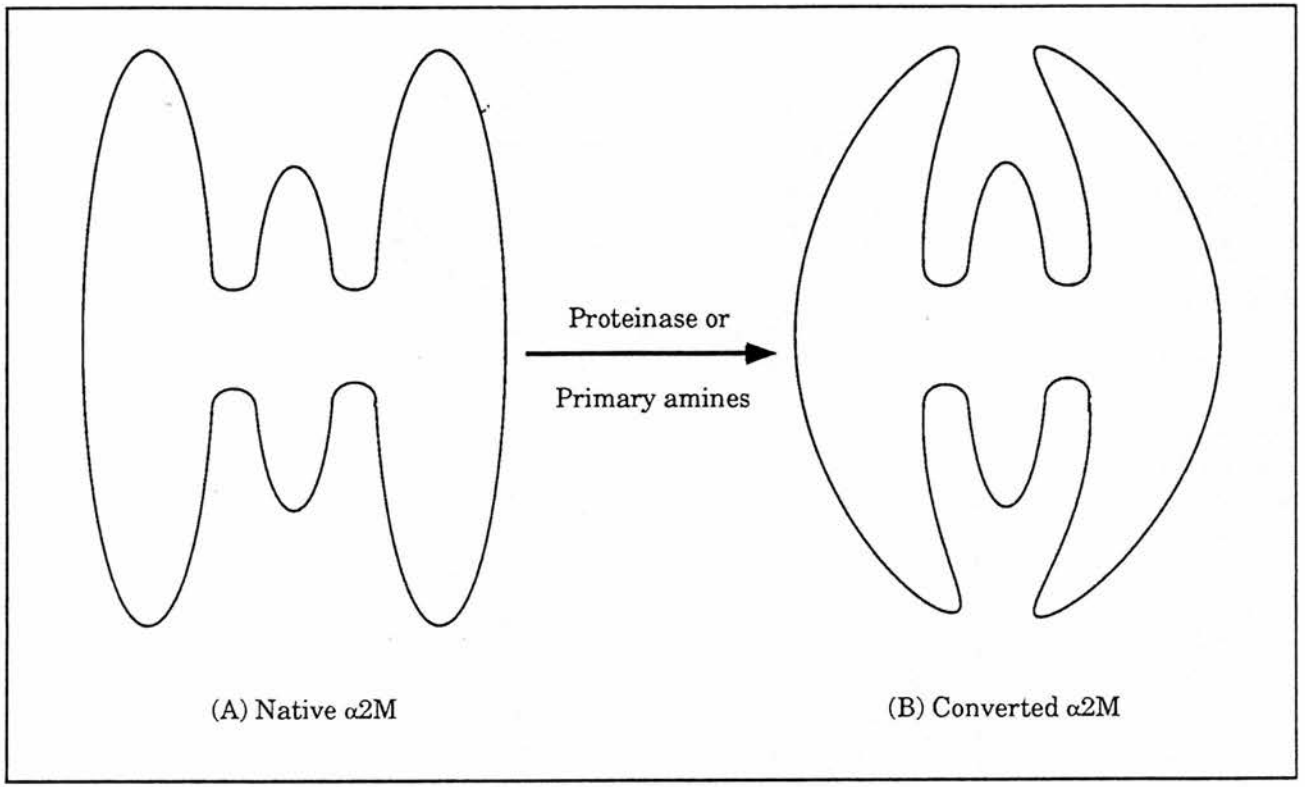


Figure 1.1.1: Models of native and converted α2M
(A) Diagram of native α2M as ascertained from electron micrographs. This form is retained in the circulation and is present as "slow" form in electrophoresis. (B) Diagram of "fast" form α2M from electron micrographs. Interaction with proteinases or primary amines conformationally alters the native molecule (A) to the "fast" form protein (B) due to cleavage of the internal thiol ester bond. The conformational change exposes a receptor recognition domain resulting in clearance *in vivo* via the α2M receptor. α2M is a hollow cylinder, proteinases interact on the internal surfaces of the native molecule and are subsequently trapped as the molecule converts to the fast form. Taken from Feldman et al 1985, and James 1990.

1.2: α2M in health and disease

1.2.1: α2M in health

In healthy adults, α2M is present at a plasma concentration of 2-4mg/ml, of which α2M-proteinase complexes normally constitute 0.2-0.8% (Abbink, Nuijens, Eerenberg, Huijbregts et al 1990; Zucker, Lysik, Zarrabi, Fiore, and Strickland 1991; Birkenmeier and Stigbrand 1993). The level of α2M varies with age and between the sexes (see Table 1.2.1.). Age variation is attributed to the increased requirement of α2M as a proteinase inhibitor during development, thus its levels are highest in the foetus at late gestation, and remain high through to the end of puberty (Ganrot and Schersten 1967; Tunstall et al 1975). Hormonal regulation of α2M synthesis may partly account for the increased concentration seen in females (Ganrot and Schersten 1967; Tunstall et al 1975). However, the difference between the sexes continues beyond the menopause.

α2M functions as a proteinase inhibitor and a binder of cytokines and growth factors (reviewed in James 1990; Borth 1992, see Sec.1.6, 1.7 and 1.9). By these roles it can contribute to the pathogenesis of a number of diseases as illustrated in Table 1.2.2., and discussed below.

1.2.2: Role of α2M in reproductive organs

In the male and female reproductive systems, α2M has a significant function as a proteinase inhibitor and a binder of growth factors. In the ovaries, such functions are required for tissue remodelling during ovulation, development of the corpus luteum and in pregnancy (Gaddy-Kurten, Hickey, Fey, Gauldie, and Richards 1989; Curry, Mann, Estes, Jones 1990). In the testes, α2M is the major proteinase inhibitor restricting the damage of proteinases produced by degenerating spermatozoa as they pass through the seminiferous tubule (Cheng, Grima, Stahler, Gugliemotti

Age group (years)	α2M (mg/ml)		
	Children	Male	Female
Birth	3	N/M	N/M
0-1	4	N/M	N/M
1-3	4.25	N/M	N/M
4-6	4.25	N/M	N/M
9		3.95	3.7
12-13		3.9	3.4
17-20		2.5	2.7
21-25		2.2	2.5
26-30		2	2.3
31-40		1.8	2.1
41-50		1.8	2.2
>50		1.8	2.2

Table 1.2.1: The mean serum concentration of α2M in different age groups
Serum α2M concentration was measured for 12 age groups. Levels in childhood and puberty were higher than in adult life possibly reflecting the use of α2M in control of growth in pre-adult life. Female adults consistently had a higher concentration than men. N/M indicates data not measured. Figures derived from Ganrot and Schersten (1967).

Disease	Characteristics
Ehlers-Danlos Syndrome	- α2M deficiency - cutaneous hyperelasticity, hyperextensible joints
Cystic Fibrosis	- functional α2M deficiency - error in bait region or in receptor recognition domain
Rheumatoid Arthritis	- increased α2M levels locally - α2M-proteinase complexes form - decreased clearance of complexes - proteinase still active leading to cartilage destruction
Adult Respiratory Distress Syndrome	- increased α2M levels locally - binds proteinase and maintains activity - leads to lung injury
Venous Ulceration	- α2M, and other macromolecules leak into dermis - inhibit wound repair and tissue maintenance
Cancer	- variable effects dependant on tissue, disease stage, individual patient - effects involve interactions with proteinase, cytokines, or cytoskeleton
Alzheimer's Disease	- increased levels locally - present in amyloid plaques and in microglial cells - function in disease pathogenesis unclear
Gingivitis	- increased levels locally - may act to inhibit proteinase activity

Table 1.2.2: The role of α2M in a range of disease states
α2M has been reported to be involved in the pathogensis or recovery from a number of diseases. Its activity may be due to association with proteinases, cytokines, or with cytoskeletal proteins as discussed in Section 1.2.

et al 1990). α2M also limits the action of proteinase dependent seminiferous tubule remodelling, and maintains TGFβ and other growth factor and cytokines in an inactive/latent state limiting their activity to a small area of epithelium.

Foetal and maternal levels of α2M have been found to be inversely proportional to foetal birth weight (Goldenberg, Tamura, Cliver, Cutter et al 1991; Cliver, Goldenberg, Neel, Tamura et al 1993). It has been proposed that the increased α2M concentration may be linked to an immune response against a bacterial infection in the foetus, the infection also resulting in growth retardation of the foetus. In addition, pregnancy zone protein (PZP), a high molecular weight α-globulin, of similar form to α2M, rises in pregnancy from trace levels to 1mg/ml by the third trimester. This protein is further discussed in Sec.1.5.

1.2.3: Deficiency of α2M

Complete absence of α2M is thought to be a lethal deletion. Examination of 100,000 different sera did not find an individual devoid of α2M (Laurell and Jeppsson 1975). It has been proposed that absence of α2M may cause death of the foetus, indicating that it is a crucial requirement for the developing embryo.

Deficiencies of α2M have been recorded (Mahour, Song, Adham, and Rinderknect 1978; Bergqvist and Nilsson 1979; Stenberg 1981). Low levels of α2M are found in some patients with Ehlers-Danlos syndrome, where it may contribute to cutaneous hyperelasticity and hyperextensible joints observed in this condition (Mahour et al 1978). In contrast to deficiencies of other proteinase inhibitors, for example α2 anti-plasmin, α1 anti-trypsin and anti-thrombin III which predispose the patient to lung disease, liver dysfunction, and thrombosis, α2M deficiency can be

asymptomatic (Stenberg 1981). However, a low concentration of α2M is observed in a number of other conditions including multiple myeloma, pulmonary embolism, and extensive trauma.

1.2.4: Cystic Fibrosis

In cystic fibrosis (CF), a pseudodeficiency occurs where, due to defective proteinase trapping or receptor recognition, α2M does not inhibit and clear proteinase effectively (Wilson and Fudenberg 1976; Shapira, Martin, and Nadler 1977; Marynen, Van Leuven and Cassiman 1983). The pseudodeficiency may arise from an inherited error in the bait region of the α2M molecule rendering proteinases unable to cleave it and resulting in decreased proteinase trapping and therefore decreased clearance (see Sec.1.6 and 1.7). Alternatively, polyamine derivatives, present in high levels in the plasma of CF patients, may bind to the thiol ester causing closure of the trap and inhibiting further interaction with proteinases (Wilson and Fudenberg 1976). In addition, the α2M-receptor recognition domain may be defective in CF patients resulting in abnormal levels of proteinase bound to α2M compared to control sera (Marynen et al 1983). In such circumstances, the level of endocytosis of α2M-proteinase can be decreased by 20%, with the result that a proportion of bound proteinase remains active and in the circulation or tissues for longer.

1.2.5: Role of α2M in disease

In normal health, the principle function of α2M appears to be as a proteinase inhibitor. It traps and inhibits members of all 4 endoproteinase classes, and may function when other inhibitors are exhausted (Barrett and Starkey 1973; Travis and Salvesen 1983, see Sec.1.6 and 1.7). The enzyme protein complex binds to receptors expressed on a range of cells,

especially hepatocytes. This binding occurs via a neoantigen receptor recognition domain on the converted protein and results in endocytosis of the complex (see Sec.1.6.7).

In disease states, the levels of α2M synthesised may change, and as levels of proteinase rise the amount complexed to α2M requiring clearance from the circulation or tissues increases. The efficient inhibition of proteinase requires not only rapid binding by inhibitor, but also rapid clearance since proteinases can remain active against small substrates when bound to α2M (Barrett and Starkey 1973; Levine, Sherry, Strickland and Ilowite 1993). The rate of clearance of α2M-proteinase is a particular problem in rheumatoid joints, and possibly in adult respiratory distress syndrome as discussed below.

1.2.6: Rheumatoid arthritis

In adult and juvenile rheumatoid arthritis (RA), the concentration of anti-proteinase activity within the synovial joint increases (Teodorescu et al 1984; Borth, Dunky and Kleesiek 1986; Levine et al 1993). Specifically, increases in α2M-proteinase complex concentration are seen, and these have been correlated with increased levels of neutrophils and of neutrophil elastase activity. The key role of neutrophils in RA is highlighted by the increase in their concentration from 5% to 90% of the cells in the RA joint, and they have been implicated as major mediators of tissue destruction in RA (Abbink, Kamp, Nieuwenhuys, Nuijens et al 1991). α2M may enhance the activity and half life of neutrophil elastase, and possibly of other proteinases in synovial fluid by two means (Abbink et al 1991; Teodorescu et al 1984; Levine et al 1993):

(i) α2M traps proteinase and unlike other inhibitors, the interaction does not inactivate the proteinase; small substrates still have access to them.

Thus, when neutrophil elastase is bound by α2M it retains some activity. In this context it should be noted that 74% of neutrophil elastase activity in the RA joint has been reported to be associated with α2M (Levine et al 1993).

(ii) α2M can undergo the aforementioned conformational change by direct cleavage of a second bond, an internal thiol-ester (see Sec.1.1.1 Fig.1.1.1, Sec.1.6 and 1.7). The cleavage of the thiol-ester can be mediated by oxygen reactive species produced by neutrophils, and apparently can account for 90% of the total conformationally altered α2M levels observed (Levine et al 1993; Shatcher, Maayan and Feinstein 1973).

Thus, neutrophils produce elastase which can be bound by α2M and remain active, and whose clearance rate as a complex with α2M is decreased because of the overload of inactive, receptor-recognised α2M produced by oxygen reactive species. It was found that up to 46% of α2M present in RA synovial fluid is inactive, arising due to interactions with proteinase or with oxygen species (Abbink et al 1991). The neutrophil elastase bound by α2M is protected from inactivation by other proteinase inhibitors.

In addition, α2M binds IL1β and IL6 retaining the cytokines bioactivity (Teodorescu, McAfee, Skosey, Wallman et al 1991; Matsuda, Hirano, Nagasawa and Kishimoto 1989). This may also contribute to the pathogenesis of RA and is further discussed in Sec.1.9.

1.2.7: Adult respiratory distress syndrome

The interaction of α2M with neutrophil elastase has also been implicated in adult respiratory distress syndrome (ARDS) (Wewers, Herzyk and Gadek 1988). There are increased numbers of neutrophils in ARDS lung lavage fluid. In normal patients less than 5% of their lavage cells are

neutrophils, but in ARDS this increases to 60-90% of the total cell count (Abbink et al 1991; Wewers et al 1988). Simultaneously, protein levels increase, and there is a marked rise in neutrophil elastase activity which has been shown to be associated with α2M. Thus, it appears that a parallel scenario to RA occurs in the lung in ARDS with an influx of neutrophils producing proteinase whose activity may be maintained by α2M. The α2M-proteinase complexes may be cleared thus defending the lungs against injury, but there is evidence to suggest that the complexes promote lung injury (White, Janoff, and Godfrey 1980; Wewers et al 1988). The increased levels of α2M could be leaking from the vascular system due to the increased permeability associated with ARDS, or may be synthesised by macrophages that infiltrate the area during ARDS (White et al 1980).

1.2.8: Cancer

It is presently uncertain what role α2M plays in the control and development of cancers. The concentration of α2M varies according to the tissue and stage of cancer, and with considerable individual deviations (Twining and Brecher 1977; Saksela, Vaheri, Schleuning, Mignatti and Barlati 1984a; Saksela, Wahlstrom, Meyer, and Vaheri 1984b; Burtin, Chavanel, Andre-Bourgaran, and Gentile 1987; Matoska, Wahlstrom, Vaheri, Bizik and Grofova 1988; Bizik, Lizonova, Grofova, Matoska et al 1989).

Changes in α2M concentration appear to be local to the tumour site, suggesting it is acting as a local defence protein remote from plasma α2M (Zucker et al 1991; Saksela et al 1984b). For example, variations in α2M levels are observed during the development of cervical cancer. Examination of cervical epithelia at a number of stages of dysplasia

reveals an inverse correlation between α2M concentration and the disease stage. Furthermore, in truly neoplastic tissue no α2M synthesis or uptake can be detected (Saksela et al 1984b). Differences between individual patients were found in human melanomas where α2M production varied considerably. In one study, α2M levels were found to correlate positively with a poor prognosis, suggesting the inhibitor may contribute to the pathogenesis of the disease (Matoska et al 1988). This may have been due to α2M maintaining the proteolytic activity of bound molecules, similar to its proposed role in RA and ARDS.

In contrast, the interaction of α2M with growth factors and cytokines may inhibit tumour development (see Sec.1.9). For example, α2M is a selective growth disadvantage in certain melanomas *in vitro* where its presence has been found to increase the tumours doubling time (Bizik et al 1989). α2M is known to bind PDGF and to restrict its activity, therefore it has been proposed that it retards the growth of tumours by inhibiting PDGF activity (Huang, Huang and Deuel 1984; Bizik et al 1989).

A loss of α2M function may occur as its synthesis is switched off in transforming cells. A decrease in α2M levels may contribute to tumour development due to a change of surface properties, and loss of cell adhesion and morphology (Saksela et al 1984a; Twining and Brecher 1977; Zardi, Carnemolla, Cagnasso and Santi 1980). A positive correlation between α2M and fibronectin was reported by Zardi et al (1980), implying a role for α2M with respect to fibronectin, a protein active in maintenance of cell morphology, cytoskeletal structure and adhesion. The physical changes observed in transformed cells may occur due to the uncontrolled activity of proteinases that function at the cell surface, and may arise due to a decreased production of α2M and a number of other inhibitors.

There is a range of proteinase inhibitors present at tumour sites, and α2M

may be part of a network. It may be acting as a "back-up" inhibitor, for example inhibiting plasmin activity when its principle inhibitor α2 anti-plasmin is at a low concentration (Burtin et al 1987). These and other proteinase inhibitors deserve further investigation as to their pleiotropic effects at tumour sites (Troll, Frenkel and Wiesner 1984).

1.2.9: Venous Ulceration

α2M, and other macromolecules, may have a role in venous ulceration (Falanga and Eaglstein 1993). It is known that venous hypertension precedes this condition, but the subsequent pathogenesis is unclear. α2M, fibrin, and other macromolecules leak into the dermis during hypertension, and ulcers are characterised by extravasation of fibrinogen and α2M. α2M and other macromolecules may "trap" growth factors and other stimulatory or haemolytic substances in the dermis. Thus, repair of the wound that forms during ulceration and maintenance of tissue integrity is restricted. TGFβ and TNFα are bound by α2M which may inhibit their activity (Danielpour and Sporn 1990; Wollenberg, LaMarre, Rosendal, Gonias and Hayes 1991, see Sec.1.9). Though abundant in ulcers, wound fluid does not contain the expected stimulatory activity attributable to TGFβ and TNFα, indicating restriction of the activity of these molecules. The effects of α2M, and other macromolecules, may therefore be implicated by binding to growth factors and altering their concentration or sequence of release, thus affecting the growth factor "language" of repair (Sporn and Roberts 1988).

1.2.10: Alzheimer's Disease

α2M may have a role in the development of Alzheimer's disease (AD). It is present at increased levels in central nervous system (CNS) tissue in

AD patients. It appears that α2M may immunomodulate microglial cells in an inflammatory response. This is supported by the observation of α2M staining in neuritic plaques, although *in vitro* production of α2M by neuroblastoma cell lines have contradicted these findings (Ashall and Goate 1994; de Strooper, Van Leuven and Van den Berghe 1992). α2M has previously been purported to be an inhibitor of secretase, a proteinase required for the normal cleavage of amyloid precursor protein, a significant protein in AD (Strauss et al 1992). Inhibition of secretase results in aberrant cleavage of the protein to form β-amyloid which is found in high concentrations in AD plaques. However, it appears that α2M and other proteinase inhibitors do not influence the disease progression at the point of secretase (de Strooper et al 1992).

1.2.11: Gingivitis

In the buccal cavity, gingivitis can be induced by cessation of brushing for 10 days (Giannopoulou, Di Felice, Andersen and Cimasoni 1990). The *in vivo* study of α2M production in the gingival sulcus was based upon an *in vitro* observation of α2M synthesis by cultured gingival fibroblasts (Condacci, Cimansoni, Rey and Baehni 1988). The study revealed a 6 fold increase in α2M levels in the gingival sulcus that corresponded to the development of inflammation. Albumin levels increased 2 fold therein, and neither protein increased in concentration in the serum. Due to its size it is unlikely that α2M could move freely from the serum to the buccal cavity membranes, indicating that α2M found locally under normal and inflammatory conditions was produced by local cells and by infiltrating leucocytes.

1.3: Synthesis of α2M

1.3.1: Hepatic synthesis of α2M

As with most plasma proteins, the primary site of α2M synthesis is believed to be the liver, specifically hepatic and Kupffer cells (Laurell and Jeppson 1975). However, a number of other cells are known to produce α2M including lymphocytes, cultured human fibroblasts, monocytes, human alveolar macrophages, gingival fibroblasts, cultured bovine adrenal cortical cells, and cells of the testis and ovaries (Tunstall and James 1974; Mosher and Wing 1976; Hovi, Mosher and Vaheri 1977; White et al 1980; Straight, Jakoi, McKee and Snyderman 1988; Condacci et al 1988; Giannopoulou et al 1990; Kirshner, Corcoran and Erickson 1989; Cheng et al 1990; Gaddy-Kurten et al 1989; Curry et al 1990).

1.3.2: Extra-hepatic synthesis of α2M

Local synthesis of α2M may be outwith the control of the hepatic system, as demonstrated in various disease states. For example, as discussed previously, in RA and in cancer α2M levels have been found to rise in synovial fluid or at the tumour site, but plasma levels remain normal (Levine et al 1993; Zucker et al 1991, see Sec.1.2). The synthesis of α2M at extra-hepatic sites serves a range of functions including local control of proteolysis (see Sec.1.2). There is also evidence that in some tissues α2M may regulate growth factor and cytokine activities (see Sec.1.9).

In bovine adrenocortical cells, TGFβ and α2M form a feedback loop each modulating the synthesis and activities of the other (Kirshner et al 1989; Shi, Savona, Gagnon, Cochet et al 1990; Keramidas, Chambaz and Feige 1992,see Sec.1.9). In addition, it appears that hormonal control of synthesis occurs in the ovaries and testis (Gaddy-Kurten et al 1989;

Curry et al 1990; Cheng et al 1990). It has been suggested that there is very little control over α2M synthesis normally (Mosher 1983). Beyond these details the factors that induce or inhibit α2M production in humans are not well documented.

It has been shown that there is a positive correlation between proteinase and α2M secretion in fibroblast like cells: it may be that α2M is synthesised to inhibit proteinase activity and limit it to the microenvironment in which it was produced. There are a range of *in vivo* sites at which this correlation is evident, such as in the RA joint, in the alveolar space, and in the buccal cavity (see Sec.1.2). Therefore, it may be proposed that at non-hepatic sites the stimulus for the production of proteinase and inhibitor may be one and the same.

Thus, it can be concluded that α2M is primarily produced by the liver, but local synthesis can occur both constitutively and in disease states. In addition, there is evidence of exchange between the vascular and tissue pools of α2M (Zucker et al 1991).

1.3.3:The α2M gene

The α2M gene is located on chromosome 12 (Kan, Soloman, Belt, Chain et al 1985). Control elements have been identified in the rat, for example the HP1 element, a metal response element, a potential Sp1 binding site and a transcription initiation site in the 5' flanking region of exon 1 which is homologous to IL6 response elements (reviewed in Borth 1992). IL6 has been identified as an acute phase stimulator of α2M synthesis in rat liver, and to induce α2M synthesis in a murine neuroblastoma cell line (Warburton, Gusterson and O'Hare 1993; Ganter, Strauss, Jonas, Weidemann et al 1991; see Sec.1.2).

1.4: α-Macroglobulins in other species

1.4.1: Detection of αM in other species

α–macroglobulins (αM's) have been recognised in a number of species (Starkey and Barrett 1982a; Starkey, Fletcher and Barrett 1982; Starkey and Barrett 1982b; Saito and Sinohara 1985; Feldman and Pizzo 1985; Bjork, Lindblom and Lindahl 1985; Sottrup-Jensen, Borth, Hall, Quigley and Armstrong 1990). Starkey and Barrett conducted a definitive study by examining a large range of vertebrates for the presence of a papain binding protein with α2M-like characteristics (Starkey and Barrett 1982a). Their premise was that due to its important role as a human proteinase inhibitor α2M would probably be found in other species therein conducting similar functions. All major vertebrate taxa were found to have a 725kDa protein that could bind papain restricting the enzyme's activity to small substrates. Invertebrates, specifically arthropods, were also examined: not all were found to be positive for α2M, although *Limulus polyphemus*, the horse shoe crab, was shown to have a form of αM. These results indicate that human α2M has homologous proteins throughout a wide range of organisms, as illustrated in Table 1.4.1.

Limulus αM was found to interact with proteinase and with methylamine in a similar manner to αM in higher organisms. However, its molecular weight is in the region of 550kDa, thus it is trimeric as compared to the 725kDa tetrameric human α2M. Fish, specifically plaice, were found to contain a 360kDa αM that can interact with proteinase or with methylamine, but which did not show a conformational change (Starkey and Barrett 1982a and b; Starkey et al 1982). Starkey and Barrett speculated that a transition of αM from a dimer to tetramer form and ability to change conformation must have occurred during evolution.

Subsequent studies have confirmed and extended the results of Starkey

αM	Form	Thiol Ester	S to F with Me?	S to F with Pr?	References
Human α2M	T	Yes	Yes	Yes	Starkey and Barrett 1982
Human PZP	T	Yes	Yes	Yes	Sand et al 1985
Rat α2M	T	Yes	No	Yes	Sottrup-Jensen et al 1990
Rat α1M	T	Yes	Yes	Yes	Sottrup-Jensen et al 1990
Rat α1I3	M	N/A	N/A	N/A	Gauthier and Ohlsson 1978
Murine αM	T	Yes	Yes	N/A	Saito and Sinohara 1985
Murinoglobulin	M	Yes	N/A	N/A	Saito and Sinohara 1985
Bovine α2M	T	Yes	No	Yes	Feldman et al 1984
Hen α2M	T	Yes	Yes	Yes	Nagase et al 1983
Hen ovostatin	T	No	No	Yes	Nagase et al 1983
Frog α1M	T	Yes	No	No	Feldman and Pizzo 1985
Frog α2M	D	Yes	N/A	No	Feldman and Pizzo 1986
Fish αM	D	Yes	No	No	Starkey and Barrett 1982
American Lobster	D	Yes	No	Yes	Spycher et al 1987
American Horseshoe Crab	520kDa	Yes	N/A	N/A	Starkey and Barrett 1982

Table 1.4.1: Characteristics of αM from a range of species
αM is present in all vertebrates examined, and in a number of invertebrates. Between the forms of αM variation is seen with respect to the presence of a thiol ester, and the conformational change, slow (S) to fast (F), observed in the presence of methylamine ("Me") or proteinase ("Pr"). The subunit number making up the protein also varies, as indicated: tetramer (T),-dimer (D), or monomer (M). For some sources of αM, information was not available (N/A). Additional references appear in the text.

and Barrett as illustrated in Table 1.4.1. The most striking features are the range of species in which αM have been found, and that many species have 2 or more distinct forms of the inhibitor.

1.4.2: Homology amongst αM's

Sequencing studies and amino acid composition analysis have been carried out on a number of αM's, and as expected homology is apparent (Sottrup-Jensen, Folkersen, Kirstensen, and Tack 1984, Feldman and Pizzo 1986, Sottrup-Jensen 1987, Spycher, Arya, Isenman, and Painter 1987). Conserved sequences have been found to be at the N terminus, bait region and thiol ester, and at other sites throughout the molecule (see Fig.1.4.1.)

1.4.3: Evolution of αM

Frog α1M and bovine α2M seem to be proteins at evolutionary crossroads in the development of the tetramer of higher vertebrates (Feldman, Gonias, Ney, Pratt and Pizzo 1984; Feldman and Pizzo 1985; Bjork et al 1985; Feldman and Pizzo 1986). They exhibit a range of characteristics that make them less efficient as proteinase inhibitors than the αM found in higher species. These various features of lower species αM's, such as dimer form, no conformational change, no receptor recognition domain, low trypsin binding efficiency, contrast to those of higher organisms such as rats and humans. In the latter, αM is usually present as a tetramer whose conformational change alters upon efficient interaction with proteinase activating the inhibitor, exposing the receptor recognition domain, entrapping the proteinase and resulting in clearance of the inhibitor from the circulation via the α2M-receptor (Feldman, Gonias and Pizzo 1985). Monomer and dimer αM are present in a number of species, as indicated in Table 1.4.1. (Gauthier and Ohlsson 1978; Esnard, Gutman, el Moujahed

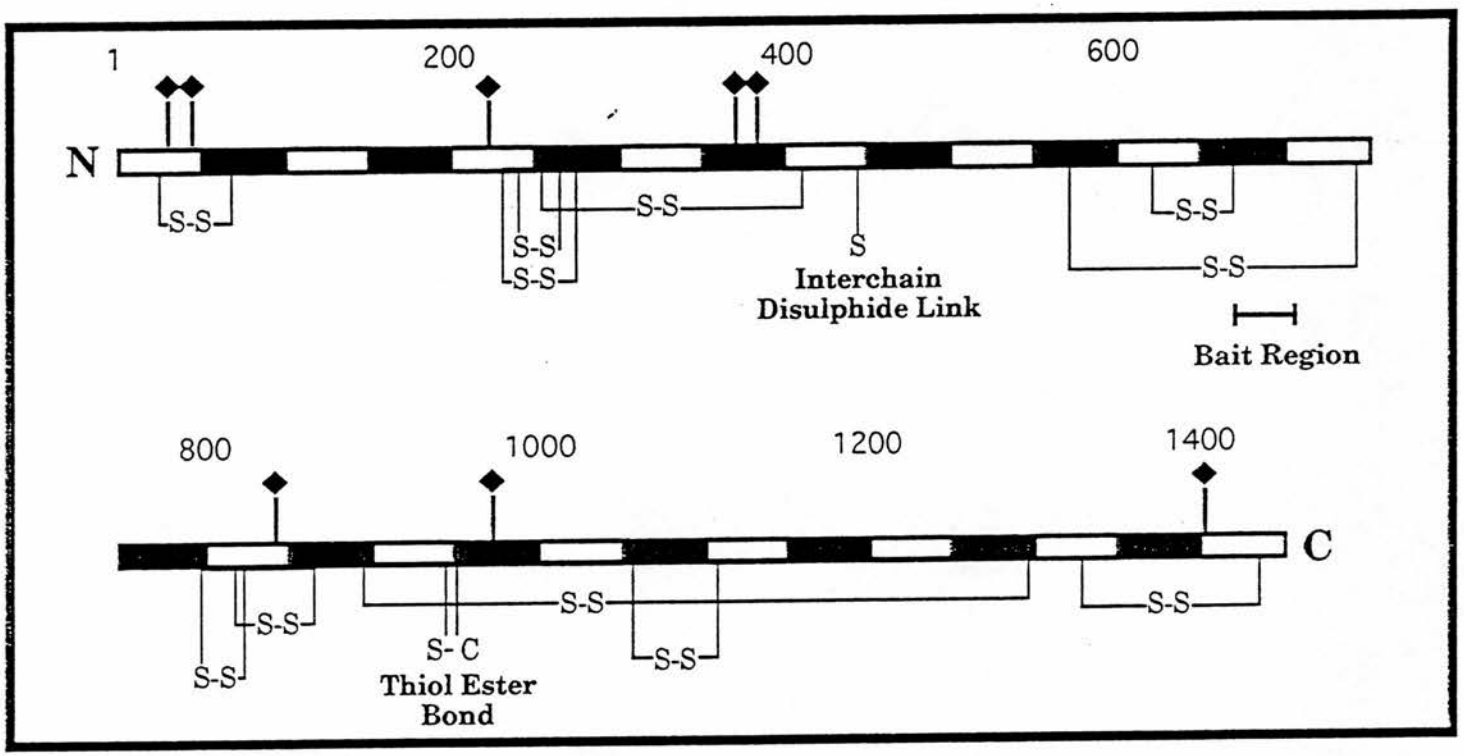


Figure 1.4.1: The primary structure of α2M monomer chain
α2M and the complement proteins C3 and C4 share a common primary structure as illustrated. Disulphide bridges are marked as ⌊S-S⌋. Carbohydrate residues are represented as ⏊. There is an interchain disulphide present at cys^{447}. The bait region (~residues 681-700) and thiol ester bond are common to the thiol ester proteins, eg PZP, C3 and C4. Each double box represents 100 residues, ie ▭▬. Approximately the first 610 residues of the sequence form the β chain of C3 and C4, the remainder constitutes the α form for each. However, pro-C4 has a third cleavage site to form a short chain, γ, from the C teminal residues. These features are further discussed in Sec.1.5. αM from a range of vertebrates and invertebrates share this structure.

and Gauthier 1985; Saito and Sinohara 1985; Moncino, Roche and Pizzo 1991). For example, rat $\alpha 1I_3$ is similar to the monomer subunit of human α2M, and has an internal thiol ester bond. It traps proteinase in a similar mechanism to human α2M but does not limit access to substrates (Esnard et al 1985). Unlike dimers and tetramers, covalent binding is essential for proteinase interaction with monomers (see Sec.1.7).

1.4.4: Ovostatin

The thiol ester bond is a characteristic feature of αM's and it is vital to the activity of the related complement proteins C3 and C4 (see Sec.1.5, 1.6, and 1.7). Nevertheless, some forms of αM exist without this bond. Hen plasma has a thiol ester containing α2M, but in hen egg white, as in other avian and amphibian eggs, there is a distinct second form (Nagase, Harris, Woessner and Brew 1983; Nagase and Harris 1983). This protein, ovostatin, has no internal thiol ester but is otherwise similar to other αM proteins and its activity against proteinases does not seem to be perturbed by this absence. The complement protein C5 also has a structure similar to the monomer chain of α2M but the cysteinyl and glutamyl residues of the thiol ester bond are absent (Sottrup-Jensen 1987, see Sec.1.5).

1.4.5: Role of αM as a defence protein

αM has been identified in diverse species and it has been suggested that it could be a primitive defence molecule for inhibiting proteinase (Barrett 1981; Sottrup-Jensen 1987; Matsuda et al 1989). Indeed, the lobster *Homanus americanus* has a trimeric form of α2M but apparently no thiol ester containing complement proteins, such as C3 and C4 (Spycher and Painter 1991). In lower species, α2M may form part of a "primitive" defence system from which its "modern day" counterparts in higher

organisms have evolved, principally α2M, its homologues, and the complement proteins C3, C4, and C5 (Sottrup-Jensen, Stepanik, Kristensen, Lonblad et al 1985). Furthermore, the *Limulus* lineage and vertebrates diverged at least 550 million years ago, indicating that the function of α2M is ancient and evolutionarily conserved (Quigley and Armstrong 1983a and b).

1.5: Thiol ester plasma proteins

1.5.1: The thiol ester plasma proteins in humans

Human blood contains two α-macroglobulins, namely α2M and PZP (see Sec.1.4 Table 1.4.1). These proteins contain an internal thiol ester bond, but they are not unique in this respect. The complement proteins C3 and C4 have also been identified as thiol ester plasma proteins (TEPP) the bond being critical to their activities (Fig.1.5.1; Tack, Harrison, Janatova, Thomas and Prahl 1980; Janatova and Tack 1981; Sottrup-Jensen 1987). These proteins can be grouped by a number of common features:

(i) Slow inactivation by amines (Gordon, Whitehead and Wormall 1926; Tack et al 1980; Janatova and Tack 1981; Sand, Folkersen, Westergaard, and Sottrup-Jensen 1985; Van Leuven, Cassiman and Van den Berghe 1982).

(ii) Rapid covalent binding of the proteinase activated form of the TEPP to another protein or to a cell surface (Law 1984; Salvesen and Barrett 1980; Sand et al 1985).

(iii) The appearance of titratable -SH groups during these reactions (Janatova and Tack 1981; Sand et al 1985; Barrett et al 1979).

(iv) Specific conformational change with recognition by a cell surface receptor (Janatova, Lorenz, Schechter, Prahl and Tack 1980a; Jantova, Tack and Prahl 1980b; Janatova and Tack 1981; Van Leuven, Cassiman and Van den Berghe 1986a; see Sec.1.6 and 1.7).

1.5.2: Homology

A comparison of available sequence data has enabled alignment of homologous regions and has lead to the conclusion that these proteins come from a single ancestral gene (Sottrup-Jensen et al 1985; Sottrup-Jensen 1987; Sand et al 1985). C3 and C4 are translated as pro-proteins

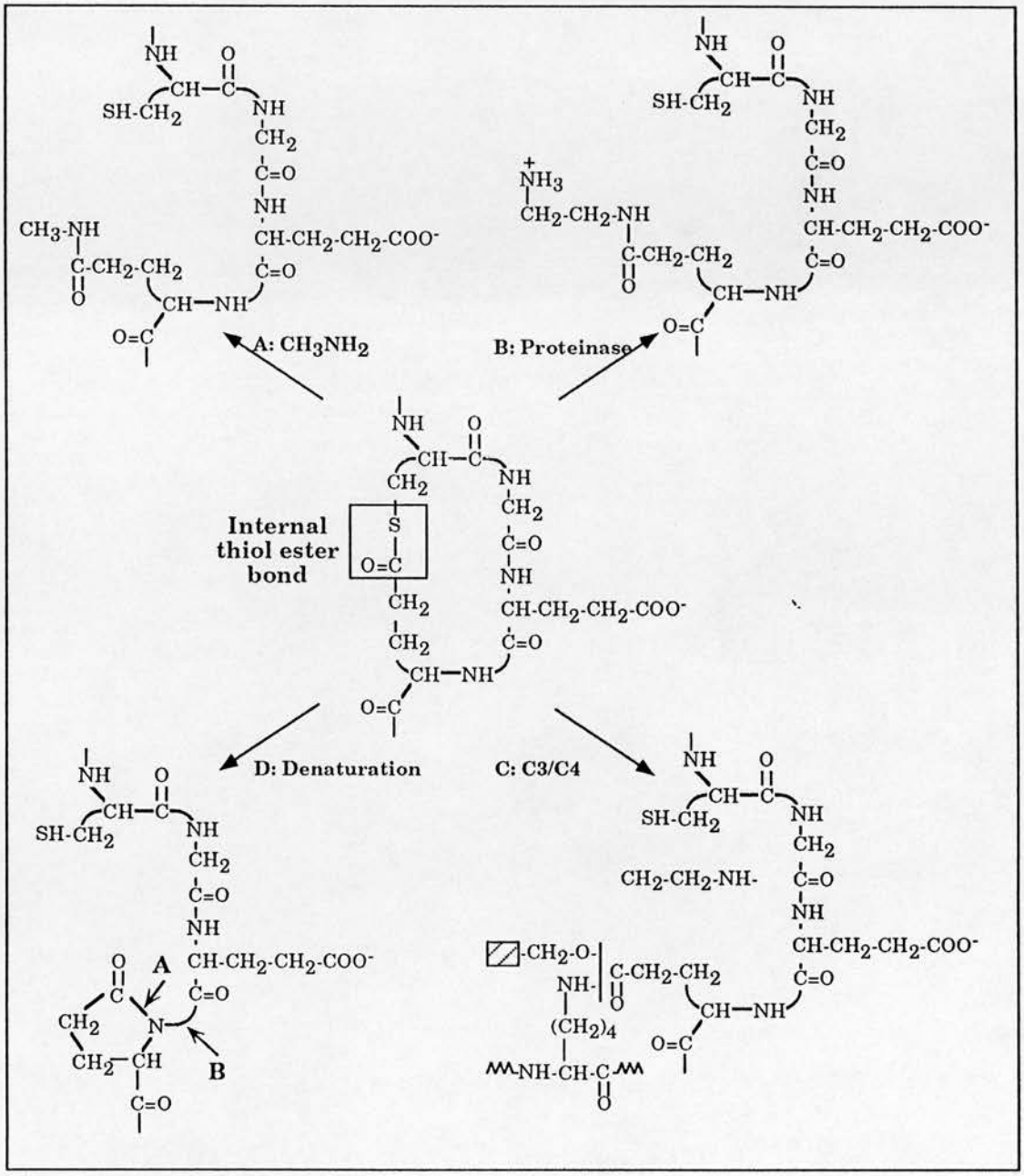


Figure 1.5.1: Chemical structure and reactions of the internal thiol ester bond in the thiol ester plasma proteins
The internal thiol ester bond of all thiol ester proteins can react with primary amines such as methylamine (A). α2M reacts with lysine sidechains of entrapped proteinases (B). Complement proteins C3 and C4 react through the cleaved thiol ester with polysaccharides, membrane components, and immune complexes (C). The thiol ester can also be denatured forming an internal pyroglutamic acid ring (D). In each case a free -SH group appears which is available to react with other proteins. Taken from Sottrup-Jensen 1987.

and subsequently cleaved to produce their secreted form. The single pro-protein chain of each complement protein compares to the primary structure of the α2M monomer at several points (see Sec.1.4. Fig.1.4.1). The key features are:

(i) N terminus: the terminal 40 or so residues are homologous.

(ii) The bait region sequence: the bait region is found in the α chain of C3 and C4. In addition, the bait region of the α2M monomer aligns with the sequence of pro-proteins of C3 and C4 within which they are cleaved to form the α–β chains of the secreted protein. C4 has a second cleavage point towards the COOH terminus and its secreted form is therefore trimeric.

(iii) Internal thiol ester bond: a residue sequence of approximately 150 amino acids containing the thiol ester bond is very homologous between the proteins (Swenson and Howard 1980; Tack et al 1980; Thomas, Janatova, Gray and Tack 1982; Sand et al 1985; Sottrup-Jensen et al 1985; Sottrup-Jensen 1987).

Overall there are long stretches of identity, interspersed with regions of low homology that provide each protein with a unique tertiary structure and function. Analysis of the primary structures of the TEPP's indicated approximately 60% homology between α2M, C3 and C4, and 68% between α2M and PZP (Sottrup-Jensen 1987). In addition, the complement protein C5 had homologous regions but did not contain the cysteine and glutamyl residues that constitute the thiol ester bond. This is similar to ovostatin the αM in hen egg white that has αM-like proteinase inhibitory activity, but lacks a thiol ester bond (Nagase et al 1983; Nagase and Harris 1983; see Sec.1.4).

1.5.3: Distinct features amongst TEPP

Despite the high degree of primary structural homology, the TEPP do have distinct features. PZP and human α2M exist as single chains that dimerise and can form tetramers (Sand et al 1985; Barrett et al 1979). C3 and C4 are a heterologous dimer and trimer respectively formed from the cleavage products of a single chain (Sottrup-Jensen 1987). In keeping with their function, C3 and C4 contain an anaphylatoxin site, and they also have an extension beyond the C terminus of the αM gene. Disulphide bridges are a key feature of human α2M which contains 11 intra and 2 inter disulphide bridges per chain, whereas C3 has 3 such bridges. Approximately 10% of the weight of α2M is carbohydrate which is distributed between 8 diverse sites on the molecule (Sottrup-Jensen, Stepanik, Kristensen, Wierzbicki et al 1984b; see Fig.1.4.1). The carbohydrate residues may be an evolutionary conserved feature being employed in lower species as a mechanism of clearance (Sottrup-Jensen et al 1984b). C3 has 3 carbohydrate attachment sites which would account for much less of its mass (Sottrup-Jensen 1989).

1.5.4: Internal thiol ester bond

For both the αM and complement TEPP's, activation of the thiol ester site is critical to their function. However, the complement proteins have a narrow substrate specificity for their activation cleavage site, whereas α2M and PZP have broad substrate ranges (Sottrup-Jensen 1987; Sand et al 1985; Barrett and Starkey 1973; Salvesen and Barrett 1980). Fig.1.5.1. illustrates the thiol ester bond and the reactions that it can undergo in αM's and in the complement proteins.

With respect to substrates, α2M and PZP diverge somewhat: their ranges do overlap but the interaction rates with particular proteinases differ between the 2 inhibitors indicating a variation in their specificity (Sand et

al 1985). The bait region sequences of α2M and PZP are not identical which explains the disparity in reactivity. Due to the variation in substrate, and also that PZP is normally only available in pregnancy, it has been proposed that PZP may have a distinct role in the body from that of α2M, possibly to clear proteolytic activity arising from increased cell turnover. PZP has been reported to be immunosuppressive and may act to protect the immunologically privileged state of the foetus (Stimson 1972; Stimson and Eubank 1972; Sand et al 1985). PZP and α2M have been found on the surface of syncytiotrophoblasts suggesting protection of the maternal-foetal interface by clearing proteinase, or by binding and eliminating growth factors therefore controlling growth of the maternal foetal unit (Lim and Halbert 1976; Lim, Halbert and Spellacy 1976; Sand et al 1985).

1.5.5: The nascent state

The activation of the thiol ester bond in TEPP's results in a nascent state in which the glutamyl residue can react rapidly with available nucleophiles (see Fig.1.5.1 and Sec.1.7). Activation results in clearance of TEPP's from tissues or from the circulation. For α2M and PZP this involves a conformational change and exposure of the receptor recognition domain (see Sec.1.6 and 1.7). The receptor recognition domain of both proteins can bind to the α2M receptor, expressed by a range of cells, resulting in receptor mediated endocytosis of the protein-proteinase complex.

Activation of the thiol ester is required for the covalent interaction of C3 and C4 with cell surface antigens such as CR1 and also with immune complexes (Sottrup-Jensen 1987; Tack et al 1980; Janatova and Tack 1981; Law 1984). As with the αM's, C3 and C4 can be inactivated by reaction with methylamine due to cleavage of the thiol ester bond by the

amine (Barrett and Starkey 1973; Sand et al 1985; Tack et al 1980; Janatova and Tack 1981). However, for the αM's, proteinase inhibition requires trapping within the dimeric functional unit of the inhibitor and does not rely upon the subsequent covalent interaction of proteinase and glutamyl residue (Sand et al 1985; see Sec.1.7). Nevertheless, the thiol ester does have a pivotal role in the conformational change of α2M and PZP that is required in trapping. Thus, the thiol ester is central to the functions of all the TEPP either for trapping of proteinases due to a conformational change in the αM's, or for covalent binding of C3 and C4 to cell surface molecules and to immune complexes.

1.6: The structure of α2M

1.6.1: The tetrameric form of α2M

Human α2M is a tetrameric protein composed of identical subunits. The monomers form disulphide bonded pairs which noncovalently bond to form the tetramer (Jones, Creeth, Kekwick 1972; Frenoy, Bourrillon, Lippoldt, and Edelhoch 1977; Barrett et al 1979). Upon interaction with proteinase or primary amine, the intersubunit interactions change, so that in the reacted form there are an increased number of noncovalent links between the monomers and the molecule appears more compacted (Fig.1.1.1, Barrett et al 1979; Feldman et al 1985).

The alteration in intersubunit interactions is due to a conformational change, which has been detected by circular dichroism, sedimentation velocity measurement, electrophoresis and electron microscopy (Barrett and Starkey 1973; Bjork and Fish 1982; Feldman et al 1985; Boisset, Taveau, Pochon, Tardieu et al 1989; Delain, Pochon, Barray and van Leuven 1992). In addition to the overall structural change certain chemical groups are revealed, as illustrated in Fig.1.4.1 and 1.5.1. It is possible to titrate α2M with trypsin or methylamine to reveal 4 sulphydryl groups per α2M tetramer, that is one sulphyryl group per cleaved thiol ester bond (Salvesen, Sayers and Barrett 1981; Swenson and Howard 1979). Within α2M is the "bait region" which is a sequence of residues that can be cleaved by most endoproteinases resulting in activation of the thiol ester bond and conformational change. Methylamine and other primary amines elicit the conformational change by activating the thiol ester bond directly. The conformational change is accompanied by exposure of the receptor recognition domain required for clearance of α2M from the circulation (Sottrup-Jensen, Gliemann and Van Leuven 1986).

Physical changes in the structure of α2M have been examined by a

number of means. The most direct technique has been electron microscopy which has revealed quite clearly the change in α2M tertiary structure upon incubation with proteinase or primary amine (Fig.1.6.1; Schramm and Schramm 1983; Feldman et al 1985; Delain et al 1992). In the native protein, α2M is an open cyrillic H form which becomes compacted during the conformational change.

1.6.2: Primary structure

The primary structure of α2M was fully elucidated by Sottrup-Jensen et al, although previously certain regions had been examined (Sottrup-Jensen et al 1984b; Swenson and Howard 1979). From the primary structure a number of key sites can be distinguished including positions of carbohydrate attachment and disulphide bridges, as illustrated in Fig.1.4.1. As previously emphasised, some 10-11% of the mass of α2M (725kDa) is attributable to carbohydrate which is bound at 8 sites on the molecule (see Sec.1.5.3). The carbohydrate chains bound are very heterogeneous. Of the 24 cysteine residues in the monomer sequence, 22 form intrachain bridges, one is required for the internal thiol ester bond (cys^{949}), and one forms an interchain bridge with a neighbouring monomer (cys^{447}). There is very little intrachain sequence homology indicating that it is unlikely that the primary sequence of α2M arose by gene duplication.

1.6.3: Secondary structure

Based on the primary sequence data, 47.8% β structure and 8.6% α helix were predicted for the secondary structure of α2M (Sottrup-Jensen et al 1984b; Welinder, Mikkelsen and Sottrup-Jensen 1984). This corroborated well with a previous report (Bjork and Fish 1982). Furthermore, the

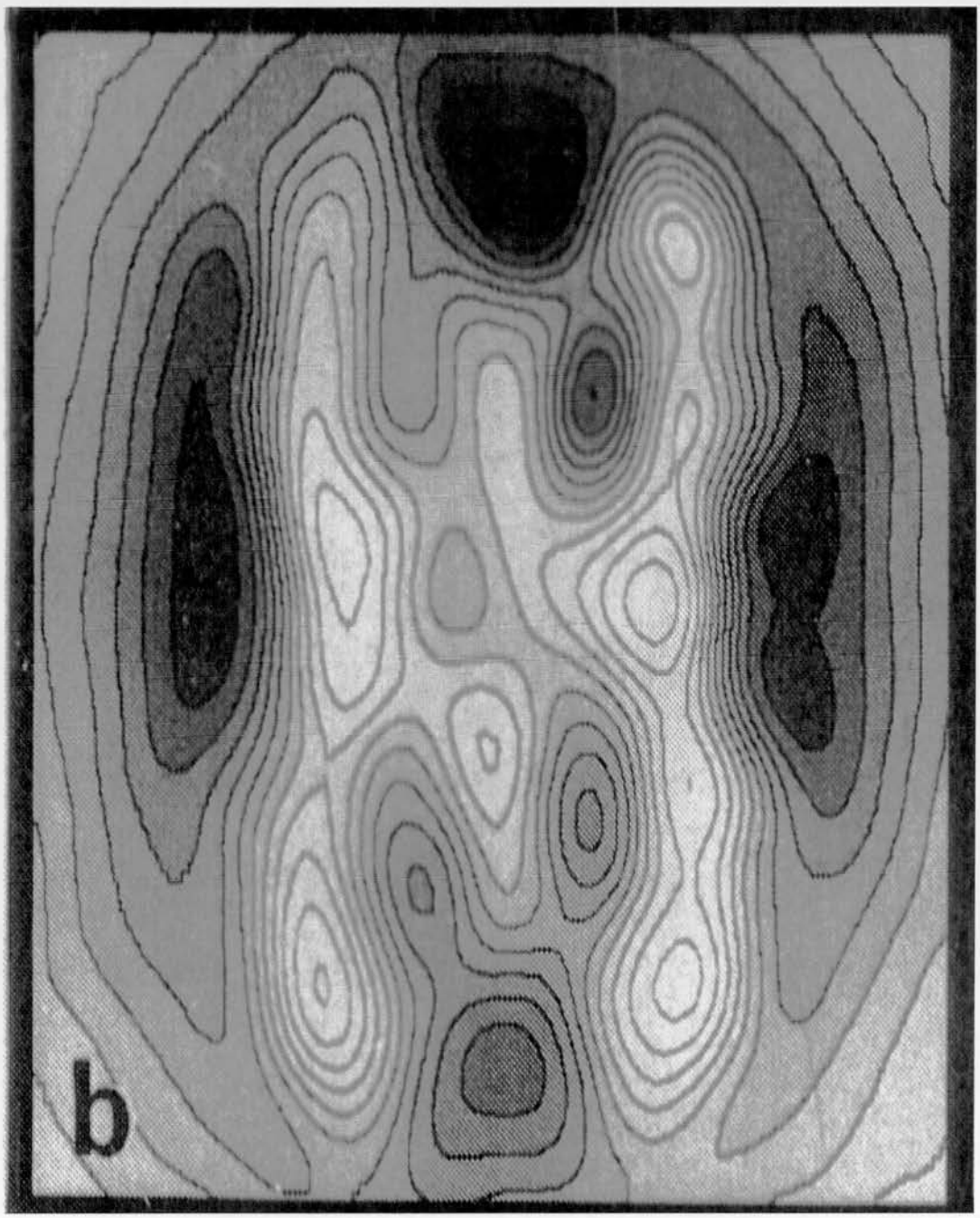


Figure 1.6.1: Electron micrograph image of alpha2M
The image presented is formed of a series of images of alpha2M-chymotrypsin by Boisset et al (1989). The long arms of the molecule can be distinctly identified; compare to Fig.1.1.1. Boisset et al indicate that the central domains represent the proteinase.

appearance of hydrophobic and hydrophilic sequences indicated a domain structure with exposed and shielded regions as expected of a macromolecule.

1.6.4: Quaternary structure

Feldman et al proposed the classical model of α2M (Fig.1.1.1; Feldman et al 1985). The model is overtly similar to images seen in EM studies, namely a hollow cylinder with lateral arms, one short pair and one long pair in each half of the molecule (Fig.1.6.1; Delain et al 1992). Each monomer contributes one long and one short arm. The dimensions suggested agree favourably with later work in which the functional unit of α2M, that is a dimer that acts to trap and inhibit a proteinase, was designated as the basket-like halves of the protein (Roche, Salvesen and Pizzo 1988; Chen, Wang, Yuan and Feinman 1992). The monomers that compose these functional units are non-covalently bonded, the disulphide bonds forming across the X-axis of the molecule (Roche et al 1988).

Feldman et al's model predicted the bait region and thiol ester to be internal, central, and adjacent to each other (Feldman et al 1985). In addition, they hypothesised that the receptor recognition domain would be on the external face of the cylinder at the base of the long arms. It was proposed that the long arms would move inwards to trap the proteinase upon activation of the thiol ester, positioned at the internal base of the hinge arms. Concomitantly, the receptor recognition domain would be revealed facilitating α2M-proteinase complex clearance from the circulation. Further investigation has confirmed certain aspects of this model and disputed others. These will be discussed below.

1.6.5: Bait region

The bait region is a sequence of approximately 27 amino acids in the middle of the primary structure that presents cleavage sequences that are suitable for all known endoproteinases (Fig.1.4.1; Travis and Salvesen 1983; Sottrup-Jensen et al 1984b). Prior to identification of the sequence, the bait regions presence was predicted due to the interaction of one proteinase precluding the interaction of a second in the same functional half molecule (Barrett and Starkey 1973). In addition, reaction with all endoproteinases followed by reductive SDS-PAGE revealed cleavage products of 85-100kDa (Barrett et al 1979; see Sec.1.7). These observations indicated the same or adjacent cleavage sites for the range of endoproteinases.

Within the bait region, there are "hot" sequences specifically Arg^{681}-Glu^{686} and Arg^{696}-His^{699}. The proteinase cleaves α2M within the bait region often within the "hot" sequences resulting in thiol ester activation and a conformational change of α2M therefore restricting access to the bait region for other proteinases. Although it is cleaved by proteinases at one and sometimes a second site within the bait region, no cleavage products are released from α2M: this is due to a disulphide bridge that spans the bait region thus keeping the molecule intact (see Fig.1.4.1; Sottrup-Jensen et al 1984b). The bait region may be able to adapt its conformation to present a more suitable substrate specificity to a particular proteinase, some proteinases binding better than others (Sottrup-Jensen 1989; Sand et al 1985).

Upon cleavage of the bait region, the "trap" is sprung: the conformation of α2M is altered and the proteinase is entrapped and its activity against large substrates is lost, although its active site is still functional (see Fig.1.1.1; Barrett and Starkey 1973). As previously stressed, the retention of proteolytic activity may be important in disease states, for

example in rheumatoid arthritis and in adult respiratory distress syndrome (see Sec.1.2).

The location of the bait region in the quaternary structure of α2M is unclear. Feldman's model predicted it to be at the central base of the interior of the cylinder, and this seems to be the most satisfactory explanation as judged by EM and fluorescence studies (Schramm and Schramm 1983; Boisset, Taveau, Pochon, Barray et al 1991; Delain et al 1992). Gonias et al reported that the bait region was at the termini of the long arms, but this result may be an artifact arising from use of an IgG-colloidal gold complex which may have been sterically hindered from accessing the cylinder interior (Gonias, Allietta, Pizzo, Castellino and Tillack 1988). Differential images of α2M after binding free chymotrypsin, immobilised chymotrypsin, and methylamine indicated a central location for the proteinases and implies a central location for the bait region, and also for the thiol ester site (Boisset et al 1989; Boisset et al 1991).

1.6.6: Internal thiol ester bond

The internal thiol ester bond is notable for two reasons: it is a unique internal bond of a glutamyl and cysteinyl residue found only in α2M and related proteins (eg C3, C4, PZP), and it allows primary amines to interact with native α2M to induce conformational changes that are essentially identical to those observed with proteinases (See Fig.1.5.1, Sec.1.4 and 1.5; Swenson and Howard 1979; Salvesen et al 1981). The bond is formed between cys^{949} and gln^{952} by post-translational modification that requires only the energy of folding for its formation (Swenson and Howard 1979; Pangburn 1992; Gettins and Crews 1993a). Primary amines act as nucleophiles attacking the electrophilic thiol ester resulting in cleavage of the bond. The amine reacts with the glutamyl residue, and concomitantly

free SH groups are observed (Salvesen et al 1981). SH groups are also observed upon proteinase interaction: bait region cleavage activates the thiol ester resulting in its cleavage. In early studies, the sites of proteinase and amine binding in the primary structure were found to be identical, ie both bind to the glutamyl residue (Swenson and Howard 1979; Van Leuven et al 1982). Lysine sidechains of the entrapped proteinase may interact to form covalent bonds with the glutamyl residue, though this is not essential to proteinase inhibition (see Fig.1.5.1 and Sec.1.7; Salvesen and Barrett 1980; Salvesen et al 1981). In addition, the glutamyl and cysteinyl residues of the proteinase-cleaved thiol ester can interact with amines, or with other proteins such as cytokines (see Sec.1.9; Swenson and Howard 1979; Chu, Rubenstein, Enghild and Pizzo 1991; Chu and Pizzo 1993). One nucleophile can interact per monomer, and 4 SH groups can be titrated per tetramer indicating that there are 4 thiol ester sites in the complete molecule (Swenson and Howard 1979; Salvesen et al 1981; Salvesen and Barrett 1980; Van Leuven et al 1982).

It has been proposed that the thiol ester is a conformational lock (Howard, Swenson and Eccleston 1983). In the native molecule, stability is maintained, but cleavage of the bait region releases tension in the molecule resulting in cleavage of the thiol ester and so conformational change. Primary amines attack the bond directly and a similar conformational change occurs, although it is much slower, taking 2-3 hours, compared to 100msecs with proteinases (Van Leuven et al 1982; Gonias, Reynolds and Pizzo 1982).

Structural predictions proposed that the thiol ester site would be located at the loop of a β hairpin, and shielded in the native molecule and therefore unable to bind large amines (Welinder et al 1984). However, in the quaternary structure of α2M, its location is unknown: the model of

Feldman et al suggested it to be at the base of the "hinge" arms. Subsequently other groups proposed the thiol ester bond to be at the distal ends of the arms (Feldman et al 1985; Osada, Nishigai and Ikai 1988; Schramm and Witke 1988). As mentioned above, electron micrograph images of α2M and proteinase indicate a central position for the proteinase, suggesting a similar site for the thiol ester bond (Fig.1.6.1; Boisset et al 1989; Boisset et al 1991). More recently, the thiol ester in native α2M has been distinguished by using activated PLA2, a small glycoprotein with 10 lysine residues and no proteinase activity which acts as a nucleophile to bind gln^{952} (Boisset, Pochon, Chwetzoff, Barray et al 1992a). In a separate study the sulphydryl group of cys^{949} has been located by cryoelectron microscopy (Boisset, Grassucci, Penczek, Delain et al 1992b). Both investigations have indicated central locations in the interior of α2M for the thiol ester.

In addition, it is interesting to note that the thiol ester is susceptible to cleavage by heat, due to freeze/thaw activity or to prolonged storage (Barrett et al 1979; Harpel, Hayes and Hugli 1979; Howard, Vermeulen and Swenson 1980; see Fig.3.1.2).

1.6.7: Receptor recognition domain

Neoantigens on α2M can be detected after conformational change (Barrett et al 1979). The increased rate of α2M clearance from the circulation is a direct result of the exposure on the molecule of the receptor recognition domain (Barrett and Starkey 1973; Imber and Pizzo 1981; see Sec.1.7). Papain extraction cleaves a 20kDa C terminal fragment that has receptor recognition activity, and is capable of blocking α2M-trypsin binding to murine macrophages (Sottrup-Jensen et al 1986). A similar fragment that binds to the α2M receptor on fibroblasts was isolated (Van Leuven,

Marynen, Sottrup-Jensen, Cassiman and Van den Berge 1986). It was found at the termini of the long arms, contrary to Feldman's earlier prediction of a position at the external base of the hinge arms (Sottrup-Jensen et al 1986; Feldman et al 1985; see Sec.1.4). Delain et al proposed that the prediction of Feldmann et al over-simplified the conformational changes that occur in α2M upon activation by proteinase or methylamine (Delain, Barray, Tapon-Bretaudiere, Pochon et al 1988). They went on to suggest that upon thiol ester cleavage globular domains may unfold to build the lateral walls of the molecule, and to expose the C terminus containing the receptor recognition domain. Furthermore, a cis-Dichlorodiamineplatinum (cisDDP) sensitive site has been found: inactivation of it with cisDDP decreases the receptor binding (Gonias and Pizzo 1981; Pizzo, Roche, Feldman and Gonias 1986; Enghild, Thogersen, Roche and Pizzo 1989). The implication is that the complete recognition domain is contained within a larger sequence than was initially identified, the total region covering the C terminal 40kDa fragment.

1.7: α2M as a binding protein

α2M is able to interact with a range of molecules. The associations are of three forms, namely trapping, covalent binding, and adherence interactions. These are discussed below.

1.7.1: Trapping interaction

α2M is unique amongst proteinase inhibitors: it does not inhibit active sites, but operates a trap mechanism to limit substrate access to the active site (see Fig.1.1.1 and Sec.1.6.). This concept was conceived by Barrett and Starkey (Barrett and Starkey 1973). They proposed that endoproteinases cleave a specific sequence within α2M resulting in a conformational change of the serum protein and irreversibly enclosing the proteinase. A range of observations were the foundation for this model:

(i) Upon interacting with α2M, endoproteinases retain their activity against small but not large substrates indicating that their active site is still functional but access is restricted.

(ii) Only active endoproteinases can be inhibited; exoproteinases, non-proteolytic hydrolases and inactive endoproteinases are not effected, implying that an internal cleavage reaction is involved in the inactivation process.

(iii) The range of endoproteinases inhibited by α2M covers all four classes which suggests a common sequence for cleavage.

(iv) Previous electron microscopy studies had indicated a conformational change of the α2M molecule upon interacting which would be consistent with a trapping mechanism involving protein rearrangement (Morelis, Ambrosioni, Got and Fontanges 1969).

(v) α2M that has interacted with one proteinase cannot bind with a second. This is due to restricted access to the cleavage site upon trap closure.

Further to the evidence of Barrett and Starkey, in subsequent investigations reductive SDS-PAGE of α2M-proteinase complexes revealed fragments of 85-100kDa, indicating adjacent or identical cleavage sites for all endoproteinases (Barrett et al 1979; Travis and Salvesen 1983; see Sec.1.6).

Barrett and Starkey thus proposed a sensitive region within α2M that is especially susceptible to cleavage by a large range of endoproteinases (see Sec.1.6). They termed it the bait region and suggested that cleavage would be accompanied by conformational change enclosing the proteinase, thus the "trap" is sprung. Subsequently, the bait region has been identified: it is composed of 2 very sensitive areas which offer a substrate specificity appropriate to most endoproteinases (Sottrup-Jensen et al 1984b; see Sec.1.6).

1.7.2: Biological consequences of proteinase trapping

The conformational change that occurs as the trap is sprung exposes the receptor recognition domain, located at the termini of the trap arms (Delain et al 1988; see Sec.1.6). The result of binding is rapid clearance of α2M-proteinase complexes via the α2M receptor which is expressed on a number of cells (Imber and Pizzo 1981; see Sec.1.6.7). Under normal circumstances, α2M-proteinase complexes will be cleared rapidly (Barrett and Starkey 1973; Imber and Pizzo 1981). However, as discussed before, in some disease states clearance can be retarded because of an excessive concentration of conformationally fast α2M (Abbink et al 1991; see Sec.1.2). The retention of proteolytic activity against small substrates as a result of trapping then assumes greater importance, as does the carrying of cytokines and growth factors (see Sec.1.9).

In addition to inhibiting proteinases and removing them from their

environment, the interaction of α2M-proteinase with the receptor may modulate the receptor cell's functions. For example, binding to macrophages induces intracellular signalling cascades with increases in intracellular calcium concentration, and changes in inositol phosphate and cAMP levels (Misra, Chu, Rubenstein, Gawdi and Pizzo 1993). There is also evidence to suggest that α2M may deliver antigen to macrophages through endocytic pathways (Chu and Pizzo 1993; Moestrup 1994). The antigen is then processed and presented as normal. From α2M delivery experiments with hen egg lysozyme, Chu and Pizzo proposed that *in vivo* α2M may target antigen in sites of inflammation (Chu and Pizzo 1993).

1.7.3: Clearance of α2M

Although native α2M is removed from the circulation, its half life is decreased from hours to minutes upon interacting with proteinases or primary amines (Imber and Pizzo 1981; Barrett and Starkey 1973). The conversion of α2M by proteinase or primary amine exposes the receptor recognition domain which can bind with available α2M receptor expressed on cells (Imber and Pizzo 1981; Delain et al 1988). The critical step for receptor recognition domain exposure is the cleavage of the thiol ester bond, the interaction of the proteinase with the bait region merely facilitating this event (Van Leuven, Cassiman and Van den Berghe 1981; Imber and Pizzo 1981; Van Leuven et al 1982). The presence of other molecules bound to α2M, for example hen egg lysozyme or insulin, does not alter the rate of α2M binding and uptake (Chu and Pizzo 1993). This may be of significance when considering the role of α2M as a cytokine binder, maintaining or clearing activity *in vivo* (LaMarre, Hayes, Wollenberg, Hussaini et al 1991)

1.7.4: The α2M receptor

As previously indicated, the α2M receptor is expressed by a range of cells, most importantly liver cells, hepatocytes and Kupffer cells, which take up 80% of injected α2M (Moestrup and Gliemann 1989). Other cells including fibroblasts, monocytes/macrophages, smooth muscle cells, neurones, and astrocytes account for local removal of α2M and ligands from interstitial and cerebrospinal fluids (Imber and Pizzo 1981; Van Leuven et al 1986b; Straight et al 1988; reviewed Moestrup 1994). In blood, the α2M receptor (CD91) is expressed only by monocytes/macrophages and is therefore a marker protein for these cells (Moestrup 1994, Moestrup 1995; James, Milne and Donaldson 1985). In addition, there is 97% identity between the murine and human receptor, and homologus residues in other species have been identified (Enghild et al 1989; Van Leuven, Stas, Raymakers, Overbergh et al 1993; Moestrup 1994).

The receptor is the largest member of the low density lipoprotein (LDL) receptor superfamily, and is probably the oldest: it is expressed in a lower organism, *Caenorhabdites elegans*, than other family members (reviewed in Moestrup 1994). The low density lipoprotein receptor, very low density lipoprotein receptor, and gp330 constitute the rest of the family.

The α2MR is composed of 2 chains: α, 515kDa, and β, 85kDa which are homologous to LDL-related protein (LRP; Herz, Hamann, Rogne, Myklebost et al 1988; Moestrup and Gliemann 1989; Kristensen, Moestrup, Gliemann, Bendtsen et al 1990). Other ligands for the α2MR are βVLDL with apoE, and α2MR-associated protein (RAP) both of which compete with α2M (fast) for binding to the receptor (Kristensen et al 1990; Strickland, Ashcom, Williams, Battey et al 1991; Hussain, Maxfield, Mas-Oliva, Tabas et al 1991). RAP is a 40kDa protein associated with the α chain of the receptor (Strickland et al 1991; Moestrup and Gliemann 1991;

Herz, Goldstein, Strickland, Ho and Brown 1991). The *in vivo* role of RAP is unclear but its inhibitory effects implies a modulatory function with respect to α2M-α2MR binding (Moestrup and Gliemann 1991; Herz et al 1991).

Binding of α2M by its receptor is calcium dependent (Moestrup and Gliemann 1989; Moestrup, Kaltoft, Sottrup-Jensen and Gliemann 1990). There are 8 or more calcium binding sites on the receptor which demonstrate positive cooperativity. In the absence of calcium no α2M can be bound: it appears that calcium is required for the appropriate receptor conformation. As the pH is decreased, calcium is released thus releasing α2M: this is important in the endocytic vesicle where α2M and its receptor will therefore be uncoupled in the acidic environment (Moestrup et al 1990).

The affinity of α2M for its receptor is increased by the binding of 2 receptor recognition domains of a single α2M molecule to 2 cellular receptors. Thus, a binding affinity of 40pM is observed when 2 receptor recognition domains are involved, and of 2nM, when 1 receptor recognition domain is bound (Delain et al 1988; Moestrup and Gliemann 1989; Moestrup and Gliemann 1991). It may be that the requirement for 2 receptors modulates binding to induce interaction with the α2MR located in coated pits where it is at a higher density, thus aiding endocytosis.

1.7.5: Covalent interactions

As previously indicated, α2M and related proteins contain a unique internal thiol ester bond (see Figs.1.4.1 and 1.5.1, Sec.1.5 and 1.6). This is an electrophilic centre that is activated during proteinase cleavage of the bait region. In the native protein, the thiol ester is sterically shielded but can be contacted by small primary amines which act as nucleophiles to cleave

the bond, and then bind to the released glutamyl residue (Swenson and Howard 1979; Van Leuven et al 1981). In a similar manner, lysine residues on an entrapped proteinase can interact with the glutamyl residue after cleavage of the thiol ester has occurred (Salvesen and Barrett 1980; Salvesen et al 1981). The bonds formed are non-disulphide covalent in nature (Salvesen and Barrett 1980). The interactions that occur at the thiol ester bond are illustrated in Fig.1.5.1. The covalent interaction with the thiol ester bond is secondary to the trapping interaction of proteinases, and does not occur with all proteinases (Barrett and Starkey 1973; Salvesen and Barrett 1980; Howard et al 1983). For αM's that are monomeric the covalent binding of proteinase is essential to its inhibition, as seen for rat α1I3 (Moncino et al 1991, see Sec.1.4). The covalent interaction involving the thiol ester residues are also important for the function of complement proteins C3 and C4 in binding to cells, and to complement substrates (Fig.1.5.1, Sottrup-Jensen 1987, see Sec.1.5).

The activation of the thiol ester by a proteinase can allow other substrates, such as bEGF and insulin, to covalently bind with either the glutamyl or thiol residues of the bond (Chu et al 1991; Chu and Pizzo 1993; Gettins and Crews 1993b). It has been suggested that the role *in vivo* for the thiol ester in dimeric and tetrameric αM's may be to capture small proteins with increased activity during the conformational change, that is when α2M is in the nascent state (Chu and Pizzo 1993, see Sec.1.6). *In vivo* both cysteinyl and glutamyl residues may be available for binding, although glutamyl is very reactive and may undergo rapid hydrolysis. Furthermore, Travis and Salvesen speculated that soya bean trypsin inhibitor (SBTI), a 21kDa inhibitor which can be degraded by trypsin, may define the limits of size or shape for access to fast α2M (Travis and Salvesen 1983). Thus, proteins of size and shape smaller than SBTI may

have access to the interior of fast form α2M and could interact with cys^{949} or gln^{952} residues. It has been suggested that in interactions in the nascent state α2M may be able to non-selectively bind proteins and peptides that express nucleophilic amino acids (Chu and Pizzo 1993). To this end, α2M has been identified as an SH-reactive agent (Schlesinger, McEntire, Wallman, Skosey et al 1989).

1.7.6: Adherence interactions

The macromolecular state of α2M enables it to interact with a range of molecules by adherence, rather than by more specific interactions (Barrett 1981). For example, α2M is the primary zinc binding protein carrying 40% of the plasma zinc (Kurecki, Kress and Laskowski 1979). In addition, the 8 carbohydrate chains can bind the mitogens concanavalin A and PHA (Sottrup-Jensen et al 1984b; Barrett 1981). Such non-specific interactions may be of importance within the immune response. α2M can also interact hydrophobically, for example with endotoxin and LPS (Barrett 1981).

1.8: α2M as an immunomodulator

Proteinase inhibitors, especially inhibitors of serine proteinases, are recognised as having immunosuppressive activity *in vitro* (Petersen, Ejlersen, Moestrup, Jensen et al 1989; Hubbard, Hess, Hsia and Amos 1981; Dickinson, Shenton, Alomran, Donnelly and Proctor 1985). α2M shows immunomodulatory effects on a number of immunogical events which may be attributed to:

(a) Its proteinase inhibitory activity

(b) Binding to its receptor

(c) Activity with respect to molecules of immunological importance including cytokines.

1.8.1: α2M synthesis and clearance within the immune system

There are a range of cells of the immune system reported to synthesize and secrete α2M, including macrophages and monocytes (see Sec.1.3). In addition, a number of cells, including monocytes, carry receptors for α2M (see Sec.1.7). Thus there exists the potential for a source of α2M and a mechanism for its clearance in the immune system and in diseased tissue. It has been suggested that α2M may dysregulate the immune response (Heumann and Vischer 1988). Various methods have been proposed as to how it may do so, or how it may play a positive role in the immune system, as discussed below.

1.8.2: Roles of native and fast form α2M in the immune response

The two forms of α2M have distinct biological properties: the native form has a half life in the circulation of 24 hours, can bind proteinases and has a very low affinity for the α2M receptor (Imber and Pizzo 1981; Barrett and

Starkey 1973). Fast α2M, inactivated by proteinase, oxygen reactive species, or by chemical means, cannot bind proteinase, has a much shorter half life in the circulation, and is removed via the α2M receptor. This distinction may be of importance with respect to the immunomodulatory effects of α2M.

There are different immunological effects attributed to each form of the protein. For example, native α2M has immunosuppressive potential as observed in LAK cell lysis assays (Petersen et al 1989). It was found that α2M bound to molecules with trypsin and chymotrypsin-like activity on the LAK cell surface. Petersen et al proposed that interaction with proteinases and their probable inhibition was the mode of suppression used.

Both forms of α2M suppress the mixed lymphocyte response (Hubbard et al 1981). However, the dose response observed was stronger with α2M-proteinase. It has been found that monocyte activity can be increased by fast α2M, and that the native protein has no effect on the chemotactic activity of the cells (Forrester, Wilkinson and Lackie 1983). α2M-proteinase interferes with the T cell proliferation response to antigen in a dose-dependent manner. This effect is partly because of residual trypsin activity against IL2, but is also mediated through macrophages (Mannhalter, Borth and Eibl 1986; Heumann and Vischer 1988; see Sec.1.9).

The effects of α2M-proteinase may be significant in cases were there are increased levels of the complexes (see Sec.1.2.6). As discussed before, in disease states where there is an excessive proteinase release there may be a resultant increase in α2M-proteinase complexes as other inhibitors become exhausted, or, as in rheumatoid arthritis, clearance may be inhibited (Harpel 1977; Burtin et al 1987; Sec.1.6 or 1.7). The change to

α2M as a principle inhibitor may result in modulation of the immune response as noted above or by other immune mechanisms, for example altering NK activity or antibody dependent cell-mediated cytotoxicity. These events have been observed to be affected by α2M-trypsin (Dickinson et al 1985).

Fast form α2M inhibits the change in cell morphology of macrophages during antigen presentation, therefore suppressing T cell activation (Roche, Hoffman and Pizzo 1990). It was reported that fast form α2M could have a specific, dose-dependent and receptor-mediated immunomodulatory effect on murine macrophages (Hoffman, Pizzo and Weinberg 1987). Subsequently, it was reported that α2M mediates its effect by preventing the normal IFNγ induced change in morphology of the macrophage (Roche et al 1990). The consequence of the α2M activity is a decrease in the density of Ia on the macrophage surface, and therefore a reduction in T cell activation (Roche et al 1990). In addition, α2M binding to the α2M receptor expressed on macrophages results in various intracellular changes, for example increased intracellular calcium levels, and changes in inositol phosphate and cAMP levels (Misra et al 1993; see Sec.1.7.2). This and antigen delivery to macrophages can increase T cell activation.

1.8.3: Influence of α2M on molecules of the immune system

Further to the proteinase and cellular effects attributed to α2M, it may also inhibit molecules of specific immunological importance. For example, α2M has been found to inhibit *in vivo* and *in vitro* the activity of deglycosylated ricin A chain (Ghetie, Uhr and Vitetta 1991). Defensins, inflammatory mediators, are bound by fast α2M (Ganz and Lehrer 1994; Panyutich and Ganz 1991). α2M may constitute a mechanism for

regulation and containment of these and other molecules in inflammation. The pathogenesis of RA may therefore be further influenced by α2M acting in this capacity, in addition to its role as a proteinase inhibitor, cytokine binder, and mediator of chemotaxis (see Sec.1.2.6).

Thus it appears that α2M can mediate a range of effects on the immune system. *In vivo*, local circumstances may dictate which effects are paramount. Furthermore, α2M has been attributed as being a carrier of cytokine activity, either to maintain or clear it from the circulation and tissues. This will be discussed in Section 1.9.

1.9: α2M-cytokine interactions

1.9.1: Early studies of α2M-cytokine binding

Early studies of α2M indicated that it has an immunomodulatory function (reviewed James 1980, see Sec.1.8). In some cases, the activity can be attributed to α2M's role as a proteinase inhibitor. For example, α2M was found to inhibit esterases resulting in increased activity of migration inhibitory factor on macrophages (Remold and Rosenberg 1975). However, there is also evidence that it has growth inhibitory properties that are not due to proteinase activity, but can be attributed to small bioactive molecules, suggesting that α2M can act as a carrier protein of biological activity (Koo 1982). α2M has been identified as a carrier molecule of a range of biological molecules apart from proteinases, including insulin and a number of cytokines and growth factors (Hoffmann, Ristow, Veser and Frank 1973; Remold and Rosenberg 1975; McDaniel, Laudico and Papermaster 1976; Koo 1982; Chu et al 1991).

Table 1.9.1 summarises the principle cytokines and growth factors examined, and the characteristics and consequences of the interactions.

1.9.2: Affects of cytokines on α2M activity

In a number of cases, the activity of α2M, rather than the associated cytokine, is affected. For example, CSF1, IL6 and TGFβ have each been reported to alter α2M activity (Hussaini, Srikumar, Quesenberry and Gonias 1990; Ganter et al 1991; Shi et al 1990). CSF1 acts on bone marrow macrophages to increase α2M receptor expression leading to increased clearance of fast α2M (Hussaini et al 1990). A change in clearance may have repercussions on other proteins or processes affected by α2M.

Cytokine	Slow/Fast α2M bound	Bonds	Bioactivity	Antigenicity	References.
MIF	N/A	N/A	Inhibited	N/A	Remold and Rosenberg (1975)
MAF	N/A	N/A	Maintained	N/A	McDaniel et al (1976)
NGF	N/A	C	N/A	N/A	Ronne et al (1979)
PDGF	S, F	DS/NC	Retained partially	Lost	Huang et al (1984)
TGFβ1, β2	S, F	NC/C	Dependant on local conditions	Lost	O'Connor-McCourt and Wakefield (1987)
Placental Lactogens	S	N/A	Maintained	N/A	Southard and Talamantes (1989)
bFGF	F	C/DS	Inactive	N/A	Dennis et al (1989)
TNFα	F	NC	Maintained	N/A	Wollenberg et al (1991)
IL6	N/A	N/A	Maintained	N/A	Matsuda et al (1989)
IL1β	F, N	DS	Maintained	Partially lost	Borth and Luger (1989)
Insulin	N	NC/C	N/A	N/A	Chu et al (1991)
EGF	N	C	N/A	N/A	Gettins and Crew (1993b)
5HT	S	C	Potentiation	N/A	Liebl and Koo (1993)

Table 1.9.1: Characteristics of α2M-cytokine interactions
α2M-cytokine interactions are reported in the literature with respect to a range of their characteristics, namely: (i) interaction with slow (S), fast (F) or nascent (N) form α2M, (ii) non-covalent (NC), disulphide (DS), or covalent non-disulphide (C) bonds used, (iii) effect of the interaction on bioactivity and antigenicity of the cytokine. Key references for each cytokine are listed, additional material is discussed in the text. Information is not available on certain cytokines, as indicated (N/A).

TGFβ1 stimulates bovine adrenocortical cells (BAC) to secrete α2M (Shi et al 1990). The response to TGFβ1 can be inhibited by an anti-RNA POL II reagent which indicates that stimulation occurs at the protein synthesis level. TGFβ1 binds to both slow and fast forms of α2M, and remains active on a range of cells including BAC when bound by α2M. Collectively these results suggest that there may be an autocrine loop operating to control or to enhance TGFβ1 activity in BAC (Keramidas et al 1992).

Like TGFβ1, IL6 is known to be bound by α2M, but the free form of the cytokine has itself been found to effect α2M production (Matsuda et al 1989; Ganter et al 1991). In the CNS, α2M may act as an acute phase protein (see Sec.1.2.10). The report that IL6 can induce α2M synthesis in a murine neuroblastoma cell line has lead to speculation that IL6 and α2M may have roles in the pathogenesis of Alzheimer's Disease (Ganter et al 1991).

Inhibition of a cytokine's activity may also be induced without contact of α2M and cytokine as reported for IFNγ and α2M on murine macrophages (Hoffmann et al 1987; Roche et al 1990; see Sec.1.8).

1.9.3: PDGF binding to α2M

PDGF is a potent polypeptide growth factor released when platelets are activated, namely during blood coagulation or after blood vessel injury (Huang et al 1984). It is also a chemoattractant for inflammatory cells and for cells required at sites of wound repair. PDGF was found to bind to a plasma protein, subsequently identified as α2M, which could clear the growth factor thus limiting its activity to the site of local injury (Huang, Huang and Deuel 1983; Huang et al 1984). It can bind to either form of α2M, using disulphide bonds, and retains about 50% of its mitogenic activity upon binding though it loses its antigenicity. Non-covalent and

reversible association of PDGF and α2M has also been demonstrated, and it is not clear which report, if either, is entirely correct (Crookston, Webb, Wolf and Gonias 1994). The binding of ^{125}-I PDGF to native or fast form α2M is inhibited by cold PDGF, and only binding to the fast form can be blocked by TGFβ, bFGF, and IL6. These results indicate that binding is specific, and that the binding site is not shared with the other cytokines tested. PDGF remains bound by α2M during and after the conformational change (Bonner, Goodell, Laskey and Hoffman 1992). Thus, if bound to slow α2M, PDGF could be maintained in the circulation until the conformational change occurs and then be cleared via the α2M receptor. By these means the growth factor is prevented from acting outwith its local site of release from activated platelets.

However, as noted above, PDGF has been reported to retain some activity when bound to α2M (see Sec.1.2.8). The consequences of this are seen with fibroblasts where the presence of α2M appears to synergistically increase their PDGF induced growth (Bonner, Badgett, Osornio-Vargas, Hoffman and Brody 1990). In contrast, in melanomas the presence of α2M has been found to decrease the rate of disease development and this effect may be due to the inhibition of PDGF activity by α2M (Bizik et al 1989; see Sec.1.2.8). In conclusion, it appears that the *in vivo* consequence of the interactions between PDGF and α2M may be more complicated than present *in vitro* findings indicate.

1.9.4: TGFβ-α2M interactions

Similar to PDGF, TGFβ is composed of 2 polypeptide chains, is localised in platelets, and has a high disulphide linkage content. Early studies indicated that TGFβ was bound covalently to α2M, resulting in a decrease in the cytokines activity and loss of its antigenicity (O'Connor-McCourt

and Wakefield 1987; Huang, O'Grady and Huang 1988). Binding of radiolabelled TGFβ can be blocked with the cold growth factor, but is unaffected by PDGF, EGF and insulin. This corroborates the earlier report that TGFβ did not effect PDGF binding (Huang et al 1984). There is evidence that TGFβ activity increases in melanoma cell lines where α2M is absent, as observed with PDGF (Lizonova, Bizik, Grofova and Vaheri 1990; Bizik et al 1989). These results may have implications *in vivo* for the control of TGFβ activity by α2M. Subsequent investigations have shown TGFβ2 to be more strongly bound by α2M than is TGFβ1 (Danielpour and Sporn 1990; LaMarre, Wollenberg, Gauldie and Hayes 1990). The bioactivity of TGFβ2 is inhibited to a much greater extent than that of TGFβ1. These results suggest that there is differential control of TGFβ1 and β2 activities *in vivo* by α2M.

As mentioned above, TGFβ1 increases α2M secretion by bovine adrenocortical cells (BAC) (Shi et al 1990). α2M-TGFβ1 complexes have been shown to be bioactive on BAC, in contrast to the negation of activity seen in other cell lines, such as lung carcinoma cells and melanomas (Keramidas et al 1992; O'Connor-McCourt and Wakefield 1987; Huang et al 1988; Danielpour and Sporn 1990). BAC cells may contain an effector that can mediate release of the active growth factor from α2M. It has been suggested that α2M may be a latent carrier of TGFβ1 activity, and that it is being released in its active form in the acidic conditions of the lysosome (Keramidas et al 1992). It is unlikely that the BAC cells are using this to provide them with active TGFβ1 since all cells have lysosomes. The unique component of BAC is unclear, but may be an enzyme in or on the surface of the cells. In addition, there is evidence of α2M enhancing TGFβ1 activity on smooth muscle cells, possibly requiring the α2M receptor for activity (Stouffer, LaMarre, Gonias and Owens 1993).

1993).

1.9.5: In vivo activity of PDGF and TGFβ

Injection of PDGF or of TGFβ results in their binding to α2M *in vivo* suggesting the *in vitro* binding and effects attributed to PDGF and TGFβ interacting with α2M may have an *in vivo* equivalent (Crookston, Webb, LaMarre and Gonias 1993). It has been found that TGFβ1 injected intravenously is removed via the liver, the principle site of α2M clearance, and that the level of clearance can be increased to more than 90% if TGFβ is injected bound to α2M (LaMarre et al 1991). However, there is contradictory evidence to suggest that endogenous TGFβ1 is cleared from the circulation via its own ubiquitous receptor (Philip and O'Connor-McCourt 1991). Binding of TGFβ by α2M may be importance to maintain the growth factor in a latent state to protect it from proteinases and also from clearance via its own receptor. It may be carried in the circulation by α2M until it is required, for example at a disease site, at which time it may be activated, possibly by the binding of proteinases by α2M leading to TGFβ1 release.

1.9.6: The binding of other cytokines to α2M

Unlike PDGF and TGFβ, some cytokines and growth factors bind only to fast form α2M, whilst others interact preferentially with nascent α2M (Table 1.9.1; Dennis, Saksela, Harpel, and Rifkin 1989; Borth and Luger 1989; Wollenberg et al 1991; Chu et al 1991; Gettins and Crews 1993b). The glutamyl residue of the activated thiol ester bond is rapidly lost by reaction with a lysine side-chain of a proteinase, a primary amine, water, or with nucleophile residues on proteins or peptides (Chu and Pizzo 1993; see Sec.1.7). There is evidence that EGF and insulin may bind to the gln^{952}

residue (Chu et al 1991; Gettins and Crew 1993b).

IL1β is believed to interact with the cysteinyl residue of the cleaved bond (Borth and Luger 1989; Borth, Scheer, Urbansky, Luger, Sottrup-Jensen 1990a; Borth, Urbanski, Proaska, Susani and Luger 1990b; Teodorescu et al 1991). For IL1β, the cysteine residue that forms a disulphide bridge with α2M was identified as cys^{8} (Teodorescu et al 1991). In addition, IL1β may use histidine to bind (Borth and Luger 1989; Borth et al 1990a). The interaction of radiolabelled IL1β can be blocked by a 20 fold excess of cold IL1β, but not by IL2 nor by IFNγ (Borth et al 1990a). Gettins and Crew proposed that the short half life of the nascent state of α2M is such that binding of IL1β, insulin, or EGF *in vivo* may not be plausible (Gettins and Crews 1993b). However, if cytokine is *in situ* during activation of α2M, binding to the nascent residues may be more feasible (Chu et al 1991; Chu and Pizzo 1993). Furthermore, the binding of IL1β by α2M appears to have a role in rheumatoid arthritis (Teodorescu et al 1991; see Sec.1.2.6): D-penicillamine, active in the treatment of rheumatoid arthritis, is able to inhibit the binding of IL1β to α2M in the reagents therapeutic range. α2M levels rise in synovial fluid of RA patients, and in the disease state fast form α2M is not rapidly cleared (Abbink et al 1991; see Sec.1.2.6). In addition, IL1β when bound to α2M retains its bioactivity (Borth and Luger 1989; Borth et al 1990a; Teodorescu et al 1991). α2M-IL1β complexes may therefore form *in vivo* in the inflammatory state, thus the complexes could actively operate in the synovial fluid. IL1β is 15-17kDa in its active form, and it may therefore be possible for it to access the trap after it has been sprung. In a similar way to IL1β, IL6 is bound by fast form α2M and retains its bioactivity (Matsuda et al 1989). The effect of such interactions may be to protect the cytokines from proteinase activity whilst allowing their continued bioactivity.

There is evidence of binding to fast form α2M still leaving a cytokine open to proteinase inhibition. Stimulation of CTLL-20 by IL2 is inhibited by addition of α2M-trypsin (Borth and Teodorescu 1986). The inhibition results from degradation of IL2 by bound trypsin. It may be that α2M acts as a catalyst bringing together IL2 and trypsin in close proximity. The binding of TNFα by α2M has been detected at a qualitative level (Wollenberg et al 1991; James, van den Haan, Lens and Farmer 1992). In addition, it was reported that the cytokine retained its bioactivity when bound. However, in these previous studies the separation of bound and free cytokine and the subsequent application of "bound only" cytokine to the cell line was not reported. This is further discussed in Sec.1.10, Sec.4.3 and Sec.4.4.

1.9.7: Overview of the literature on α2M-cytokine studies

The most obvious characteristic of the interactions of α2M with cytokines is that there is no distinct pattern of binding: some bind to slow form α2M, others to fast form, some to both, using non-covalent, disulphide covalent, or other covalent interactions, with or without the loss of bioactivity and antigenicity. It is apparent that α2M does have the capacity, due to its size and to its hollow trap formation, to interact with a range of proteins, in a diverse manner and with a range of consequences.

1.10: The current project background and strategy

1.10.1: Background to the current study

As outlined above, the binding of a diverse range of cytokines by α2M has been examined (see Sec.1.9). However, many of the previous publications report studies of α2M-cytokine complexes at a qualitative level only. Furthermore, in examining their bioactivity they did not remove cytokine not bound by α2M. Thus, in attempting to confirm and extend previous studies on α2M-cytokine interactions, quantitative and bioactivity studies were of importance.

The cytokines examined herein were IL2, TNFα and IFNγ. All three had been examined in association with α2M previously, but in limited studies (see Sec.1.8.3 and Sec.1.9.6). Investigations of TNFα were the most extensive, but had no quantitative analysis and an incorrect assay of bioactivity (Scuderi, Dorr, Liddil, Finley et al 1989; Wollenberg et al 1991; James et al 1992). IL2 and IFNγ had been examined on the basis of interference by α2M on their bioactivity, but not on the premise of protein-cytokine interactions (see Sec.1.8.3). These cytokines have key roles in the immune response. In addition, these cytokines are of special interest to the Wilkie Laboratories with respect to their roles in anti-tumour immunity. They have well defined structures and functions, and associations with their receptors, as outlined below.

1.10.2: Interleukin 2

Interleukin 2 (IL2) is a 15.5kDa T cell derived cytokine. It is an O-glycosylated globular protein, with a tertiary structure of 4 α helical bundles similar to those for GM-CSF and IL4, and the recently discovered IL15 (Robb, Kutny, and Chowdhry 1983; Bazan 1992). IL2 is produced by antigen or mitogen activated T cells, and can by autocrine or paracrine

means stimulate T cell proliferation (Morgan, Ruscetti and Gallo 1976; Gillis, Ferm, Ou and Smith 1978; Leonard, Depper, Kanehisa, Kronke et al 1985). It also affects monocytes by increasing their cytotoxic activity, and it can stimulate resting B cells to proliferate and differentiate to form activated B cells producing IgM (Malkovsky, Loveland, North, Asherson et al 1987; Tigges, Casey, and Koshland 1989).

The IL2 receptor is composed of at least three chains: α (tac, p55), β (p75), and γ (p65) which can associate in different ways to form a receptor of variable affinity and activity (Ringheim, Freimark and Robb 1991; Takeshita, Asao, Ohtani, Ishii et al 1992). In structure, the α chain is homologous to complement control protein, while β and γ are members of the cytokine receptor family (Leonard et al 1985; Takeshita et al 1992). The α chain binds IL2 with a K_d of ~10^{-8}M, but is unable to transduce the signal (Ringheim et al 1991; Semenzato, Pizzolo and Zambello 1992; Takeshita et al 1992). β chain binds IL2 with a K_d of 10^{-9}M, and an αβ dimer can form with a K_d of 10^{-10}M for IL2. The γ chain alone cannot bind IL2 but can associate with the β chain to form a receptor with a K_d of ~10^{-9}M. An αβγ receptor with a K_d of ~10^{-11}M appears to be the complete receptor (Ringheim et al 1991; Takeshita et al 1992; Nakamura, Russell, Mess, Friedmann et al 1994). The γ subunit is found in other cytokine receptor complexes, for example in those for IL4, IL7 and IL15 (Bazan 1990; Giri, Ahdieh, Eisenmann, Shanebeck et al 1994; Noguchi, Nakamura, Russell, Ziegler et al 1993a). The IL15 receptor also contains the β subunit, and the cytokine has been found to share structural features, such as the 4 α helical bundle, and biological properties with IL2 (Grabstein, Eisenman, Shanebeck, Rauch et al 1994). The importance of the γ receptor chain was highlighted by Noguchi et al who showed that its gene was at the same locus as that for X-linked severe combined

immunodeficiency (Noguchi, Yi, Rosenblatt, Filipovich et al 1993b).

Soluble IL2 receptors have also been identified in culture, such as those of HTLV-I positive cell lines, B cell lines, normal PBMC activated with plant lectins, and also *in vivo* in patients with Hodgkin's disease, with non-Hodgkin's lymphoma, or with metastatic solid tumours (Rubin, Kurman, Fritz, Biddison et al 1985; Lissoni, Barni, Rescaldani, Rovelli and Tancini 1989). It is not clear why receptor secretion occurs, but it has been proposed that it could serve an immunoregulatory function during growth and development.

1.10.3: Tumour necrosis factor α

Active TNFα is trimeric formed of 17kDa monomers placed edge-to-face in a "jelly roll" motif similar to those observed with TNFβ and CD40L (Aggarwal, Kohr, Hass, Moffat et al 1985a; Farrar and Smith 1992; Eck and Sprang 1989). It is produced as a pro-protein with a membrane anchor, from which the active protein is cleaved (Kriegler, Perez, DeFay, Albert and Lu 1988). The membrane protein is biologically active and may bind with a TNFα receptor expressed on another cell to activate the receptor's cell. Alternatively, the membrane bound TNFα could be bound by a soluble TNF receptor and transduce a signal itself.

TNFα was originally named because of its necrotic effects on tumours such as that observed with a methylcholanthrene induced murine sarcoma (Carswell, Old, Kassel, Green et al 1975; Gray, Aggarwell, Benton, Bringman et al 1984). However, it is now recognised as having a wide range of effects (reviewed by Le and Vilcek 1987; Vilcek and Lee 1991). It is a pro-inflammatory cytokine, along with IL1 and IFNγ (Dinarello, Cannon, Wolff, Bernheim et al 1986). In the inflammatory state, it is produced by infiltrating macrophages (Aggarwal et al 1985a). It can also

be synthesised by T cells, B cells, NK cells, LAK cells etc. (reviewed in Vilcek and Lee 1991).

TNFα can have beneficial effects; for example, it protects the host against bacterial infections, and acts as a mediator of inflammatory reactions and necrosis of tumours (Vilcek and Lee 1991). However, in sepsis and in chronic infections it is also known to mediate wasting (Cerami, Ikeda, Le Trang, Hotez and Beutler 1985; Beutler, Greenwald, Hulmes, Chang et al 1985). The biological activities of TNFα include T cell activation, activation of neutrophils, and induction of IFNβ2, of c-myc and c-fos, and of IL1 synthesis (reviewed in Vilcek and Lee 1991). It is able to activate a range of genes and proteins including those for a number of cytokines and their receptors, cellular adhesion molecules, and MHC proteins. This spectrum of activities and the presence of its receptors on many cell types enables TNFα to have a central role in the immune system. Furthermore, it can regulate its own production, and can act synergistically with IFNγ (Halloran 1993).

There are two receptor types, p55 and p75 both of which bind TNFα and β, with a K_d in the region of 10^{-10}M (Aggarwal, Eessalu, and Hass 1985b; Smith, Davis, Anderson, Solam et al 1990; reviewed in Le and Vilcek 1987; and in Vilcek and Lee 1991). Unlike the IL2 receptor, p55 and p75 operate independently (Vilcek and Lee 1991). Thus, p55 is expressed more widely, p75 is restricted to cells of haematopoeitic lineages. Both are members of the NGF receptor family characterised by extracellular cysteine repeats (Farrar and Smith 1992; Mallett and Barclay 1991). However, TNFα forms a separate ligand family from NGFβ, along with TNFβ and CD40L (Farrar and Smith 1992).

Soluble receptors are present in normal human urine and serum, possibly to control TNF bioactivity *in vivo* (Olsson, Lantz, Nilsson, Peetre et al

1989; Engelmann, Aderka, Rubinstein, Dalia et al 1989). The trimer cone form of active TNFα inserts its narrow end into the soluble receptor, each subunit contributing crucial residues in association with its adjoining subunits (Banner, D'Arcy, Janes, Gentz et al 1993). It is thought that the membrane bound receptor will operate in the same manner.

1.10.4: Interferon γ

IFNγ is a 17kDa glycosylated protein present as a dimer in its active form (Ealick, Cook, Vijay-Kumar, Carson et al 1991). It is synthesised by activated T cells and by NK cells, and has a range of effects *in vivo* (reviewed in Halloran 1993). It induces MHC I and II expression on macrophages indicating a specialised role for it in the immune response (Basham and Merigan 1983; Langer and Pestka 1988). IFNγ is a pro-inflammatory cytokine involved in inhibition and activation of macrophage migration. It can induce synthesis of TNFα, also a pro-inflammatory cytokine, and synthesis of the TNF receptor. It was initially identified as an anti-viral agent, and hence was classified as an interferon (Wheelock 1965). In addition, IFNγ can augment its own production by T cells (Morris 1988).

The active IFNγ dimer is composed of 2 identical subunits to form a globular protein (Ealick et al 1991). Each subunit comprises six α helices, and no β sheet. There are extensive intersubunit contacts in the dimer: for example, the first four α helices of one subunit form a cleft to accommodate the COOH terminus of the other subunit. The NH_2 and COOH termini of opposing subunits are adjacent and together contact the receptor.

The IFNγ receptor is composed of an α and a β subunit (Ucer, Bartsch, Scheurich, Berkovic et al 1986; Auget, Dembic and Merlin 1988; Soh,

Donnely, Kotenko, Mariano et al 1994). The α subunit binds IFNγ, and the β "accessory" subunit transduces the signal. Both receptor subunits are members of the type II cytokine receptor family (Bazan 1990). The IFNγ receptor is expressed on most cell types, and K_d values in the range 10^{-9}-10^{-11}M have been identified for various tissues (Ucer et al 1986; Langer and Pestka 1988; Fischer, Wiegmann, Bottinger, Morens et al 1990). Fischer et al (1990) observed post-translational regulation of the IFNγ receptor by GM-CSF, and proposed that GM-CSF may enhance membrane associated proteinases resulting in release of soluble IFNγ receptor. Soluble receptors have been identified in urine (Novick, Engelmann, Wallach and Rubinstein 1989).

1.10.5: Investigations on the qualitative binding of cytokines by α2M

Qualitative studies have previously been employed to examine α2M-cytokine interactions. Within the present investigations, qualitative binding analysis was carried out using three column techniques, namely gel filtration on G200 Sephadex, fast performance liquid chromatography (FPLC) on Superose6B and zinc affinity chromatography on Sepharose, in corroboration with native polyacrylamide electrophoresis (PAGE). The bonds involved in interactions were examined by SDS-PAGE. IFNγ was found to bind poorly, and it was noted in column work and by TCA analysis of IFNγ samples that it was of poor quality. Thus, subsequent work focused on IL2 and TNFα.

1.10.6: Quantitative investigations of α2M-cytokine interactions

Almost all previous studies of α2M-cytokine interactions have been carried out by qualitative analysis (see Sec.1.9). In this study two novel quantitative assays were established. A radioimmunoassay (RIA) was

developed in which α2M, preincubated with radiolabelled cytokine, was bound by a rabbit anti-α2M polyclonal antibody. Separation of α2M-bound cytokine from free was achieved by addition of a donkey anti-rabbit IgG polyclonal antibody attached to solid phase followed by separation on a sucrose gradient.

α2M can be isolated in highly purified form from serum or plasma on zinc affinity columns, and this methodology was also used for qualitative analysis (Kurecki et al 1979). The zinc affinity method was adopted to create a tube based assay system for the quantitative study of α2M-cytokine binding. As with the RIA, the zinc affinity "tube method" used α2M as the separating molecule. Thus, corroborative values for the binding of IL2 and TNFα with α2M were available, and the assays may subsequently be used to examine more cytokines.

1.10.7: The effect of complex formation with α2M on cytokine immunoassays

It has been noted by a number of groups that cytokines may lose their antigenicity upon complexing with α2M, that is they lose their capacity to interact with specific antisera (see Sec.1.9 Table 1.9.1). In diagnostics and in analysis of tissue culture supernatant any such decrease in antigenicity due to the presence of α2M could cause a depression of the observed cytokine concentration. To investigate this further, cytokine assay kit standards were incubated with α2M prior to application to the assay. The resultant change in the position of the standard curve, due to a specific α2M effect or to a more general protein effect was observed in a number of instances. The implications of the change in the cytokine standard curve will be discussed.

1.10.8: The effect of complex formation on the bioactivity of cytokines

The effect of the binding of cytokines by α2M on their bioactivity is of importance as regards their activity *in vivo*, and also for possible effects in culture. In this study, in contrast to previous work published by other groups, α2M-cytokine complexes were separated from unbound cytokine on G200 or zinc affinity columns prior to application to prepared cells. Bioactivity of α2M-cytokine complexes were examined on cultures of peripheral blood lymphocytes and the L929 cell line for IL2 and TNFα respectively.

1.10.9: A brief summary of the findings of the present study

Qualitative studies indicated an interaction of IL2, TNFα and IFNγ with α2M. Development of quantitative assays revealed K_d values in the micromolar range for IL2 and TNFα binding with α2M. Furthermore, it was found that the bioactivity of IL2 was maintained upon binding, whereas that of TNFα was negated. With respect to cytokine immunoassays, α2M had positive and negative effects upon a number of assays, and different observations were made depending on the form of α2M present.

Section 2: Materials and Methods

2.1: Materials

2.1.1: Alpha 2 macroglobulin

α2M was donated by Behringwerke, Marburg, Germany, and was 82.6% pure as judged by electrophoresis, the principle impurities being albumin and immunoglobulins.

Alternatively, α2M was prepared from serum or plasma by zinc chromatography according to the method of Kurecki et al (1979). Briefly, fast-flow chelating Sepharose 6B (Pharmacia) was prepared by washing with 100mM phosphate buffer pH6.5 to remove preservative, and packed into a 16x180mm glass column. The Sepharose was equilibrated to pH8.0 using 100mM phosphate buffer containing 800mM NaCl and was then charged by applying $ZnCl_2$ at 5mg/ml until zinc was detectable in the eluate by precipitation with 2M Na_2CO_3. Zinc that was not bound to the column matrix was washed off using 100mM phosphate buffer pH6.5 containing 800mM NaCl.

Serum or plasma was then applied to the column. Serum was obtained by allowing fresh human blood to clot overnight at 4°C. Plasma was separated from the cellular components of fresh heparinised blood (sodium heparin 50U/50ml of fresh blood) by centrifugation at 800g for 30 minutes. Unbound protein was removed using the pH6.5 buffer containing NaCl until the optical density of the eluate at 280nm read zero. Bound α2M was recovered using 100mM pH4.5 acetate buffer containing 800mM NaCl. The fractions containing protein were pooled as the α2M peak and concentrated approximately 20 fold to 20-30mg/ml using an Amicon™ concentrating chamber. Aliquots were stored at -70°C until required. The Sepharose was regenerated by stripping the column of $ZnCl_2$ with 50mM EDTA pH7.0 containing 800mM NaCl, then re-equilibrated and charged with $ZnCl_2$ as before.

2.1.2: α2M-methylamine preparation

α2M-methylamine (α2Mm) was employed in binding investigations due to its reported similarity to proteinase converted α2M (Bjork and Fish 1982; Boisset et al 1991).

Behringwerke α2M (50mg/ml; 1-2ml) was dialysed for 6 hours or overnight at room temperature in 1 litre of 50mM TrisHCl pH8.2 containing 200mM methylamine (Sigma Chemical Co.). The methylamine-treated α2M was subsequently dialyzed twice overnight against 60mM PBS pH7.2 containing 150mM NaCl to remove the unbound methylamine. The optical density at 280nm of the α2Mm was read. It was then aliquoted and stored at -70°C.

2.1.3: Preparation of α2M-plasmin

α2M (Behringwerke, 4mg) was incubated at 37°C with plasmin (Kabi, 0.2mg). After 21 hours, Trasylol (Bayer, 20μl) was added to inhibit the enzymic activity of plasmin. α2M-plasmin was stored at -70°C until required.

2.1.4: Radiolabelled cytokines

Radiolabelled cytokines were purchased from a number of sources as follows:

(^{125}I) interleukin2 (^{125}I IL2) with a specific activity of >600Ci/mmol was purchased from Amersham International, UK. It had been prepared by iodination of the natural sequence of recombinant human IL2 using sodium (^{125}I) iodide by the chloramine-T system, and had a radiochemical purity at the time of production of 5% free (^{125}I) iodide as determined by reverse phase HPLC.

3-(^{125}I)-iodotyrosol IFNgamma (^{125}I IFNγ), purchased from Amersham International, UK, had a specific activity of >675Ci/mmol. It had been prepared from recombinant human IFNγ by the chloramine-T system using sodium (^{125}I) iodide, and had a radiochemical purity of <5% free (^{125}I) iodide at initial analysis.

Human recombinant tumour necrosis factor α (^{125}I TNFα), that had been labelled by the Bolton-Hunter method, was purchased from New England Nuclear Research and had a specific activity of >30μCi/μg. The manufacturer indicated that free iodine was less than 5% at the time of preparation, as determined by thin layer chromatography.

These cytokines were diluted in 0.1% (w/v) BSA, aliquoted and stored at -70°C (^{125}I IFNγ and ^{125}I TNFα) or -20°C (^{125}I IL2), as recommended by the manufacturers. Cytokines were tested by TCA precipitation for the presence of free iodine both upon arrival and at the end of storage of a given batch (as Sec.2.2.2). Batches were used within four weeks of their production date.

2.1.5: Unlabelled cytokines

Unlabelled cytokines, for use in competition studies and in bioassays, were obtained from a number of sources, as follows:

Recombinant des-alanyl-1, serine-125 IL2 was a gift from Euro-Cetus BV. It was provided at a specific activity of 1.8×10^7U/mg. One unit was designated as the amount of IL2 required to produce half maximal tritiated-thymidine incorporation in cultured HT-2 cells.

Human recombinant TNFα, of specific activity 6×10^5U/ml and protein concentration of 20μg/ml, was also a gift from Euro-Cetus. One unit was defined as the amount of TNFα required to mediate half maximal cytotoxicity with L929 cells in the presence of actinomycin D.

Recombinant IFNγ, of specific activity >$2x10^7$U/mg, was purchased from Boehringer Mannheim. It was judged >99% pure by HPLC. One unit was defined as the amount of IFNγ required to produce the equivalent antiviral activity to that expected by one unit of the NIH IFNγ reference standard. These unlabelled cytokines were aliquoted and stored at -20°C.

2.1.6: Radioisotopes

Iodine-^{125} supplied as sodium (^{125}I) iodide in dilute sodium hydroxide solution with a specific activity of 14.4mCi/μg of ^{125}-I at the activity date, and methyl ^{3}H-thymidine with a specific activity of 85Ci/mmol were purchased from Amersham International

2.1.7: Antibodies

Rabbit anti-human α2M IgG was purchased from Sigma. It was reconstituted at a protein concentration of 9mg/ml in deionised water. Aliquots were stored at -20°C.

Donkey anti-rabbit IgG serum was supplied by the Scottish Antibody Production Unit (SAPU), (Law Hospital, Lanarkshire, Scotland). Immediately upon arrival, the IgG component of the serum was extracted as outlined below (see Sec.2.2.4).

2.1.8: Tissue culture media

All tissue culture media were purchased from Gibco BRL.

RPMI medium, pH 6.9-7.4, was supplemented with 10% (v/v) heat inactivated foetal calf serum. The RPMI contained 1.2g/100ml of α globulins. It was supplemented with the following factors:

1mM sodium pyruvate

100IU/ml penicillin streptomycin

2mM glutamate

2.2: General Methods

2.2.1: Incubation of α2M with cytokines

α2M, derivatives of α2M, or control proteins were mixed at the required concentrations with labelled and/or unlabelled cytokine, and incubated for 2 hours at 37°C prior to use in all systems.

The time and temperature of incubation were determined by PAGE (see Sec.2.3.4 - 2.3.6 for methodology). To examine the required time for incubation, a series of samples were set up at intervals over 4 hours prior to application to PAGE. To find the optimal temperature, protein-cytokine mixtures were incubated at a range of temperatures for 2 hours, then applied to the gel. For each study, the positions of proteins on the gels were detected by Coomassie blue staining after electrophoresis, and the positions of the cytokines were determined from autoradiographs of the gels. From this information, protein bands were cut from the dried gel and counted for the presence of radiolabelled cytokine using an LKB Wallac 1260 Multigamma II gamma counter.

2.2.2: TCA precipitation

Once a protein has been labelled with radioisotope a number of events may occur which result in breakdown of the labelled product. It is therefore necessary to know the stability of the radiolabelled molecule in order to assess the accuracy of results. To this end all radiolabelled material (commercially and in-house prepared) was routinely tested to assess the percentage of label bound to the protein. This involved mixing 10μl of sample of known counts per minute (cpm), 100μl of foetal calf serum as a carrier protein (Gibco BRL) and 3ml of 20% (w/v) tri-chloroacetic acid (TCA). The precipitate was recovered by centrifugation at 350g for 10 minutes and the percentage of counts associated with the precipitate were

determined using a gamma counter.

2.2.3: Iodination of α2Mm

Radiolabelled α2Mm was required for establishing a radioimmunoassay that was used for quantitative analysis of α2M-cytokine binding.

Preswollen Sephadex G25 (~50ml; Pharmacia) was allowed to settle and the fines removed by gentle pipetting. The Sephadex was resuspended in 50mM phosphate buffer pH7.5 and packed into a 16x250mm glass column. The same buffer was used throughout unless otherwise indicated. α2Mm was diluted to a concentration of 10mg/ml in 250mM phosphate buffer pH7.5 and mixed with 5µl of chloramine T (2.5mg/ml) and 2µl of ^{125}iodine (sodium (^{125}I) iodide solution) for 15 seconds in a Nunc cryotube. Cysteine-HCl, 100µl at 0.625mg/ml, was added to stop the reaction, and 883µl of potassium iodide (5mg/ml) applied to increase the volume to 1ml. This solution was pipetted directly onto the column. The reaction tube was washed twice with 1ml volumes of buffer, each time the 1ml was applied to the column, thus providing a cold carrier. A constant head of pressure was then maintained using the buffer. An initial void volume (Vo) of 3ml of eluate was collected, and thereafter 1ml fractions. A sample of the reaction mixture and all fractions from the column were assessed for the presence of ^{125}iodine using the LKB gamma counter. In addition, fractions were examined by TCA precipitation for the level of binding of iodine to the protein. Those fractions containing high levels of radiolabelled protein were pooled, aliquoted and stored at -70°C. The radiolabelled α2Mm was used within 4 weeks of production.

2.2.4: Preparation of solid-phase bound secondary antibody

Donkey anti-rabbit IgG bound to Sephacryl was employed to facilitate the

separation of bound and free cytokine in the aforementioned radioimmunoassay, and was prepared thus:

(i) Donkey anti-rabbit IgG fractionation: Donkey anti-rabbit IgG serum was heat inactivated at 56°C for 30mins. To one volume of the serum, one volume of prewarmed (37°C) 28% (w/v) Na_2SO_4 was slowly added while stirring. The precipitate formed was centrifuged at 5000g for 15 minutes, and resuspended in half the original serum volume of 150mM NaCl. An equal volume of 28% (w/v) Na_2SO_4 was added and the precipitate centrifuged as before. The pellet was resuspended in 150mM NaCl and the solution dialysed overnight against 100 volumes of 100mM $NaHCO_3$ pH9.0. This prepared serum could be stored for up to 6 months at 4°C without losing its activity.

(ii) Preparation of oxidised Sephacryl: Superfine Sephacryl S1000 (Pharmacia; 30ml) was allowed to settle and the fines removed by gentle pipetting. It was washed with 2 litres of distilled water on a sintered glass funnel (number 2 porosity). The washed gel was resuspended in 100ml of 5mM sodium m-periodate (Sigma Chemical Co.) in 100mM sodium acetate pH5.0 in a glass stoppered tube. It was rotated for 1 hour at room temperature. The reaction was quenched by the addition of 5ml of 10% (v/v) glycerol with further mixing for 1 hour. The oxidised Sephacryl was then washed with 2 litres of 100mM $NaHCO_3$ pH9.0 and stored in 1 volume of the buffer at +4°C for up to 6 months.

(iii) Binding of donkey anti-rabbit IgG to oxidised Sephacryl: Oxidised Sephacryl (15ml) was resuspended in 20ml of the prepared donkey anti-rabbit IgG solution and rotated overnight at room temperature. The Sephacryl with bound IgG was recovered on a glass sinter, resuspended in 50mM PBS pH7.5, and the fines removed after settling. Following washing with the same buffer, the precipitate was resuspended in 100ml of 50mM

PBS pH7.5 containing 500mg of sodium borohydride and allowed to react for 30 minutes. The Sephacryl was then washed 4 times with 250mM citrate pH4.0, and 5 times with 250mM Tris-HCl pH8.5 containing 1% (v/v) Tween20, and 2% (v/v) horse serum. The Sephacryl with bound donkey anti-rabbit IgG was resuspended in 20ml of PBS pH7.2 containing 1% (v/v) Tween20, 2% (v/v) horse serum, and 0.01% (v/v) merthiolate, and stored at +4°C for up to 4 weeks.

2.2.5: Preparation of α2Mm-cytokine complexes for use in bioassays

α2Mm (17.5nmoles), preincubated with IL2 (175pmoles), was applied to G200 Sephadex columns (outlined in Sec.2.2.1 and 2.3.2). α2Mm was eluted in the void volume in PBS pH7.2. Bound IL2 co-eluted with it. Subsequently, free IL2 was eluted from the column. All fractions were retained at -70°C until required. Prior to use, samples were filtered through a 0.22μm Acrodisc filter.

α2Mm (80.1mg), preincubated with TNFα (~4ug) as indicated above, was applied to columns of zinc charged Sepharose (see Sec.2.2.1 and 2.1.1). Free cytokine was washed off the column in pH6.5 phosphate buffer with 800mM NaCl, and the eluate collected in fractions of 3ml for subsequent analysis. Acetate buffer, pH4.5 100mM with 800mM NaCl was then used to strip the column of bound α2Mm. Eluate was collected in fractions for subsequent analysis for the presence of cytokines. All fractions were stored at -70°C for up to 6 months. The column was regenerated as previously described (Sec.2.1.1).

2.2.6: Preparation of peripheral blood mononuclear cells

10ml of fresh heparinized blood was gently layered onto 7ml of Ficoll (density: 1.077) and centrifuged at 450g for 20 minutes. The interface of

Ficoll and plasma, composed of the buffy coat containing mononuclear cells, was removed using a sterile Pasteur pipette. These cells were washed twice in 20ml of sterile 60mM PBS pH7.2 containing 150mM NaCl by centrifugation at 350g for 10 minutes. The supernatant was discarded and the cells resuspended in medium and counted for subsequent use.

2.3: Analysis of qualitative binding of cytokines by α2M

2.3.1: Methods employed in qualitative analysis

Within the course of these studies, the binding of cytokines to α2M was analysed on two levels employing a range of techniques. This section details the examination of the qualitative nature of the interactions by means of gel exclusion chromatography using G200 Sephadex, fast performance liquid chromatography (FPLC) on Superose 6B, and by PAGE. These systems separate molecules on the basis of size: they are therefore ideal techniques for assessing the binding of cytokines to α2M due to the very different masses of these molecules (15-25kDa compared to 725kDa). Zinc affinity chromatography on Sepharose was also used to qualitatively assess binding on the premise that it separates α2M from all contaminants, including unbound cytokine. In addition, sodium dodecyl sulphate- (SDS-) PAGE was used to study the types of bonds involved in the binding of cytokines by α2M.

2.3.2: G200 Sephadex gel filtration

G200 Sephadex (20g dry weight) was preswollen in 500ml of 60mM PBS pH7.2 containing 150mM NaCl by heating to 100°C for 5 hours. This buffer was used throughout. The Sephadex was washed in buffer, allowed to settle, and the fines removed by gentle pipetting. It was then degassed. The gel was packed into glass columns (dimensions: 25x520mm) in the presence of the same buffer. Alternatively, the swollen gel could be stored indefinitely at +4°C until required.

α2M-cytokine preparations were applied to the column using a Pharmacia LKB Pump P-1 in the presence of buffer. Elution was performed with the PBS buffer, the eluate being automatically monitored by a UV detector.

Fractions (1-5ml volume) were collected and subsequently analysed for the presence of labelled cytokine and for protein content.

2.3.3: FPLC

FPLC works on a similar principle to the G200 system but employs higher pressures and therefore operates more rapidly. Thus, the time between application of the sample to the column and the time of collection of the final tube containing radioactive material was one hour compared to twenty hours with the G200 system. It was hoped that this much faster separation would enable the increased throughput of α2M-cytokine complex samples.

The particular system employed was a Pharmacia Superose™6 column connected to a Pharmacia Liquid Chromatography Controller LCC 500. The column was equilibrated with 2 column volumes of degassed 0.22μm filtered 60mM PBS pH7.2 containing 150mM NaCl. All separations were performed in this buffer. Protein-cytokine complexes, pre-incubated as indicated above (Sec.2.2.1), were passed through a 0.22μm filter to remove aggregates which might block the column. Approximately 200μl of the complex solution was applied to the column. Buffer was applied to the column at a rate of 0.5ml/minute for 1 hour with a maximum pressure of 1.5MPa. Eluate was collected in one minute fractions using a Pharmacia Frac100, and was monitored for the presence of protein by use of an integral UV unit and pen tracer. Fractions were subsequently analysed for the presence of protein and radiolabelled cytokine.

2.3.4: PAGE and SDS-PAGE

Polyacrylamide gel electrophoresis was chosen for the study of α2M-cytokine interactions for a number of reasons. In addition to providing the

effective separation of α2M-cytokine complexes and free cytokine, this technique enabled the resolution of the native and fast forms of α2M, as previously discussed (Sec.1.1.1). Furthermore, in the presence of SDS and mercaptoethanol, the bonds involved in the α2M-cytokine interactions could be investigated.

2.3.5: PAGE equipment

All equipment used was from Pharmacia UK. Buffers and stock solutions were prepared as indicated in Appendix A. Glass plates were thoroughly cleaned with detergent, wiped down with alcohol to remove any contaminants and then clamped together with 1.5mm spacers.

2.3.6: Native gel electrophoresis

5% running acrylamide gels were prepared by the recipes listed in Appendix A. Atmospheric oxygen inhibits the polymerisation of acrylamide. To eliminate this effect, solutions were degassed prior to the addition of the polymerisation catalyst, ammonium persulphate (10% w/v; Sigma Chemical Co.). The running gel solution was gently pipetted into the gap between the upright plates to within 3cm of the top of the plates. A layer of distilled water (~2mm deep) was gently applied on top of the running gel to further block the effects of atmospheric oxygen. Polymerisation was complete within 1 hour. The layer of water was discarded and a comb inserted. The stacking gel was then applied by pipette. The stacking gel was prepared as in Appendix A. A narrow band of distilled water was layered on top of the gel. After 1 hour, the comb was removed and simultaneously distilled water was pipetted into the wells. The water was removed using a syringe and the wells flushed with fresh distilled water. This process cleared the wells of any unpolymerised

acrylamide. The wells were then filled with Tris-glycine electrode buffer (pH8.3) in preparation for sample loading.

Samples of protein (0.5-2mg/ml; 7-28pmoles) and/or cytokine (20μCi/ml; 0.1pmoles) were prepared as required. Loading buffer (see Appendix A) was added to each sample as 4μl per 20μl of sample. Samples were then applied to the wells. The gel was submerged in electrode buffer pH8.3 (as above) and a constant current of 60mA was applied for 2.5 hours.

Proteins were fixed to the gel by incubation for 1 hour in a 25% (v/v) methanol, 10% (v/v) acetic acid solution. The gel was stained using a 0.25% (w/v) Coomassie blue (Sigma Chemical Co.), 10% (v/v) methanol solution for 30 minutes. Excess dye was removed over an 8 hour period at 37°C using a 10% (v/v) acetic acid solution with several changes of fluid. The gel was then dried onto filter paper using a Bio-Rad slab gel drier.

For the detection of radioactive cytokines, the dried gel was applied to an x-ray plate (Agfa Curix RP1 X-Ray Film) at -70°C in a film tray (Appligene, Illkirch). The autoradiograph was developed and fixed using the solutions described in Appendix A. The positions of radiolabelled cytokine on the gel were located by comparison with the autoradiograph.

2.3.7: SDS-PAGE

SDS-PAGE reducing gels were prepared in a similar manner to native gels. However, the composition of the buffers and the recipes for the running and stacking gels differed from those used for the native gels. These are listed in Appendix A. Proteins were diluted in sample buffer containing 2% (w/v) SDS (Sigma Chemical Co.) and 5% (v/v) mercaptoethanol (Sigma Chemical Co.). The technical aspects of the preparation and performance of both gel types were the same. Where required, samples were suspended in boiling water for 3 minutes.

2.4: Quantitative analysis of α2M-cytokine binding

2.4.1: Methods employed in quantitative analysis

In order to study the interaction of α2M with the cytokines at a quantitative level, it was necessary to develop methods that could process large quantities of samples to allow subsequent mathematical analysis of the binding. Two approaches were adopted which permitted the separation of bound and free ligand. These involved either the use of antibody or of zinc affinity chromatography to separate bound and free radiolabelled cytokine from α2M. These methods are outlined below. All buffers are listed in Appendix A.

2.4.2: Radioimmunoassay protocol

RT30 tubes (Costar) were incubated overnight at 37°C with 1%(w/v) BSA, 0.02% (v/v) Tween20 to block protein binding sites on the plastic. Radiolabelled cytokine was added to these preblocked tubes and incubated for 2 hours at 37°C with 10μg of α2Mm. 100μl of rabbit anti-human α2M antibody at 1/100 dilution was then added (Sigma, protein concentration 9mg/ml). After overnight incubation at +4°C, 100μl of Sephacryl bound donkey anti-rabbit IgG antibody (prepared as Sec.2.2.4) was added followed by incubation for one hour on a shaking platform (1000+/-100rpm).

To each assay tube, 1ml of prewash was added to suspend the Sephacryl, followed by 2ml of wash (see Appendix A). The wash was underlayered through the prewash layer thus suspending the prewash-Sephacryl layer above it. After 30 minutes, the Sephacryl had settled under gravity forming a pellet in the base of the tubes. The supernatant was removed and the washing procedure repeated once. The pellet was then transferred

to clean tubes and assessed for the presence of radiolabelled material using the LKB gamma counter.

2.4.3: Zinc affinity tube-based batch assay

Sepharose was equilibrated and charged with zinc by the method of Kurecki et al as outlined in Sec.2.1.1 (Kurecki et al 1979). The process was performed overnight at room temperature in a rotating 50ml centrifuge tube. The charged gel was recovered by centrifugation (200g, 10 minutes), and washed with 100mM pH6.5 phosphate buffer containing 800mM NaCl. Sepharose was subsequently used in a 1:1 ratio with this buffer.

α2M-cytokine complexes were applied to 1ml of the zinc charged Sepharose slurry in Eppendorf tubes. These were rotated for 1 hour to ensure complete adsorption of α2M to the Sepharose. The tubes were centrifuged as above, and the pellet retained. The gel was further washed by resuspension in the same phosphate buffer and centrifuged as before. The supernatant formed in each wash was collected and the radioactivity measured using the LKB gamma counter.

The α2M-cytokine complexes were then eluted from the zinc affinity gel by rotating the Eppendorf tubes for 1 hour in the presence of 100mM acetate buffer pH4.5 with 800mM NaCl, and centrifuged as before. The presence of eluted α2M with bound cytokine in the resultant supernatant was determined using the LKB gamma counter. The Sepharose was regenerated as before (see Sec.2.1.1).

2.5: Effects of α2M on cytokine assays

2.5.1: Assays examined

The techniques outlined above were designed for the study of possible interactions between α2M and cytokines. It was also desirable to examine the influence such interactions would have on cytokine assays. To this end, the effect of α2M on commercial cytokine assays was determined, and the activity of α2M-cytokine complexes in bioassays was assessed.

2.5.2: Influence of a2M in cytokine immunoassays

Cytokine immunoassay kits were purchased from or donated by a number of sources as indicated in results (see Sec.3.7 Table 3.7.1). All were two site assays, and the protocols followed were as specified by the manufacturers.

Assay standards were prepared as per the normal protocol and sufficient reserved for forming the standard curve. The remaining solution for each standard was incubated for 2 hours at 37°C in a 1:1 ratio with α2M, its derivatives or with a control protein (BSA), the final concentration for the proteins being in the range 0.5-5mg/ml. The standard-protein mixtures were applied simultaneously and in the same manner as the normal standards to the immunoassay plates. All samples were applied in duplicate. Results were read as optical densities using a Dynatech MR700 Microplate reader set at the appropriate wavelength.

2.5.3: Assay for IL2 activity by lymphoproliferation tests

IL2 bioactivity was assessed by LPA assays on human peripheral blood lymphocyte preparations isolated as described (see Sec.2.2.5). The cells were suspended at 10^6 cells per ml in complete RPMI-10%FCS. The cell

suspension was applied at 100µl per well to a 96 well plate (flat bottom, Costar). The volume of each well was made up to 200µl by addition of medium (RPMI-10% FCS), with or without stimulants such as PHA (Sigma, 5µg/ml), IL2 or IL2 complexed with α2Mm at the appropriate concentrations. The cells were incubated for 4 days at 37°C in a 5% CO_2 atmosphere, then pulsed for 18 hours with methyl ^{3}H-thymidine (^{3}HT) at 0.5µCi/well. The cells were harvested using a semi-automatic cell harvester (Skatron). The discs which bound the released DNA were then placed in individual scintillation vials with 2ml of Vickers scintillation cocktail and the quantity of ^{3}HT incorporation measured in a Tri-Carb 1900CA liquid scintillation counter.

2.5.4: TNFα bioassay

L929 cells are fibroblast cells that are particularly sensitive to TNFα, and are routinely used in the bioassay of this cytokine. For the purposes of testing the effects of α2M on the bioactivity of TNFα, L929 cells were grown according to the following protocol.

For passage, cells were trypsinised using trypsin-EDTA ($0.5gl^{-1}$ trypsin and $0.2gl^{-1}$ disodium EDTA) in 60mM PBS pH7.2 containing 150mM NaCl at 37°C for 90 seconds and resuspended in RPMI-10%FCS. They were recovered by centrifugation at 130g for 5 minutes, and resuspended to 10mls. 4mls of these cells were added to 6mls of complete medium and grown for 2 days to confluence.

Prior to each assay, the confluent cells were trypsinised as before, resuspended to a density of $4x10^6$ cells/ml, and seeded into the inner wells of 96 well plates at 100µl per well. Cells and medium were omitted from 5 wells, which were used as blanks in assessing the optical density of wells at the end of the assay. The plates were incubated overnight at 37°C to allow

the cells to form a monolayer. Medium was then aspirated from the wells. For the formation of a TNFα standard curve, 50μl of medium was placed in all wells required, then 50μl of TNFα of known concentration in the first wells of the series. The TNFα was then diluted across the plate from these initial wells by doubling dilution, and 50μl of fluid discarded from the final wells of the series. For the testing of samples of unknown TNFα concentration, aspirated medium was replaced with 50μl of unlabelled TNFα and/or α2Mm, prepared as above (see Sec.2.2.5). All conditions were tested at least in duplicate. Control wells in which 50μl of medium alone was applied to the cells were also set up. 50μl of actinomycin D (Sigma, 8μg/ml), an RNA polymerase inhibitor, was added to all wells to prevent cell proliferation allowing a clearer assessment of the anti-proliferative effects of TNFα. The cells were incubated for 18 hours at 37°C, 5% CO_2. To each well 10μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, 5μg/ml) was applied, and the plates incubated for a further 2 hours at 37°C. The supernatant was then discarded, and prewarmed (37°C) propan2ol-0.4% (v/v) 1N HCl applied at 100μl/well and the plates incubated for 45 minutes at 37°C. The optical density in each well was then read at 570nm using a Dynatech MR700 Microplate reader.

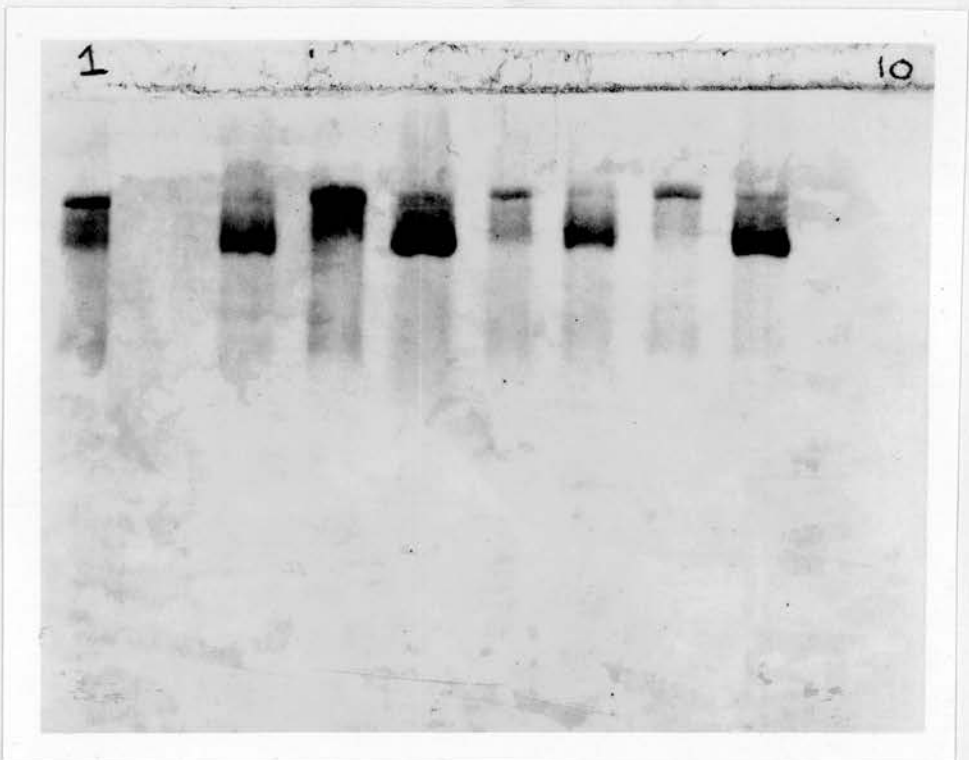
Section 3: Results

3.1: Sources and purity of α2M

3.1.1: Sources and purity of α2M and its derivatives

As previously described in Sec.1.1.1, α2M exists in 2 conformations that are differentiated by PAGE, as illustrated in Fig.3.1.1. It is evident that the native protein (lanes 1 and 6; purchased from Behringwerke) and that derived from plasma (lanes 3 and 7) migrated more slowly than the serum derived (lanes 5 and 9) and methylamine-treated forms (lanes 4 and 8). As mentioned before, it is on the basis of such migration patterns that classically the slower proteins are referred to as "slow form", and the others as "fast form". In Fig.3.1.1, no contaminants are seen in the preparations indicating a high level of purity for each of the preparations. Nevertheless, at higher concentrations of α2M some contaminants may be visible.

Storage of the Behringwerke α2M for 6 months at -70°C resulted in the conversion of some of the protein to the fast conformation (Fig.3.1.2). This protein had not been subjected to freeze-thawing. This phenomenon has been observed elsewhere and is proposed to be due to the autolytic cleavage of the thiol ester bond (Barrett and Starkey 1973; see Sec.1.6). When this age-converted protein was examined by FPLC on Superose 6B for its potential to bind to ^{125}-I IL2, it bound similar levels of the cytokine as fast forms of α2M (see Sec.3.3 and Fig.3.3.3d).



1 α2M (Behringwerke)
2
3 α2Mm
4 α2M (plasma)
5 α2M (serum)
6 α2M (Behringwerke)
7 α2Mm
8 α2M (plasma)
9 α2M (serum)
10

Figure 3.1.1: Slow and fast forms of α2M on PAGE
Samples of α2M (10µl, 2mg/ml in lanes 1, 3, 4, and 5, 1mg/ml in lanes 6, 7, 8, and 9) were applied to a 5% PAGE gel, run at 50mA for 2 hours. Note the migration pattern: α2M and α2M (plasma) ran slower than α2Mm and α2M (serum).

3.2: Time course and temperature studies

3.2.1: Investigation of the optimal α2M-cytokine incubation conditions

Prior to binding studies, native PAGE was used to determine optimal conditions for the interaction of α2M and cytokines. An initial time of incubation of 2 hours was established based on previous investigators' studies (Wollenberg et al 1991; Teodorescu et al 1991), and a range of temperatures analysed (-70°C to +65°C). Fig.3.2.1 illustrates the resultant autoradiograph for the study with IFNγ. It is apparent that +37°C provided the optimal temperature with this cytokine. A similar finding was established with IL2 (Figure not shown).

Time courses were set up with incubations commencing up to 4 hours prior to running reactants on a gel: Fig.3.2.2 indicates that 2 hours was sufficent time for the interaction of ^{125}-I IL2 with α2Mm; a similar result was found for IFNγ (Figure not shown).

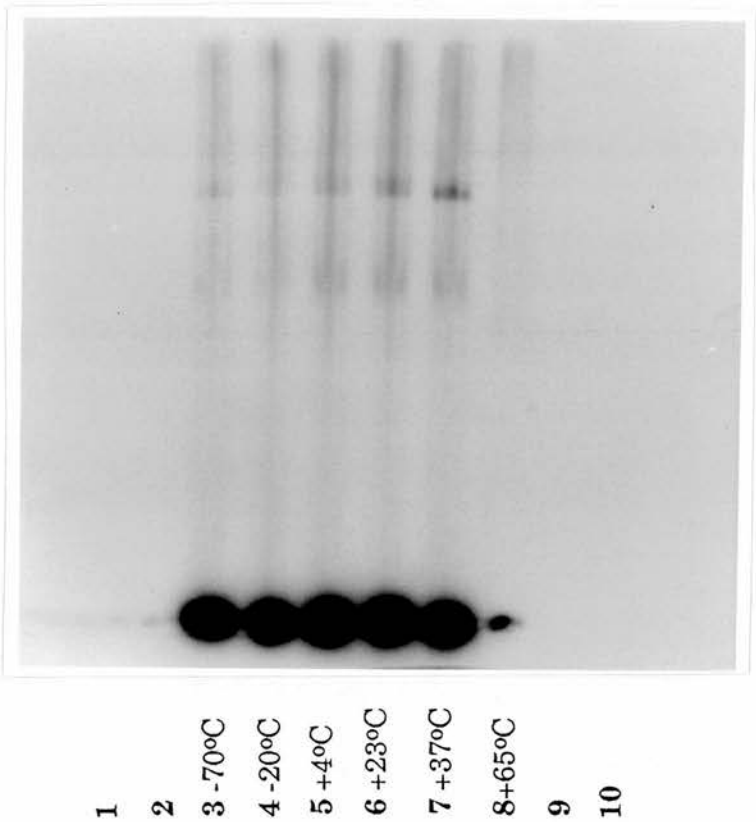


Figure 3.2.1: Temperature study for the interaction of ^{125}-I IFNγ with α2Mm

Samples of α2Mm (10µl, 2mg/ml) and ^{125}-I IFNγ (~10^5cpm) were incubated for 2 hours at a range of temperatures, applied to a 5% PAGE gel, and run for 2 hours at 50mA. After staining for proteins, the gel was dried and laid down with an x-ray plate at -70°C for 7 days, then the x-ray was developed. Note the maximal intensity of staining at 37°C.

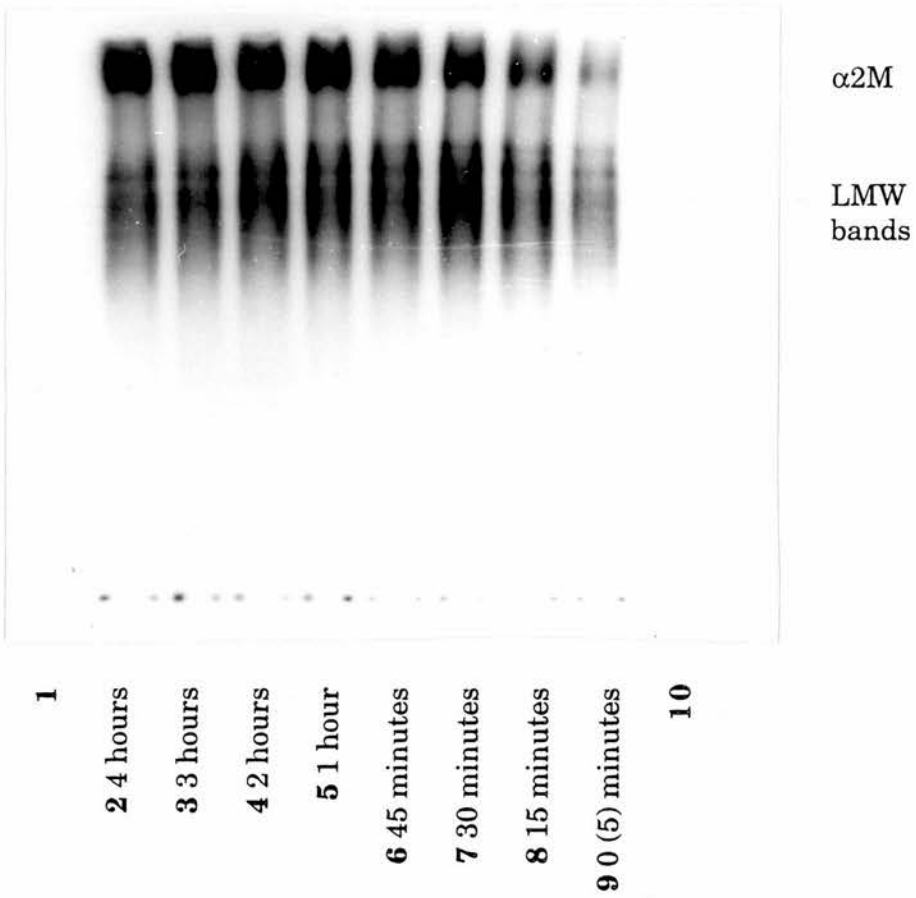


Figure 3.2.2: Time course study for the interaction of ^{125}-I IL2 with α2Mm

Samples of α2Mm (10µl, 2mg/ml) and ^{125}-I IL2 (~10^5cpm) were incubated for 2 hours, applied to a 5% PAGE gel and run for 2 hours at 50mA. The gel was stained for proteins, dried and laid down with an X-ray plate for 7 days at -70°C, then the gel was developed. Note the increase in intensity of the band associated with α2Mm as time of incubation increased.

3.3: Qualitative column studies of the binding of cytokines by α2M

3.3.1: G200 Sephadex and FPLC Superose 6B Studies

The binding of radiolabelled IL2 and IFNγ by α2M was examined by applying incubated mixtures of protein and cytokine to G200 Sephadex and FPLC Superose 6B columns. The columns permit separation of free cytokine from that bound to protein on the basis of size. The columns were calibrated with molecular weight marker proteins in order to assess the molecular weights of eluting material (see Appendix C)

3.3.2: G200 studies of the binding of cytokines by α2M

In the G200 studies, it was apparent that the various forms of α2M examined bound IL2 and IFNγ (Figs.3.3.1 and 3.3.2). Cytokine binding by albumin could also be detected but at a lower level. Transformation of the data to indicate the percentage of eluted protein-associated cytokine revealed a distinct binding pattern relating to the form of α2M: the fast forms of the protein (ie methylamine treated, or serum derived) bound more cytokine than the slow native form (illustrated in peak A in Table 3.3.1 and 3.3.2). For example, it was found that α2M bound 8.2% of the IFNγ that eluted from the column, whereas the fast forms of α2M bound 16.7-25.9% of the eluted cytokine (Table 3.3.2). In contrast, albumin bound only 6.7% of the available cytokine. A similar pattern was observed with IL2, with serum derived α2M 67.9% of the eluted cytokine was bound, with native α2M 28.6%, while albumin bound only 24.6% (Table 3.3.1).

Contrary to expectations, plasma derived α2M also bound levels of cytokine similar to the fast forms of α2M. For example, α2M from plasma bound 32.4% of eluted IFNγ as compared to the serum derived form of the protein which bound 25.9% (Table 3.3.2). The results with α2M prepared

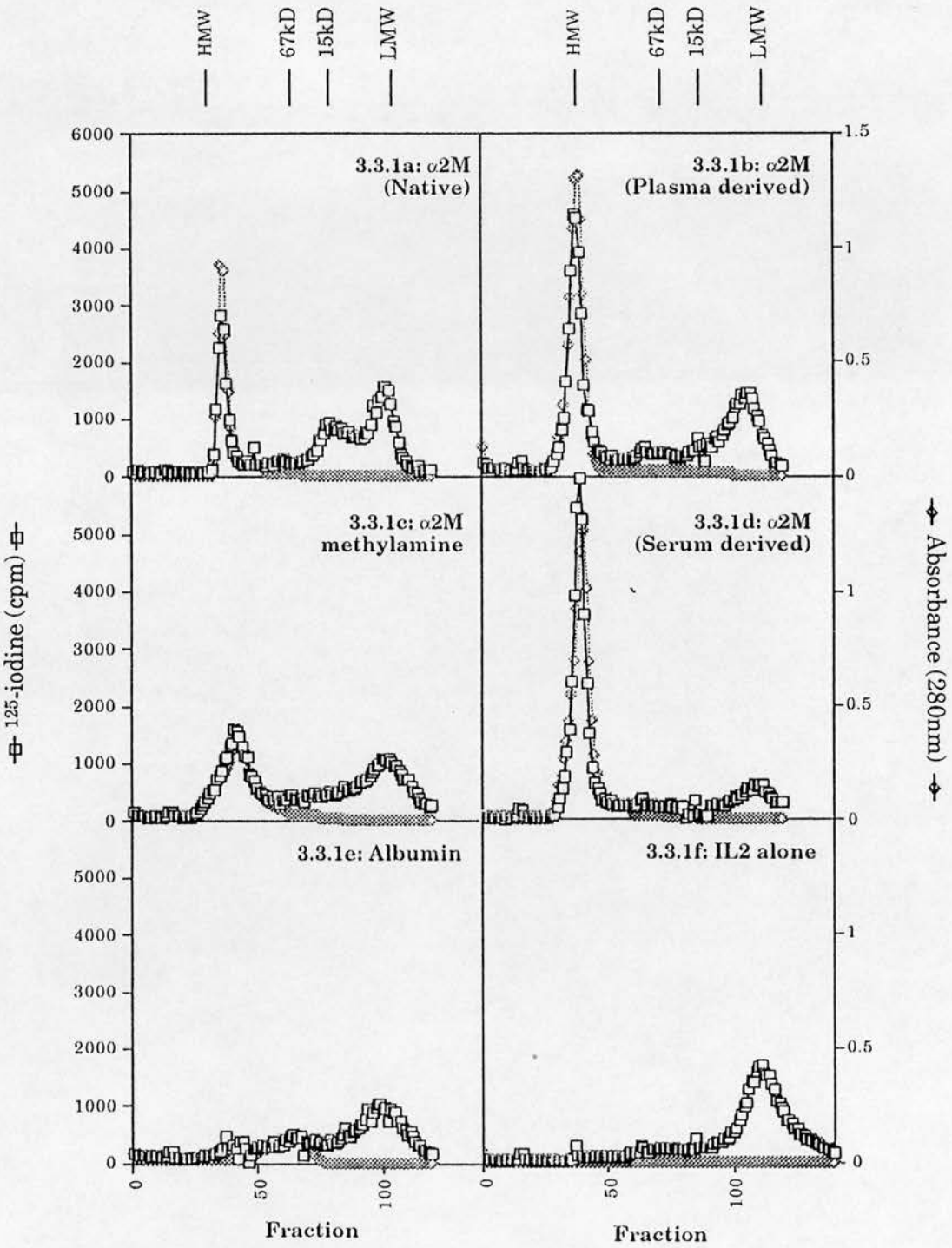


Figure 3.3.1: The binding of ^{125}-I IL2 to human α2M as revealed by filtration on G200 Sephadex

^{125}-I IL2 (94fmoles) preincubated with protein (21nmoles α2M, 224nm BSA) (2 hours, 37°C) was applied to a G200 Sephadex column (16x52mm, flow rate 6.6ml/hour) for the separation of bound and free cytokine. The presence of radioactivity and of protein in the resultant fractions was measured. Note the association of radioactivity with the heavy molecular weight α2M peak.

Protein	Protein Amount (nm)	% of total ^{125}-Iodine eluted		
		Peak A	Peak B	Peak C
α2M	5.2	28.6	23.8	35.3
α2M (plasma)	10.1	43.8	6.8	32.6
α2Mm	5.9	39.5	17.6	34.4
α2M (serum)	11.7	67.9	4.9	19.5
Albumin	55.2	24.6	10.7	48.8
IL2 alone	/	1.5	8.1	75.1

Table 3.3.1: The binding of 125-I IL2 by α2M as revealed by gel filtration on G200 Sepahdex
Preincubated IL2 (94fmoles) and protein (α2M 21nmoles, BSA 224nmoles) (2 hours, 37°C) were applied to a G200 sephadex column (16x52mm,flow rate: 6.6ml/hour) to separate bound and free cytokine. The amounts of protein and of radiolabel in the eluate were assessed. Protein came off as one peak, peak A. Cytokine eluted in 3 groups: peak A where it co-eluted with protein, peak B in the molecular weight region of whole cytokine, and peak C was degraded cytokine or free ^{125}-iodine.

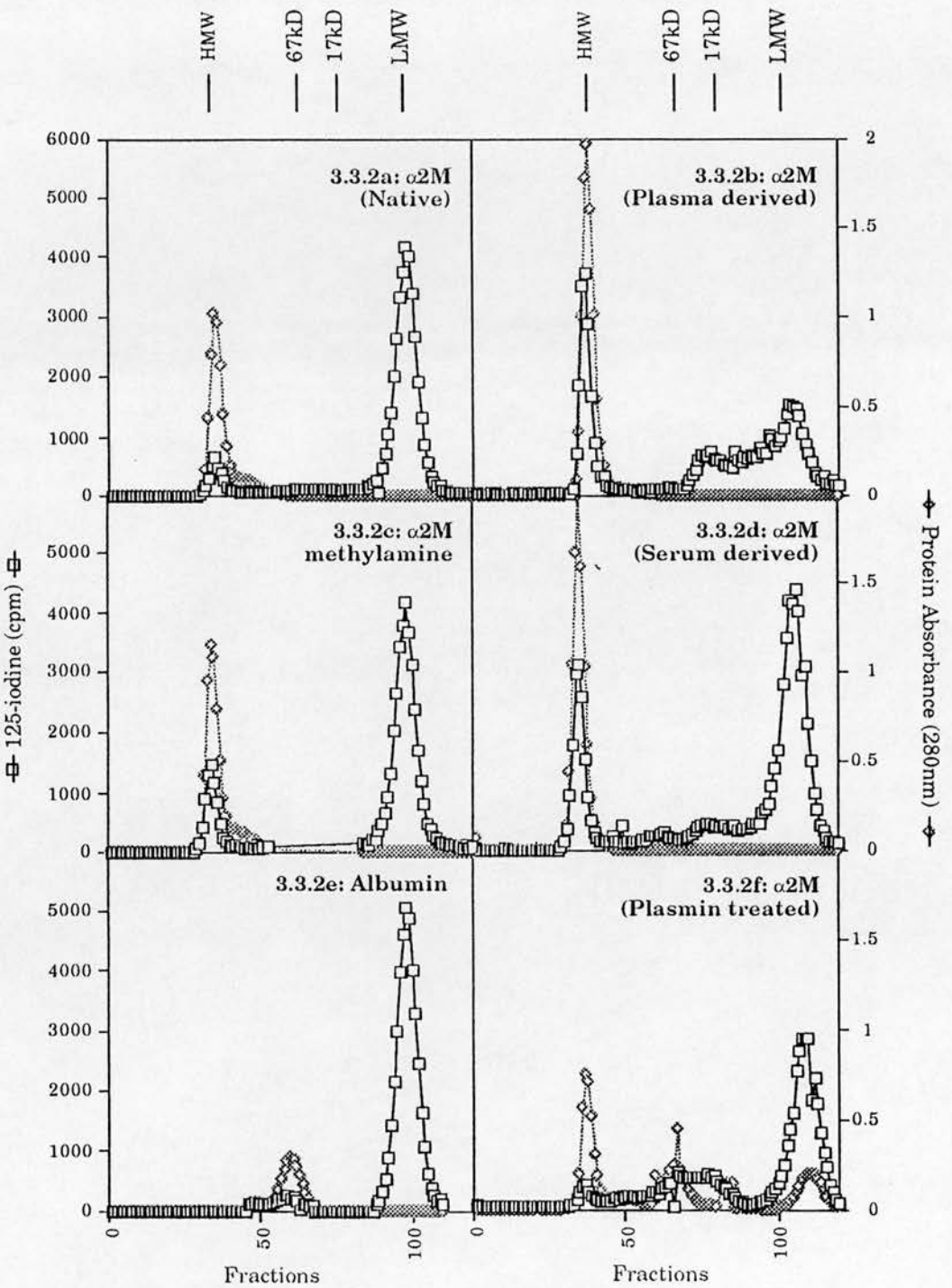


Figure 3.3.2: The binding of ^{125}-I IFNγ to human α2M as revealed by G200 Sephadex.

^{125}-I IFNγ preincubated with protein (21nm α2M, 224nm BSA; 2 hours, 37°C) was applied to a G200 column (16x52mm, flow rate 6.6ml/hour). The eluate was assessed for the presence of protein and of radiolabel. Note the association of radioactivity with the heavy molecular weight α2M peak.

Protein	Protein Amount (nm)	% of total ^{125}-Iodine eluted		
		Peak A	Peak B	Peak C
α2M	6.4	8.2	1.8	82.7
α2M (plasma)	9.8	32.4	21.8	43.1
α2Mm	7.2	16.7	2.4	74.3
α2M (serum)	9.9	25.9	/	67.9
Albumin	67	6.7	/	86.6
Plasmin	/	/	/	/

Table 3.3.2: The binding of ^{125}-I IFNγ with α2M as revealed by gel filtration on G200 Sephadex.
Preincubated IFNγ (84fmoles) and protein (21nmoles α2M, 224nm BSA) (2 hours, 37oC) were applied to a G200 Sephadex column (flow rate 6.6ml/hour) to separate bound and free cytokine. The amounts of protein and of radiolabel in the eluate were assessed. Protein came off as one peak. Cytokine eluted in 3 groups: peak A where it co-eluted with protein, peak B in the molecular weight range of whole IFNγ, and peak C was degraded cytokine or free ^{125}-iodine.

from plasma may have arisen by age-conversion of the protein due to autolytic cleavage of the thiol ester bond (see Sec.3.1 Fig.3.1.2).

Fast form α2M generated by incubation with plasmin was also examined (Fig.3.3.2f). However, G200 studies with that preparation revealed that considerable degradation had occurred yielding polypeptides of α2M of various molecular weights and binding variable quantities of ^{125}-I labelled cytokine. On the basis of this observation, subsequent work was performed with fast form α2M prepared by methylamine treatment, or derived from serum as described in Sections 2.1.2 and 2.1.1.

3.3.3: FPLC studies of the binding of α2M and cytokines

FPLC studies corroborated the G200 findings indicating that IL2 coeluted with α2M (see Fig.3.3.3). A similar binding pattern was found to that seen with G200 (Table 3.3.3): for example, native α2M was found to bind 10.0% of eluted IL2, whereas the fast forms of the protein bound 34.1-36.7% (peak A in Table 3.3.3). This was observed with methylamine treated, serum derived and age-converted α2M. In contrast, albumin bound only 3.4% of the cytokine.

3.3.4: Elution profiles of protein and radioactivity from columns

Calibration of the columns, see Appendix C, indicated that when α2M was run on G200 or on FPLC it eluted as a protein peak in the void volume, and that albumin eluted later as would be expected: this can be seen in the protein profiles in Figs.3.3.1-3.3.3. The ^{125}-iodine radiolabelled cytokine eluted in 3 principle peaks: peak A co-eluted with protein (ie either α2M or albumin) and was presumably due to the interaction of protein with whole radiolabelled cytokine. Peak B eluted in the molecular weight band of whole cytokine (ie 13.7-25kDa as indicated by G200). Peak C eluted in the

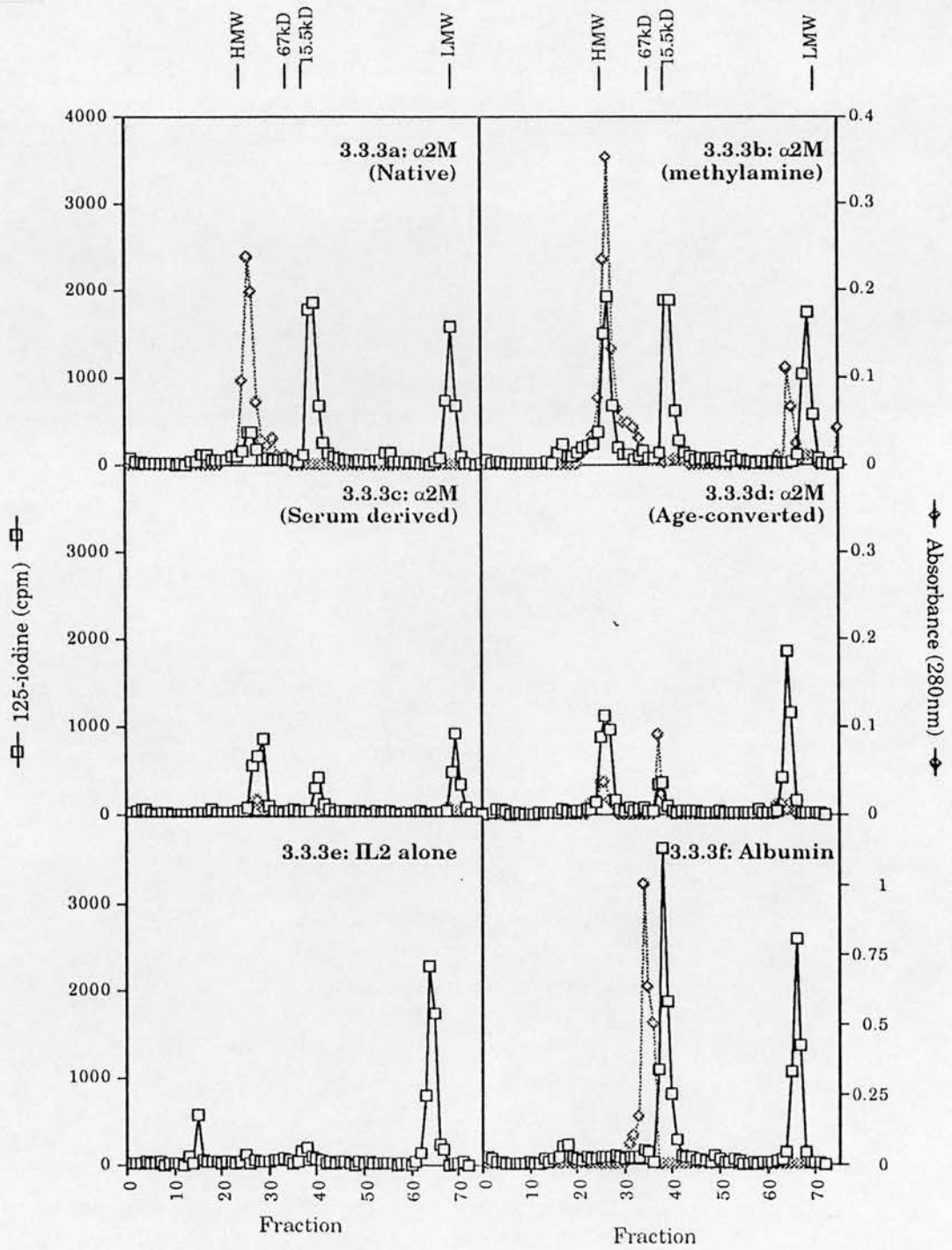


Figure 3.3.3: The binding of ^{125}-I IL2 to human α2M as revealed by filtration on Superose 6 B FPLC

^{125}-I IL2 preincubated in the presence of protein (α2M 21nmoles, BSA 224nmoles) was applied to a Superose 6B FPLC column (flow rate 0.5ml/min) to separate bound and free cytokine. Resultant fractions were assessed for the presence of radioactivity and for protein concentration. Note the association of radioactivity with the heavy molecular weight α2M peak.

Protein	Protein Amount (nm)	% of total ^{125}-Iodine eluted		
		Peak A	Peak B	Peak C
α2M	0.65	10.0	43.5	26.1
α2Mm	1.1	34.1	30.9	21.4
α2M (serum)	0.036	35.5	14.9	29.7
α2M (age-converted)	0.11	36.7	8.8	40.4
Albumin	64	3.4	47.7	32.4
IL2 alone	/	1.5	6.2	68.3

Table 3.3.3: The binding of ^{125}-I IL2 with α2M as revealed by gel filtration on FPLC Superose 6B

Preincubated IL2 (94fmoles) and protein (2 hours, 37°C) were applied to an FPLC Superose 6B column (flow rate 0.5ml/min) to separate bound and free cytokine. The amounts of protein and of radiolabel in the eluate was assessed. Protein came off as one peak. Radiolabel eluted in 3 groups: peak A where it co eluted with protein, peak B in the molecular weight fractions of whole cytokine , and peak C was degraded cytokine or free ^{125}-iodine.

low molecular weight region and would have consisted of degraded cytokine and free ^{125}-iodine. Variation in the size of peaks B and C is evident in Fig.3.3.1-3.3.3 and Tables 3.3.1-3.3.3. This was due to degradation of the radiolabelled cytokine during storage. This pattern of three peaks was confirmed by TCA precipitation studies which revealed that counts in the third peak were not associated with intact protein (see Sec.3.9 Fig.3.9.1). It should also be noted that routine studies on stored labelled cytokines indicated that degradation of these products regularly occurred (see Sec.3.9. Table 3.9.1).

3.3.5: Zinc affinity Sepharose column studies

α2M and cytokine interactions were further investigated by separation of mixtures of these materials by zinc affinity chromatography on Sepharose. A phosphate buffer was used to elute material that did not bind with the zinc, specifically cytokine. Acetate buffer was then used to elute α2M that had interacted with the zinc. Any α2M-associated cytokine was also eluted at this point.

From Fig.3.3.4, it can be seen that an appreciable amount of labelled IL2 coeluted with α2M and with α2Mm in the acetate buffer. In the absence of protein, no IL2 was present in the acetate wash. These findings imply that the ^{125}-I IL2 eluted in the acetate wash had interacted with the serum protein. Analysis of the amounts of protein and cytokine eluted revealed once more that α2M bound less of the available IL2 than α2Mm (Table 3.3.4). The general finding was that 10 fold less α2Mm, than α2M, was required to bind 1fmole of IL2. For example, for the cases illustrated, 0.24nmoles of α2M bound 1fmole of IL2 whereas 0.035nmoles of α2Mm were required to bind the same amount of cytokine.

The binding of TNFα with α2M was also examined by zinc affinity

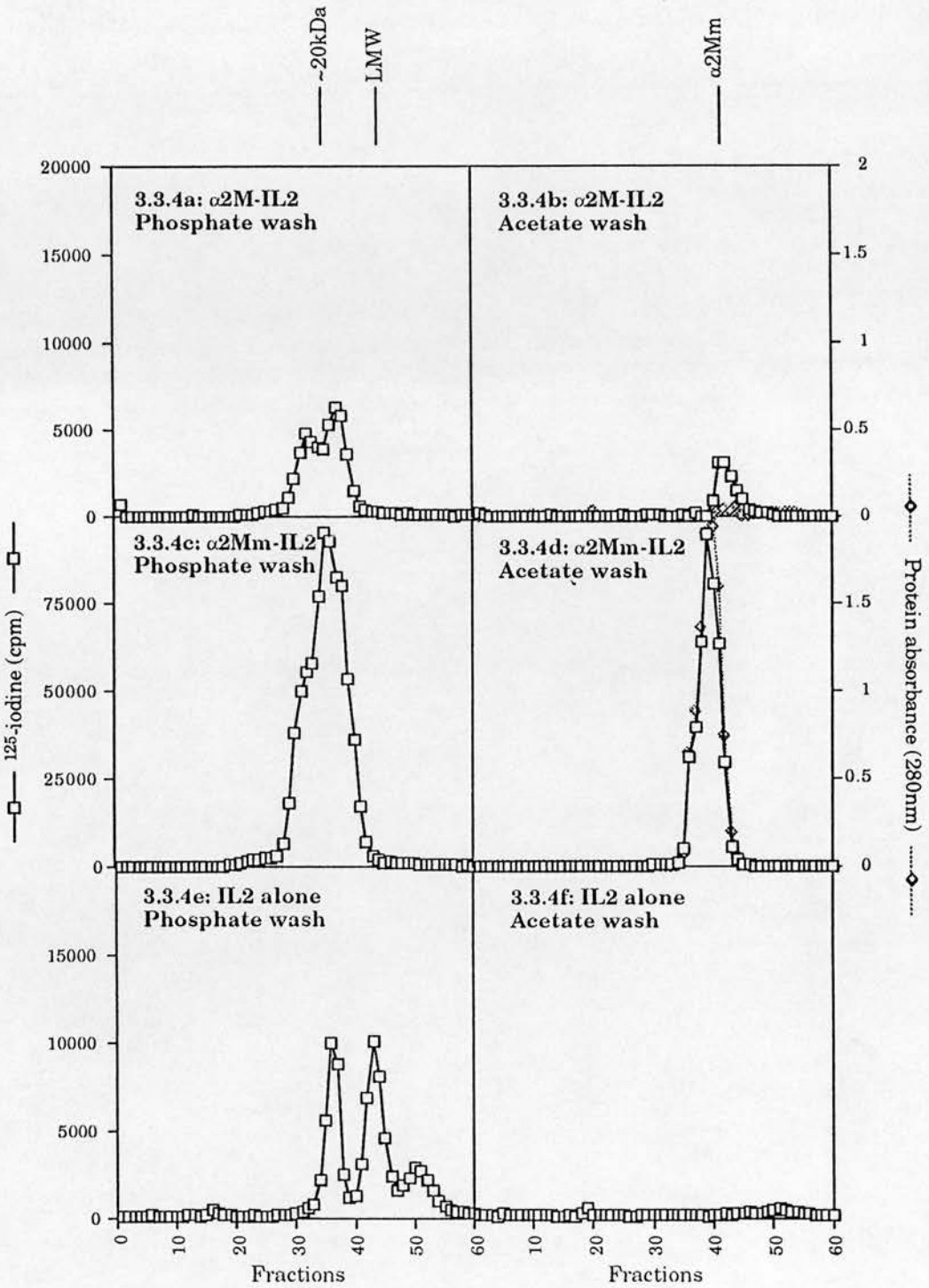


Figure 3.3.4: Zinc affinity Sepharose study of the binding of ^{125}-I IL2 by α2M

Preincubated ^{125}-I IL2 and protein (variable amounts, 2 hours, 37°C) were applied to a Sepharose column to which zinc ions had been bound. In the presence of phosphate buffer pH6.5, α2M chelated with the zinc, all other material was eluted (panels a, c, and e). Acetate buffer pH4.5 was then applied to elute the α2M, and any cytokine that had bound to it (panels b, d and f). It is apparent that α2M and α2Mm bound IL2 (panels b and d).

Conditions	Protein Amount (nmoles)	% of total ^{125}-I eluted			^{125}-I IL2 (fm): α2M (nm)
		Phosphate wash peak 1	Phosphate wash peak 2	Acetate wash	
α2M-IL2	2.04	37.8	57.3	4.9	1:0.24
α2Mm-IL2	12.56	63.3	/	36.7	1:0.035
IL2 alone	/	38.1	44.9	0	/

Table 3.3.4: Study of the binding of ^{125}-I IL2 with α2M using zinc affinity Sepharose for separation
Preincubated IL2 and protein (variable amounts, 2 hours, 37°C) were applied to a Sepharose column to which zinc ions had been chelated. ^{125}-iodine eluted in three peaks: 2 in the phosphate buffer pH6.5 of molecular weights equivalent to whole cytokine (peak 1) and cytokine fragments/free iodine (peak 2). With α2M or α2Mm present, cytokine coeluted with the protein in an acetate buffer pH4.5. The molar ratio of protein and cytokine coeluted indicated that α2Mm bound 10 fold more IL2 than did α2M.

separation on Sepharose (Fig.3.3.5, Table 3.3.5). Once again it was found that cytokine eluted in the acetate wash only in the presence of α2M (Fig.3.3.5b and d compared with Fig.3.3.5f) indicating an interaction of TNFα with α2M. In addition, α2Mm showed stronger binding to the cytokine than did the native protein, 28% of eluted TNFα associating with the methylamine converted protein while only 6.3% bound to native α2M (Table 3.3.5). Limited OD values were recorded during these studies and it is therefore not possible to make a more detailed comparison of the binding of α2M and α2Mm with TNFα from this work. Breakdown products were found in the double peak in the phosphate wash and were further investigated by TCA precipitation (see Scc.3.9 Fig.3.9.2).

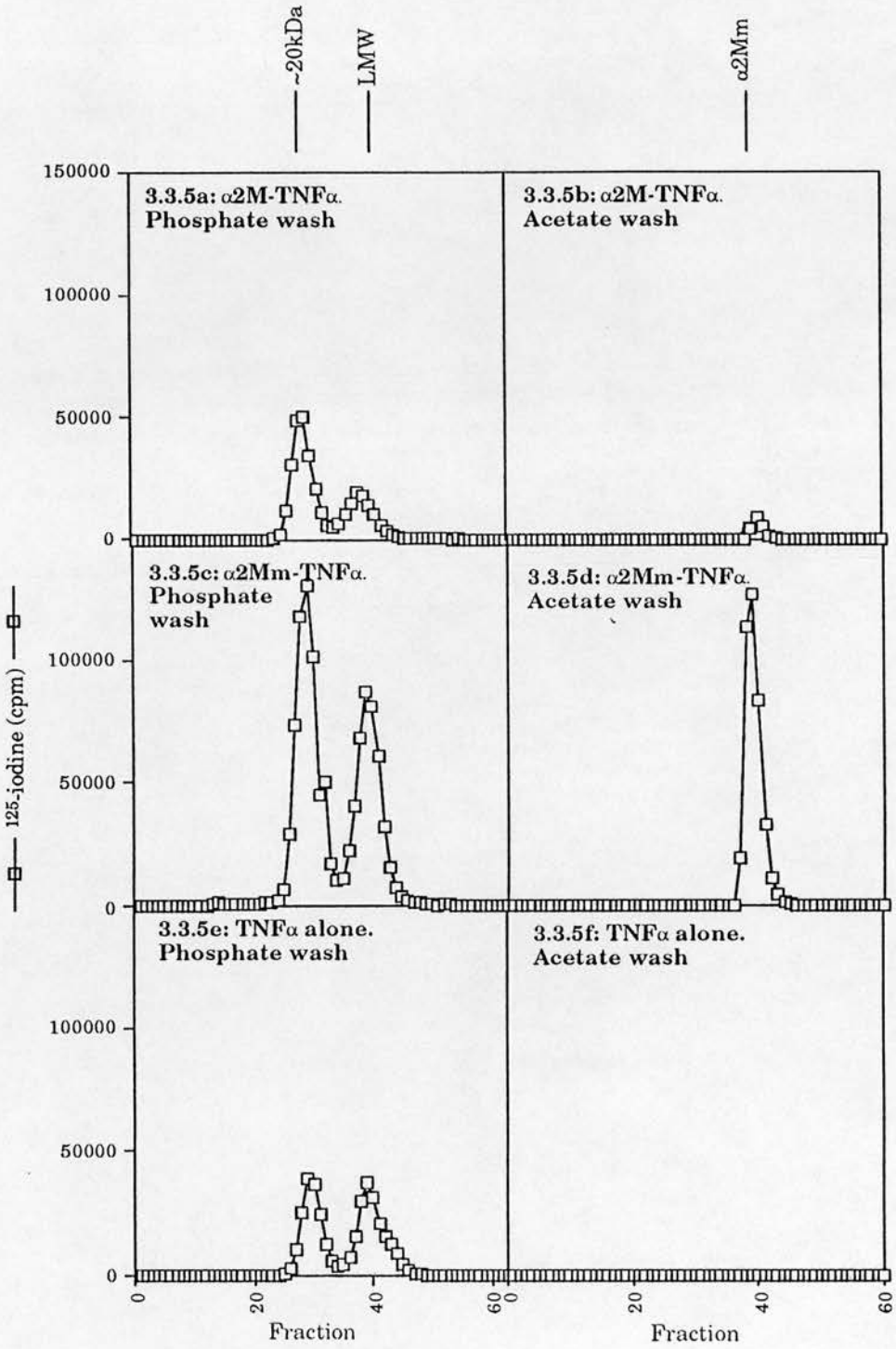


Figure 3.3.5: Study of the binding of ^{125}-I TNFα by α2M using zinc affinity Sepharose separation

Preincubated ^{125}-I TNFα and protein (variable amounts, 2 hours, 37°C) were applied to a zinc chelated Sepharose column. In the presence of phosphate buffer pH6.5, α2M bound to the zinc and cytokine was eluted (panels a, c and e). Acetate buffer pH4.5 was then used to elute the protein and any associated TNFα (panels b, d and f). It can be seen that TNFα associated with the α2M and α2Mm.

Conditions	% of total ^{125}-I eluted		
	Phosphate wash peak 1	Phosphate wash peak 2	Acetate wash
α2M-TNFα	62.3	31.4	6.3
α2Mm-TNFα	41.4	30.6	28
TNFα alone	45.7	54.1	0.2

Table 3.3.5: Study of the binding of α2M with TNFα using zinc affinity Sepharose for separation
Preincubated TNFα and protein (variable amounts, 2 hours, 37°C) were applied to a zinc chelated Sepharose column. In the presence of phosphate buffer pH6.5, α2M bound to the zinc and all other material, eg cytokine was eluted: peak 1 consisted of whole cytokine (as judged by G200), and peak 2 of cytokine fragments/free iodine. Acetate buffer pH4.5 was then used to elute the protein and any associated TNFα. It was evident that TNFα did associate with the α2M and α2Mm.

3.4: Polyacrylamide gel electrophoresis studies of α2M-cytokine interactions

3.4.1: General conclusions of the electrophoresis studies

In addition to gel filtration chromatography, qualitative analysis of the binding of the radiolabelled cytokines IL2, IFNγ, and TNFα to α2M was carried out by native and SDS polyacrylamide gel electrophoresis (PAGE and SDS-PAGE). The gels and autoradiographs illustrate that the protein and these cytokines do bind (Figs.3.4.1-3.4.3). The effects of SDS and mercaptoethanol on the interaction of the cytokines with α2Mm indicate that the interaction of IFNγ with α2Mm employs non-disulphide covalent bonds, that of IL2 with the protein uses covalent bonds, and that TNFα is bound to α2M by non-covalent bonds.

3.4.2: Binding studies with PAGE

Figs.3.4.1-3.4.3 reveal the binding of IFNγ, IL2 and TNFα to α2M and its derivatives. Binding of IFNγ is to fast form α2M in all three samples (Fig.3.4.1). It is apparent that α2M derived from serum and from plasma was composed of both slow and fast forms of the protein, contrary to that observed previously (see Sec.3.1 Fig.3.1.1). Moreover, the presence of fast form α2M in the plasma preparation explains the high levels of binding observed with this preparation in G200 studies with ^{125}-I IFNγ compared to the expected lower levels (see Sec.3.3 Fig.3.3.2b and Table 3.3.2).

^{125}-I IL2 was bound by fast form α2M. Binding was observed with the lower part of the slow-fast α2M band (Fig.3.4.2). It was not bound by an equivalent component of plasma, but did co-migrate with a 470kDa protein in both the α2M and plasma preparations. However, there is not a distinct protein band at ~730kDa in either plasma preparation, therefore it would be unlikely that any ^{125}-I IL2 bound to α2M in those preparations would be

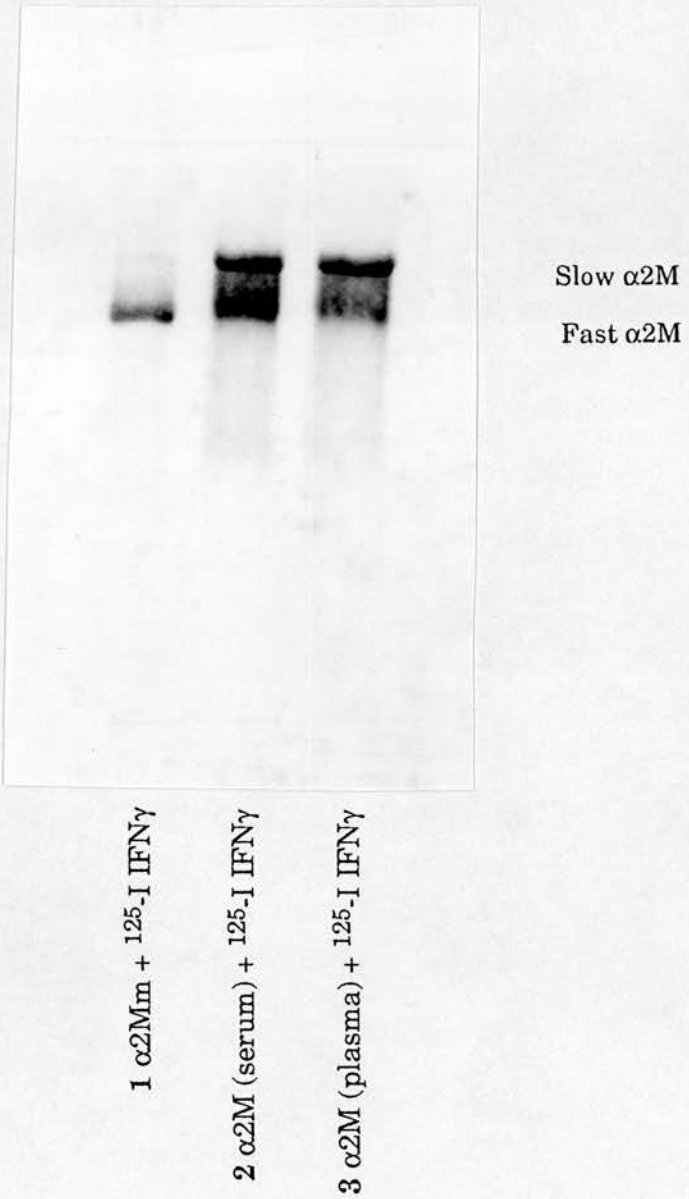


Figure 3.4.1a: Study of the binding of IFNγ by α2M using PAGE
α2M samples (0.5mg/ml, 16µl) were preincubated with ~10^5cpm of ^{125}-I IFNγ for 2 hours at 37°C, then run on a 5% PAGE gel for 2 hours at 50mA. The gel was stained for proteins (Fig.3.4.1a), laid down with an x-ray plate at -70°C for 7 days then the x-ray was developed (Fig.3.4.1b). ^{125}-I IFNγ was bound by the faster migrating bands of α2M.

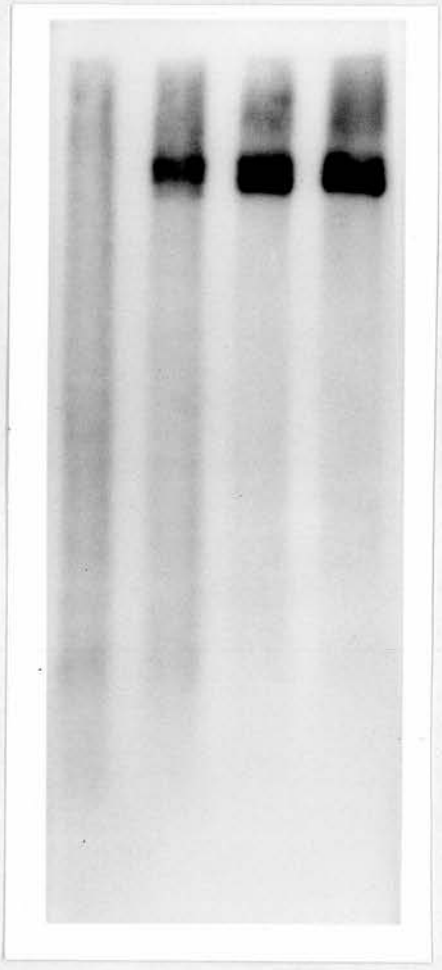


Figure 3.4.1b: Study of the binding of IFNγ by α2M using PAGE
α2M samples (0.5mg/ml, 16μl) were preincubated with ~10^5cpm of ^{125}-I IFNγ for 2 hours at 37°C, then run on a 5% PAGE gel for 2 hours at 50mA. The gel was stained for proteins (Fig.3.4.1a), laid down with an x-ray plate at -70°C for 7 days then the x-ray was developed (Fig.3.4.1b). ^{125}-I IFNγ was bound by the faster migrating bands of α2M.

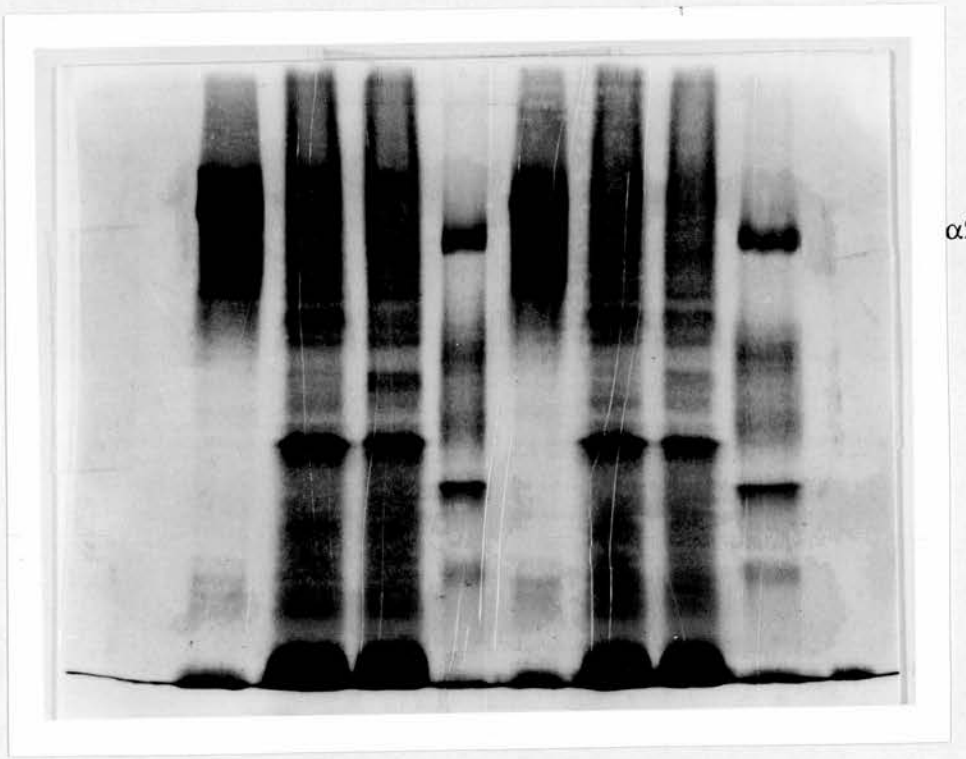


Figure 3.4.2a: PAGE study of α2M-IL2 binding
Samples of α2M (2mg/ml, 20μl/well) or of plasma (fresh or stored for 1 month at -70oC, 10μl) were incubated with 125-I IL2 at 37°C for 2 hours, then run on a 5% PAGE gel for 2 hours at 50mA. The gel was stained for proteins (Fig.3.4.2a), laid down with an x-ray plate at -70°C for 7 days and the x-ray plate developed to reveal radioactivity bands. 125-I IL2 bound to purified α2M, and also to protein bands of ~430kDa seen in the α2M and plasma preparations.

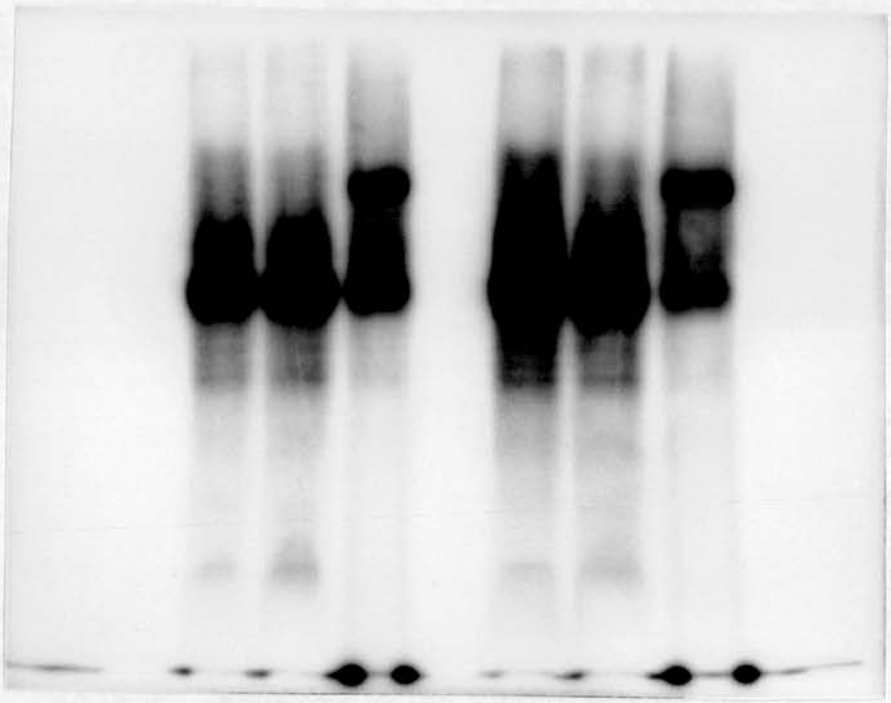


Figure 3.4.2b: PAGE study of α2M-IL2 binding
Samples of α2M (2mg/ml, 20µl/well) or of plasma (fresh or stored for 1 month at -70°C, 10µl) were incubated with ^{125}-I IL2 at 37°C for 2 hours, then run on a 5% PAGE gel for 2 hours at 50mA. The gel was stained for proteins (Fig.3.4.2a), laid down with an x-ray plate at -70°C for 7 days and the x-ray plate developed to reveal radioactivity bands. ^{125}-I IL2 bound to purified α2M, and also to protein bands of ~430kDa seen in the α2M and plasma preparations.

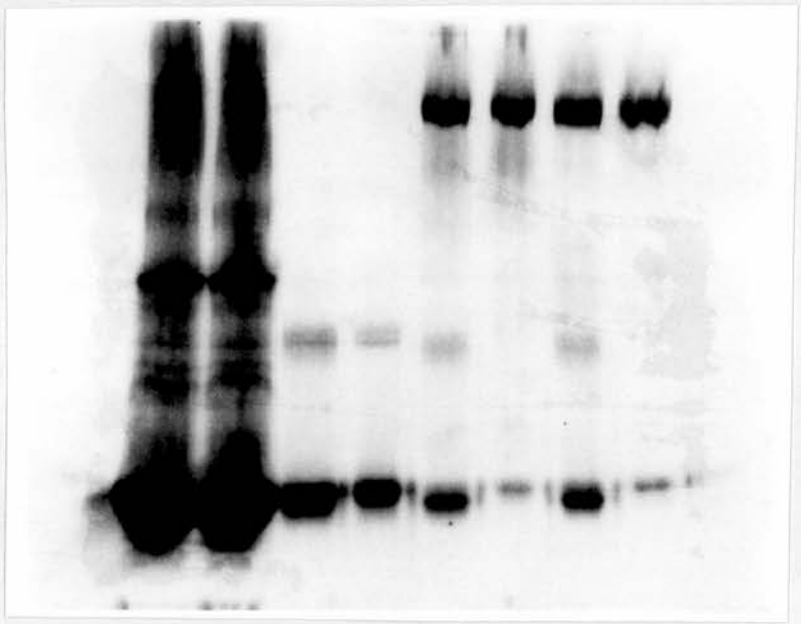


Figure 3.4.3a: PAGE study of α2M and ^{125}-I TNF α
Samples of α2M, albumin (both at 2mg/ml 20μl/well) or of fresh plasma (20μl/well) were incubated with ^{125}-I TNFα (~10^5cpm) at 37°C for 2 hours, then run on a 5% PAGE gel. The gel was stained for protein (Fig.3.4.3a), laid down with an x-ray plate at -70°C for 7 days, and then the x-ray was developed (Fig.3.4.3b). Note the interaction of α2M and ^{125}-I TNFα, and an equivalent molecular weight protein in the plasma preparations also bound the cytokine.

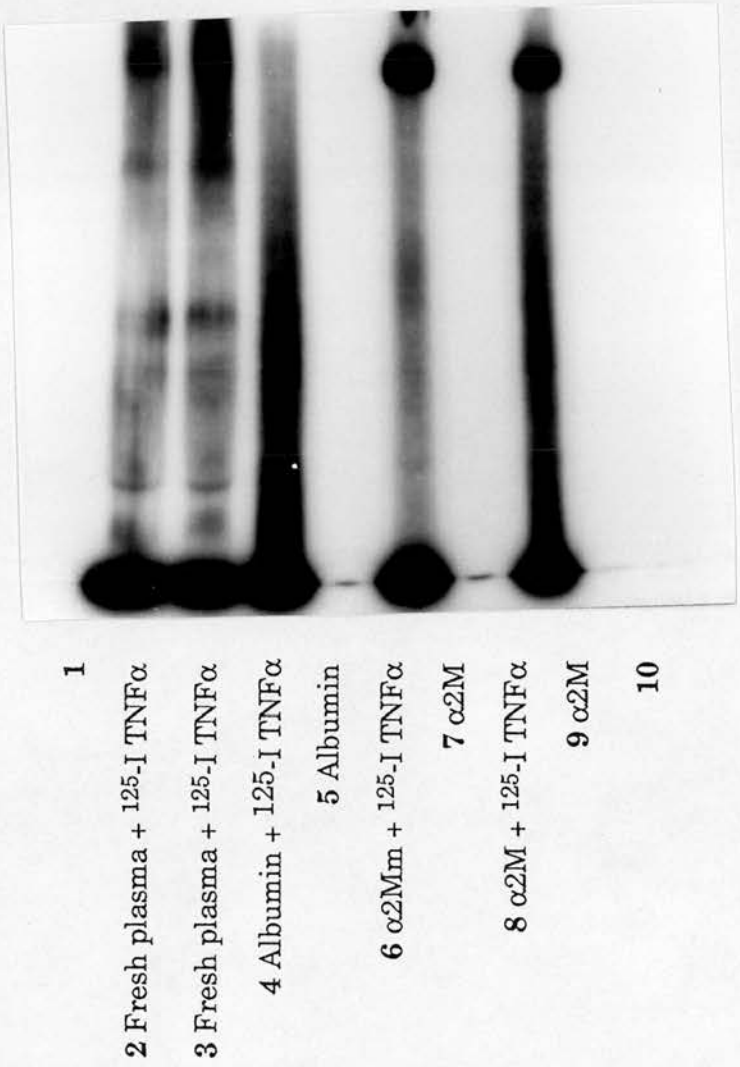


Figure 3.4.3b: PAGE study of α2M and ^{125}-I TNF α
Samples of α2M, albumin (both at 2mg/ml 20µl/well) or of fresh plasma (20µl/well) were incubated with ^{125}-I TNFα (~10^5cpm) at 37°C for 2 hours, then run on a 5% PAGE gel. The gel was stained for protein (Fig.3.4.3a), laid down with an x-ray plate at -70°C for 7 days, and then the x-ray was developed (Fig.3.4.3b). Note the interaction of α2M and ^{125}-I TNFα, and an equivalent molecular weight protein in the plasma preparations also bound the cytokine.

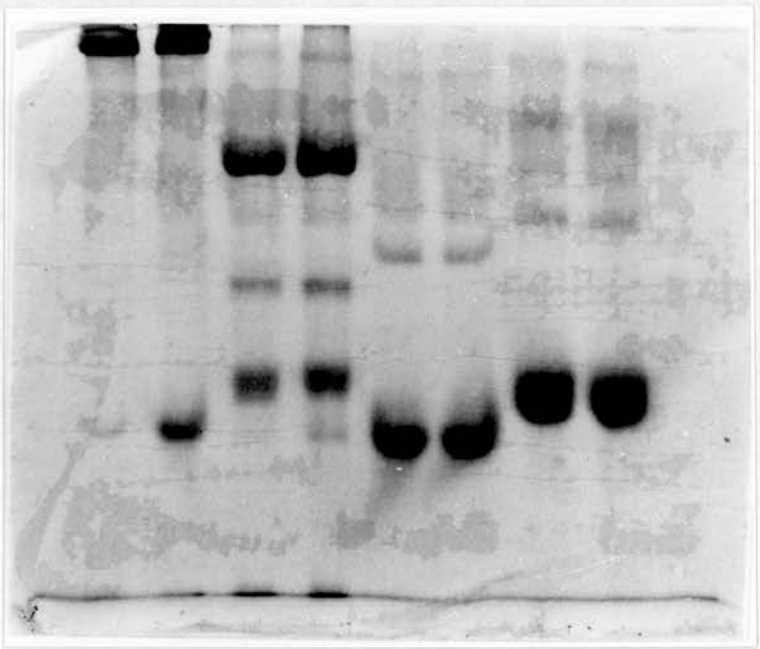
visible on the autoradiograph.

As with IFNγ and IL2, ^{125}-I TNFα was found to be bound by α2M (Fig.3.4.3). However, as has been observed elsewhere and within these studies (Barrett and Starkey 1973; see Sec.3.1.1), native α2M can undergo age-conversion during storage as seen in lanes 8 and 9 of Fig.3.4.3. The plasma preparations do show a very faint protein band of equivalent molecular weight to α2M, and there is an increased density of radioactivity on the autoradiograph at those bands (lanes 2 and 3).

3.4.3: Study of the bonds involved in α2M-cytokine interactions using SDS-PAGE

The bonds involved in these interactions were examined by use of the mild detergent sodium dodecyl sulphate (SDS) to cleave non-covalent bonds, and of mercaptoethanol to cleave disulphide bonds. It appears that IL2 did not dissociate from α2Mm even in the presence of mercaptoethanol indicating that the bonds formed had been non-disulphide covalent (Fig.3.4.4). However, if the gel is overlaid with the autoradiograph the bands of protein and radioactivity do not appear to align, even in the presence of denaturant alone which implies that the binding is non-covalent. This is contradictory to an SDS gel run in the absence of mercaptoethanol where IL2 was found to remain bound (Fig.3.4.5). Thus, the bonds between IL2 and α2Mm appear to be covalent in nature, though it is unclear if they are disulphide or not.

As with the IL2 study, the α2M-TNFα complex appeared to remain intact in the presence of SDS and mercaptoethanol indicating a covalent non-disulphide interaction (Fig.3.4.6). However, once again the protein and radioactivity bands are misaligned when the gel is overlaid with the autoradiograph. Fig.3.4.5 indicates that in the presence of SDS alone,



1

2 α2Mm

3 α2Mm, ^{125}-I IL2, SDS

4 α2Mm, ^{125}-I IL2, SDS, mercap.

5 α2Mm, ^{125}-I IL2, SDS, mercap., boiling

6 α2Mm

7 α2Mm, ^{125}-I IL2, SDS

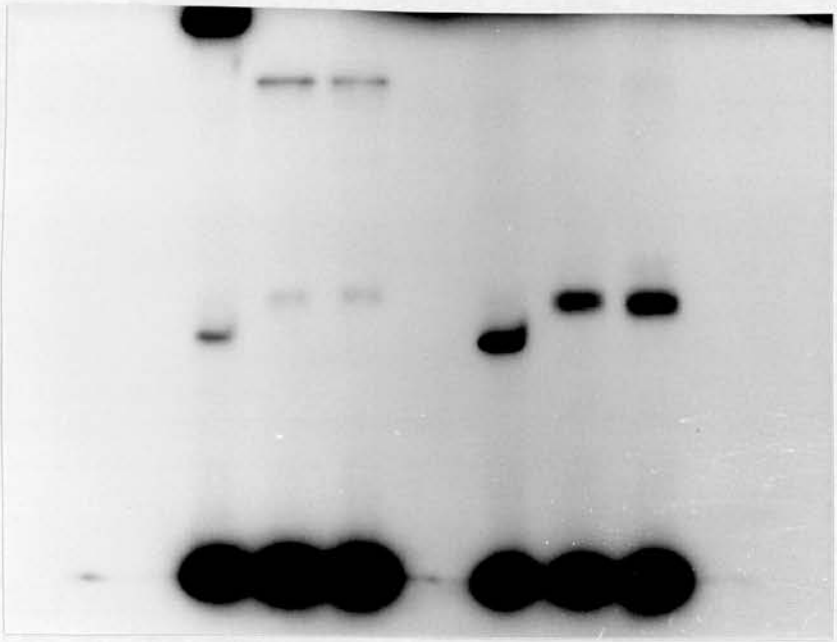
8 α2Mm, ^{125}-I IL2, SDS, mercap.

9 α2Mm, ^{125}-I IL2, SDS, mercap., boiling

10

Figure 3.4.4a: SDS-PAGE study of the interaction of α2Mm and ^{125}-I IL2

Samples of α2Mm (2mg/ml, 10μl) and ^{125}-I IL2 (~10^5cpm) were preincubated for 2 hours at 37°C. SDS and mercaptoethanol were added as appropriate, and if required samples boiled for 3 minutes prior to loading onto a 5% SDS-PAGE. The gel was run for 2 hours at 50mA, stained for proteins (Fig.3.4.4a), then laid down with an x-ray plate at -70°C for 7 days. The x-ray was then developed (Fig.3.4.4b). Note that the radioactivity does not align with the positions of the subunit bands of α2Mm in the presence of mercaptoethanol.



1

2 α2Mm

3 α2Mm, ^{125}-I IL2, SDS

4 α2Mm, ^{125}-I IL2, SDS, mercapt.

5 α2Mm, ^{125}-I IL2, SDS, mercapt., boiling

6 α2Mm

7 α2Mm, ^{125}-I IL2, SDS

8 α2Mm, ^{125}-I IL2, SDS, mercapt.

9 α2Mm, ^{125}-I IL2, SDS, mercapt., boiling

10

Figure 3.4.4b: SDS-PAGE study of the interaction of α2Mm and ^{125}-I IL2

Samples of α2Mm (2mg/ml, 10μl) and ^{125}-I IL2 (~10^5cpm) were preincubated for 2 hours at 37°C. SDS and mercaptoethanol were added as appropriate, and if required samples boiled for 3 minutes prior to loading onto a 5% SDS-PAGE. The gel was run for 2 hours at 50mA, stained for proteins (Fig.3.4.4a), then laid down with an x-ray plate at -70°C for 7 days. The x-ray was then developed (Fig.3.4.4b). Note that the radioactivity does not align with the positions of the subunit bands of α2Mm in the presence of mercaptoethanol.

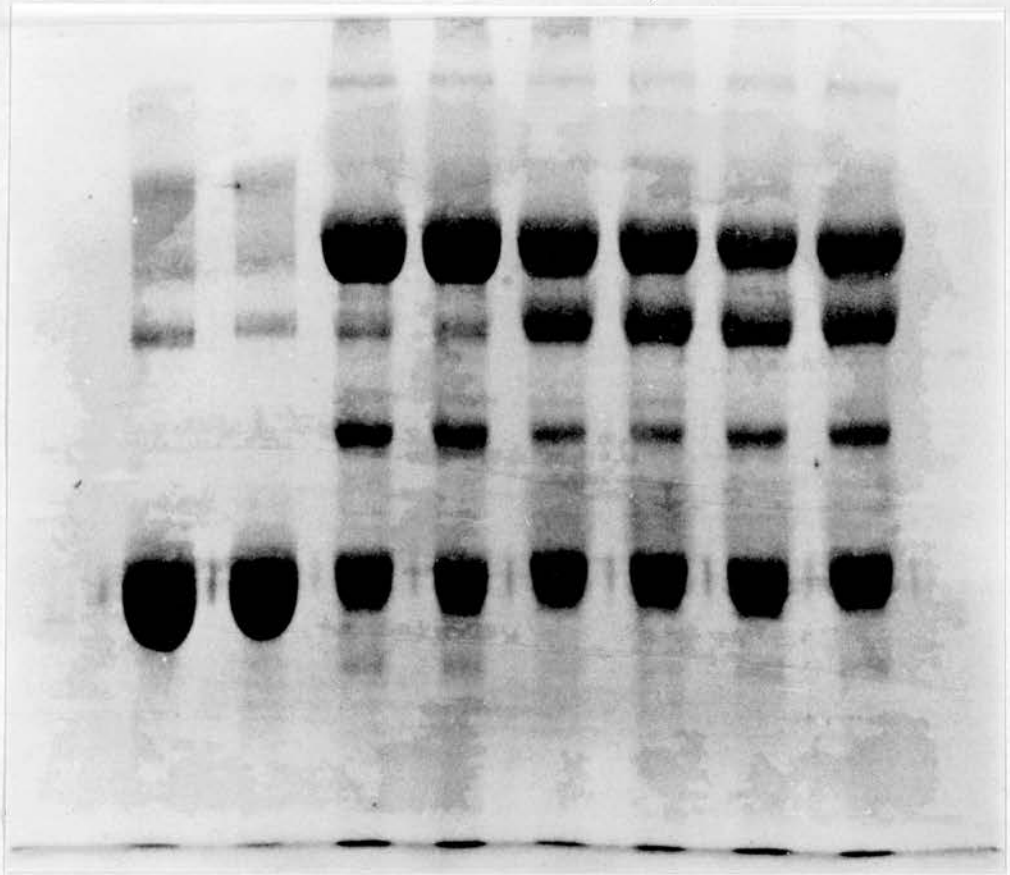


Figure 3.4.5a: SDS-PAGE study of the interaction of α2M with TNFα and with IL2

Samples of α2M or albumin (2mg/ml, 10μl) were incubated with ^{125}-I IL2 or ^{125}-I TNFα (~10^5cpm) for 2 hours at 37°C. SDS was added then samples were loaded onto a 5% SDS-PAGE gel. The gel was run for 2 hours at 50mA, then stained for proteins and dried (Fig.3.4.5a). It was laid down with an x-ray plate at -70°C for 7 days and then developed (Fig.3.4.5b). IL2 remained bound to α2Mm dimers formed in the presence of SDS, TNFα did not.

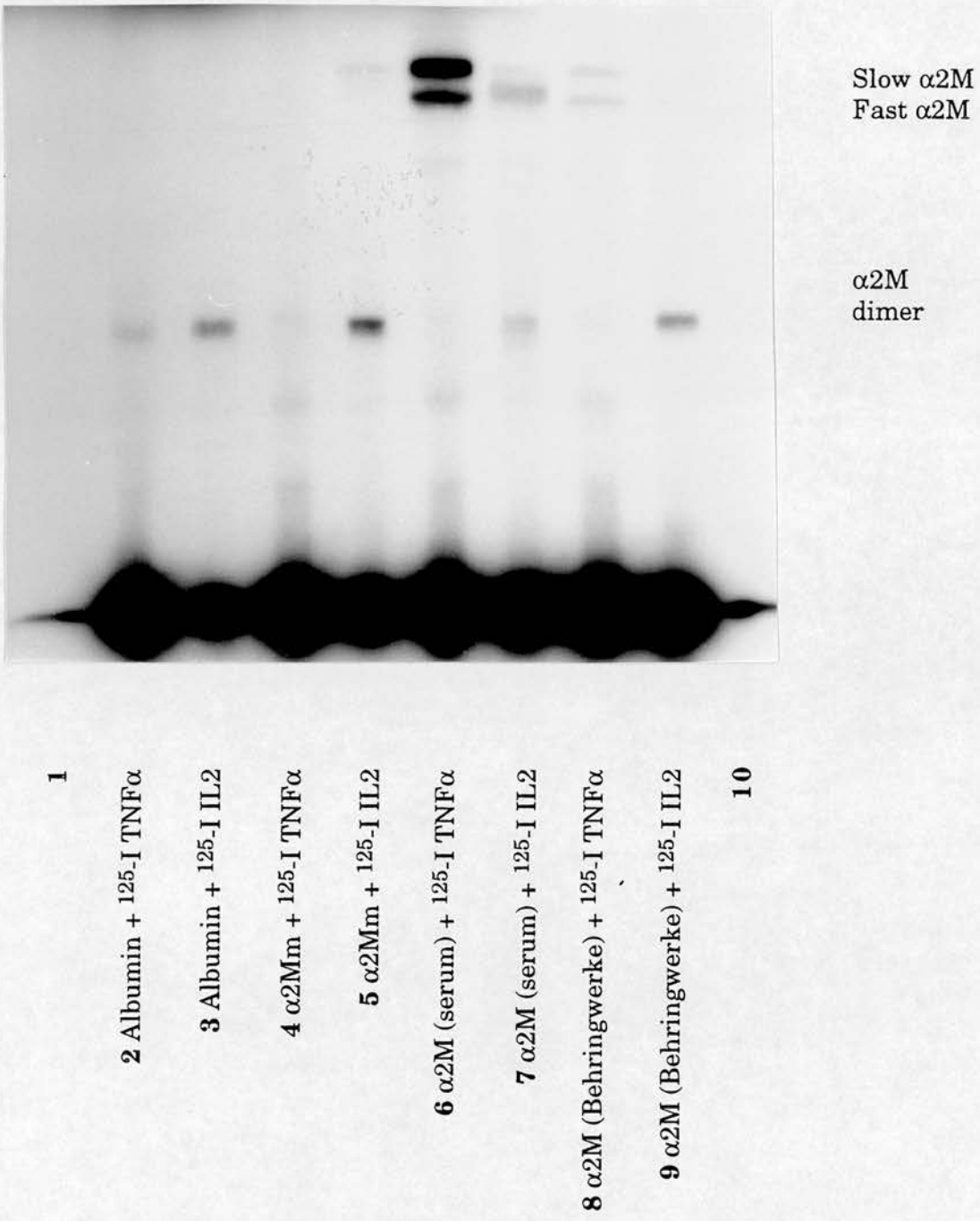
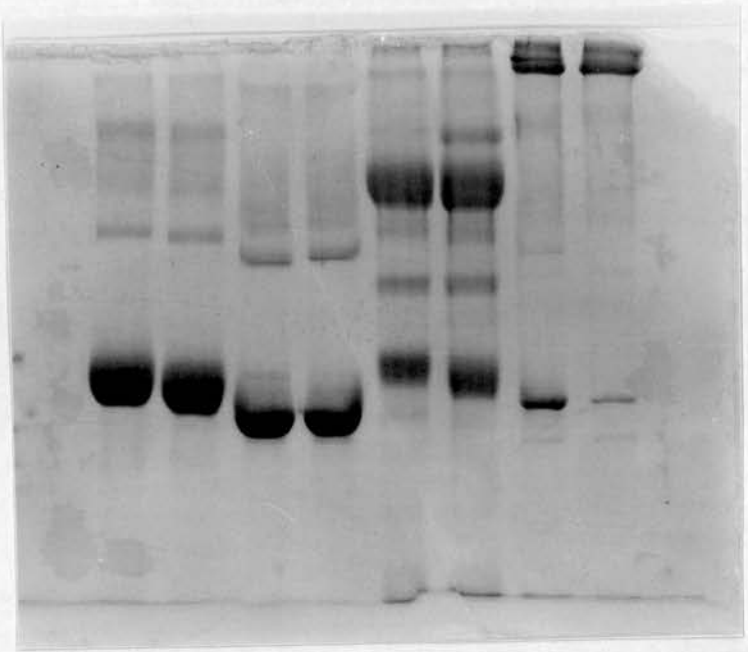


Figure 3.4.5b: SDS-PAGE study of the interaction of α2M with TNFα and with IL2

Samples of α2M or albumin (2mg/ml, 10μl) were incubated with ^{125}-I IL2 or ^{125}-I TNFα (~10^5cpm) for 2 hours at 37°C. SDS was added and samples loaded onto a 5% SDS-PAGE gel. The gel was run for 2 hours at 50mA, then stained for proteins and dried (Fig.3.4.5a). It was laid down with an x-ray plate at -70°C for 7 days and then developed (Fig.3.4.5b). IL2 remained bound to α2Mm dimers formed in the presence of SDS, TNFα did not.



1

2 α2Mm, ^{125}-I TNFα, SDS, mercapt., boiling

3 α2Mm, ^{125}-I TNFα, SDS, mercapt.

4 α2Mm, ^{125}-I TNFα, SDS

5 α2Mm, SDS

6 α2Mm, ^{125}-I TNFα, SDS, mercapt., boiling

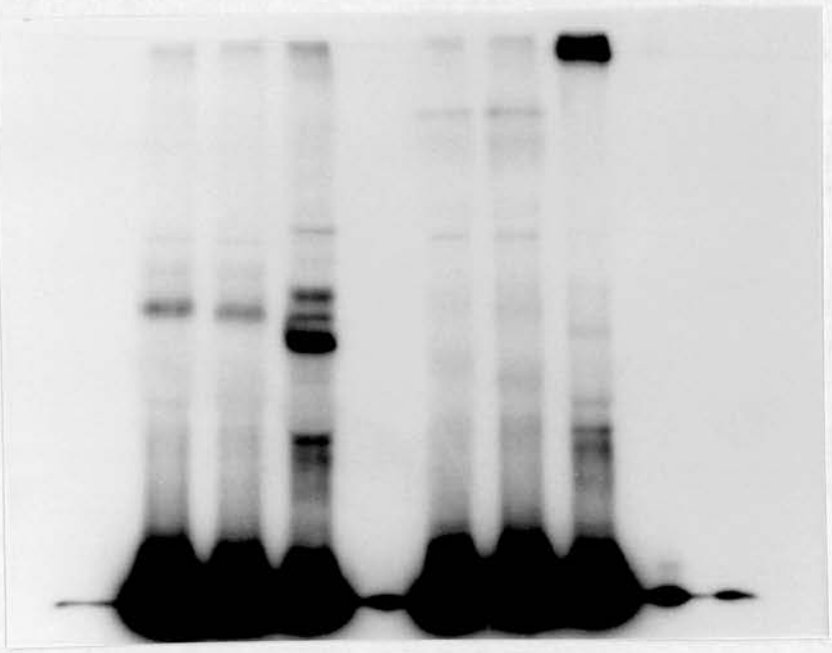
7 α2Mm, ^{125}-I TNFα, SDS, mercapt.

8 α2Mm, ^{125}-I TNFα, SDS

9 α2Mm, SDS

10

Figure 3.4.6a: SDS-PAGE study of α2Mm-TNFα binding
Samples of α2Mm (2mg/ml, 10µl) were incubated with ^{125}-I TNFα for 2 hours at 37°C. SDS and mercaptoethanol were added as appropriate, and if required samples boiled for 3 minutes. Samples were then run on a 5% SDS-PAGE gel for 2 hours at 50mA, followed by staining for proteins (Fig.3.4.6a). The gel was then dried and laid down with an x-ray for 7 days at -70°C. The x-ray was developed (Fig.3.4.6b). Note that the protein and radioactivity bands do not align between these two figures. This is discussed in the text.



1

2 α2Mm, ^{125}-I TNFα, SDS, mercapt., boiling

3 α2Mm, ^{125}-I TNFα, SDS, mercapt.

4 α2Mm, ^{125}-I TNFα, SDS

5 α2Mm, SDS

6 α2Mm, ^{125}-I TNFα, SDS, mercapt., boiling

7 α2Mm, ^{125}-I TNFα, SDS, mercapt.

8 α2Mm, ^{125}-I TNFα, SDS

9 α2Mm, SDS

10

Figure 3.4.6b: SDS-PAGE study of α2Mm-TNFα binding
Samples of α2Mm (2mg/ml, 10µl) were incubated with ^{125}-I TNFα for 2 hours at 37°C. SDS and mercaptoethanol were added as appropriate, and if required samples boiled for 3 minutes. Samples were then run on a 5% SDS-PAGE gel for 2 hours at 50mA, followed by staining for proteins (Fig.3.4.6a). The gel was then dried and laid down with an x-ray for 7 days at -70°C. The x-ray was developed (Fig.3.4.6b). Note that the protein and radioactivity bands do not align between these two figures. This is discussed in the text.

TNFα binding to α2M was only very slightly maintained in the presence of the denaturant implying that the interaction was principally through non-covalent bonds.

IFNγ remained bound to α2Mm in the presence of SDS and mercaptoethanol, from which it can be concluded that IFNγ binds to α2Mm by means of non-disulphide covalent bonds. The gel and graph are not shown because the radioactive bands were extremely faint.

It is interesting to note the mobility shift of proteins seen in the presence of mercaptoethanol as revealed in Fig.3.4.4 and 3.4.6: this may have occurred because of the protein subunits assuming a different conformation as disulphide bonds are cleaved.

3.5: Quantitative analysis of a2M-cytokine binding by radioimmunoassay

Radioimmunoassay Development

3.5.1: Iodination of α2Mm

Freshly produced α2Mm was iodinated by the Chloramine T method and used to establish optimal conditions for the radioimmunoassay (RIA). Labelled α2Mm was separated from unbound ^{125}-iodine on a Sephadex G25 column. TCA precipitation revealed that the labelled fractions 4-7 were associated with α2Mm (Fig.3.5.1). Those fractions were aliquoted, stored at -70°C and used within 1 month.

3.5.2: Blocking of nonspecific binding in assay tubes

Plastic RT30 tubes were employed throughout development and application of the assay. In order to reduce non-specific adherance of radiolabelled material to the plastic two blocking solutions were examined (Table 3.5.1). With both solutions it was apparent that less than 10% of the radiolabelled protein remained bound to the BSA-treated plastic after transfer of the reactants to fresh tubes and 2 washings of the original tubes. One wash provided efficient transfer of material. In all further experiments, tubes were blocked by treatment with 1% BSA containing 0.02% (v/v) Tween 20.

3.5.3: Optimising primary antibody dilution

A range of dilutions of the primary antibody were tested to establish the optimal titre. The optimal antibody dilution for subsequent use in the RIA was that which bound 50% of available radiolabelled ligand. It can be seen that this occured in the 1/100 dilution range (Fig.3.5.2).

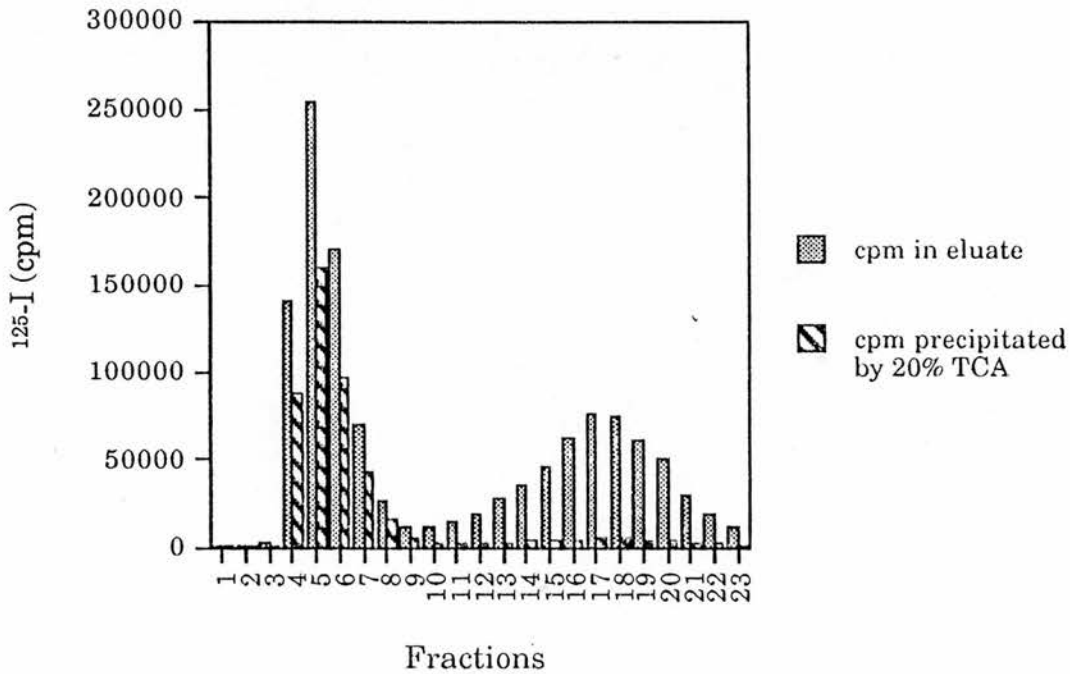


Figure 3.5.1: Protein and radioactivity profile from the iodination of α2Mm

Freshly prepared α2Mm was iodinated by the Chloramine T system and separated from free ^{125}-iodine on a G25 Sephadex column. Fractions were assayed for total and TCA precipitable radiolabel.

Conditions	^{125}-I α2Mm	cpm after 1 wash x100 / original cpm	cpm after 2 washes x 100 / original cpm
5% BSA + 0.02% Tween 20	Batch 1	95.96%+/-4.5	93.75%+/-2.75
	Batch 2	89.06%+/-3.5	92.84%+/-1.55
1% BSA + 0.02% Tween 20	Batch 1	95.74%+/-2.5	95.58%+/-2.78
	Batch 2	88.06%+/-5.8	92.48%+/-1.13

Table 3.5.1: Examination of effective blocking media for use in the coating of RT30 tubes in the RIA. RT30 tubes were incubated overnight at 37°C in the presence of two blocking solutions as indicated. The tubes were then thoroughly washed with PBS pH7.2 followed by washing with distilled water. Two different batches of freshly prepared ^{125}-I α2Mm (approximately 30,000cpm) were applied to the tubes which were then incubated overnight at 4°C. All fluid was transferred to clean RT30 tubes and the original tubes washed out twice with PBS pH7.2, the cpm present in the new tubes being counted after each transfer.

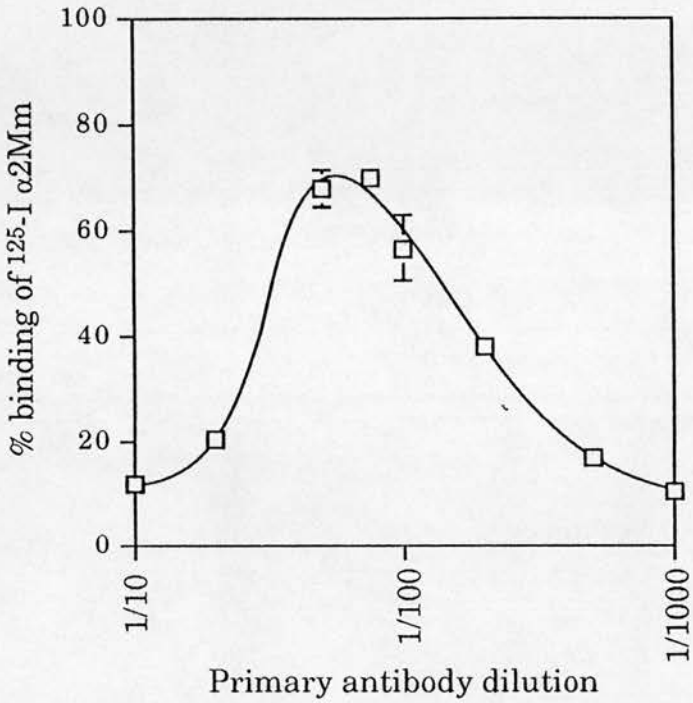


Figure 3.5.2: Study of the optimal dilution of primary antibody for use in the radioimmunoassay.
Primary antibody (rabbit anti-human α2M antibody) at a range of dilutions (1/10-1/1000) was incubated overnight with α2Mm (10μg) and ^{125}-I α2Mm (100ng), following which the components were incubated with secondary antibody (donkey anti-rabbit IgG bound to Sephacryl). Bound radiolabelled α2Mm was then separated on a sucrose gradient. Optimal sensitivity is obtained with an antibody dilution that binds 50% of available radiolabelled protein. Here it occured in the region of a 1/100 dilution. All conditions were examined in triplicate, and the study conducted three times with the same result. Values are mean +/- standard deviation; error bars are included in all cases.

3.5.4: Protein amount

A concentration series of α2Mm was examined to ensure that the conditions of the RIA were optimal. Thus, the assay was set up as described in Fig.3.5.3. The optimal amount of protein for the assay conditions should occur at the steepest part of the resultant curve: Fig.3.5.3 indicates that 10μg of cold α2Mm fulfilled this criterion.

3.5.5: Checking for non-specific binding

The various components of the RIA were examined for their non-specific influence upon the assay (Fig.3.5.4). There appeared to be very little non-specific binding due to the secondary antibody binding directly with the radiolabelled ligand. Furthermore, a non-related primary antibody did not interact. However, it should be noted that when myosin was employed as the control protein replacing unlabelled α2Mm, the binding of ^{125}-I α2Mm observed was 17.7%+/-4.6. The low binding may have arisen due to the antibodies crossreacting with the myosin and thus blocking the binding of the radiolabelled protein. Alternatively, it may have been caused by the presence of contaminants in the myosin preparation.

Use of the radioimmunoassay

3.5.6: Binding studies

Radioimmunoassays were carried out as previously described (see Sec.2.4.2). In brief, this involved incubating a range of concentrations of labelled cytokine with a fixed amount of α2M. Binding analysis revealed dissociation constants (K_d) for the interaction of α2Mm with ^{125}-I IL2 and with ^{125}-I TNFα of 2.1-2.5x10^{-6}M and 0.96-1.36x10^{-6}M respectively. These figures were derived by plotting the quantities of radiolabelled cytokine

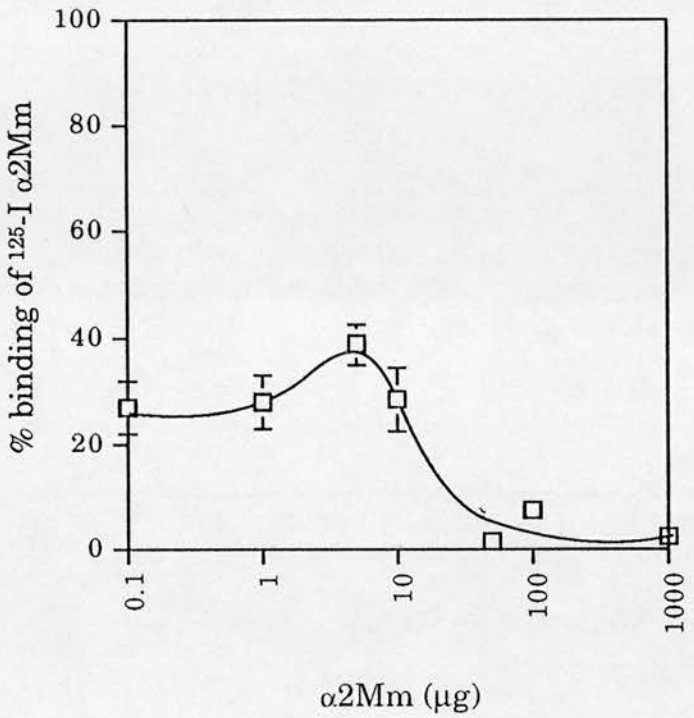


Figure 3.5.3: Protein titration curve for the development of the radioimmunoassay.
α2Mm (0.1-1000μg) was incubated with primary antibody at 1/100 dilution (rabbit anti-human α2M) overnight at 4°C in the presence of ^{125}-I α2Mm (100μg), followed by a 1 hour incubation at room temperature with secondary antibody (donkey anti-rabbit IgG bound to Sephacryl). Bound and free α2Mm were separated on a sucrose gradient, and the cpm of ^{125}-I α2Mm associated with the Sephacryl pellet counted. The presence of 10μg of the protein resulted in the binding of ~28.3% of the available ^{125}-I α2Mm this occuring on the steepest part of the curve; thus subsequent assays were carried out with this amount of protein (ie 10μg) present. All conditions were tested in triplicate, and the assay carried out twice. Values are mean +/- the standard deviation; error bars are present in all cases.

^{125}-I α2Mm	Protein	Primary Antibody	Secondary Antibody
yes	α2Mm	~α2M	yes
yes	α2Mm	NRS	yes
yes	myosin	~α2M	yes
yes	myosin	NRS	yes
yes	α2Mm	none	yes
yes	myosin	none	yes
yes	α2Mm	~α2M	none
yes	α2Mm	NRS	none
yes	myosin	~α2M	none
yes	myosin	NRS	none
yes	α2Mm	none	none
yes	myosin	none	none
yes	none	none	none
yes	none	~α2M	none
yes	none	NRS	none
yes	none	none	yes

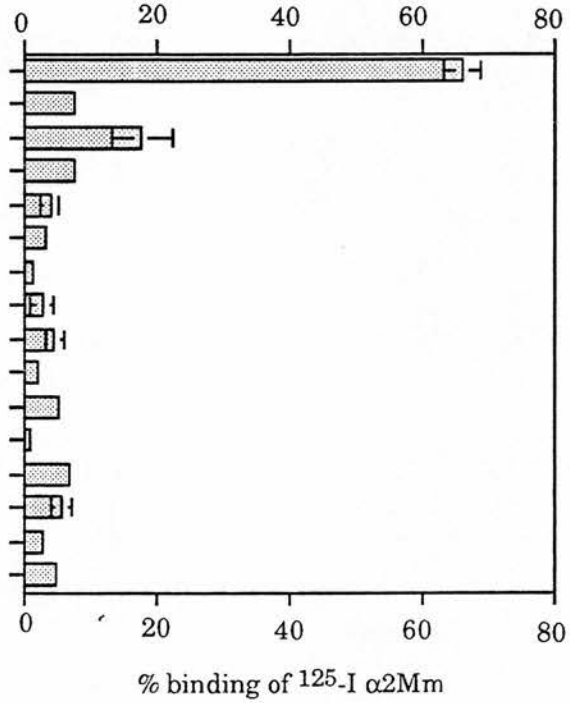


Figure 3.5.4: Testing of the components of the radioimmunoassay system for non-specific binding.
The radioimmunoassay was performed as normal but various components were missing or were replaced as noted. It can be observed that appreciable binding only occured when α2Mm, anti-α2Mm and secondary antibody were all included in the assay.

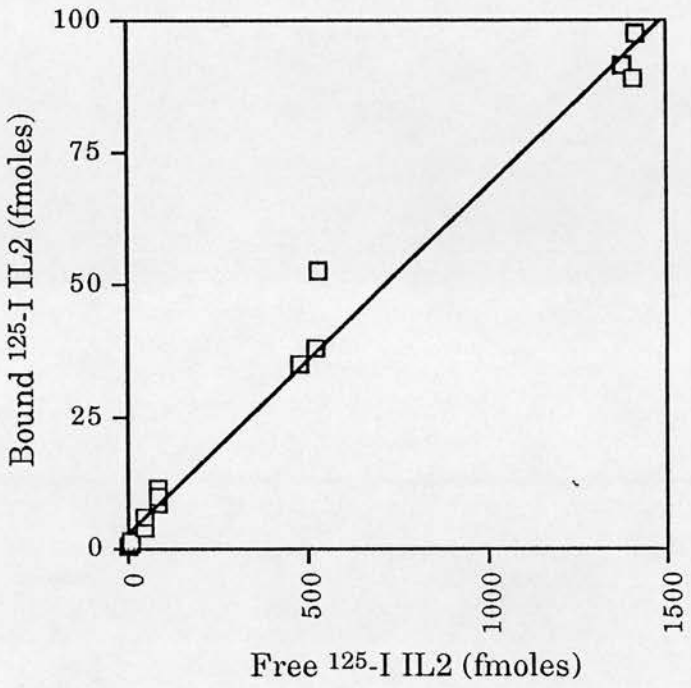


Figure 3.5.5: Radioimmunoassay for the binding of ^{125}-I IL2 with α2Mm

α2Mm (10μg) and ^{125}-I IL2 (variable amounts) were incubated for 2 hours at 37°C in pre-blocked RT30 tubes (1% BSA + 0.02% Tween 20) followed by an overnight incubation at 4°C with rabbit anti-human antibody. Subsequently, donkey anti-rabbit IgG bound to Sephacryl S1000 (100μl) was added and the tubes shaken for 1 hour at room temperture (1000+/-100rpm). Material bound to the Sephacryl was then separated from the unbound by sedimentation through a sucrose gradient. The presence of radioactivity in the pellet formed was assessed and from this the quantities of bound and free ^{125}-I IL2. A strong positive correlation (r^2=0.984) was detected for the binding as studied; a K_d of 2.1μM was calculated for the interaction as presented above.

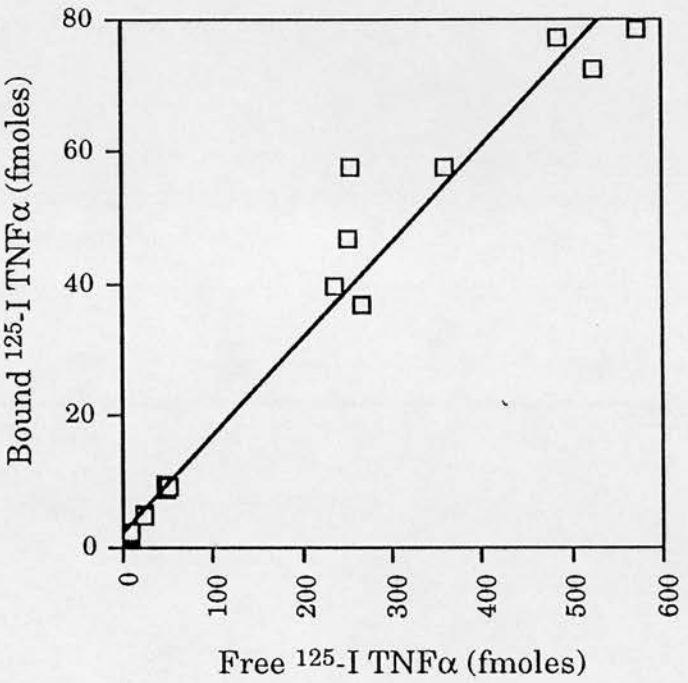


Figure 3.5.6: Radioimmunoassay for the binding of ^{125}-I TNFα with α2Mm.
α2Mm (10μg) and ^{125}-I TNFα (various amounts) were incubated for 2 hours at 37°C prior to an overnight incubation at 4°C with rabbit anti-human α2M antibody (1/100 dilution, 100μl Sigma). Donkey anti-rabbit IgG bound to Sephacryl (100μl) was applied followed by shaking for 1 hour (1000rpm+/-100). Material bound to the Sephacryl was separated from the unbound using a sucrose gradient and the radioactivity in the pellet counted to determine the amount of bound and free ^{125}-I TNFα. A strong positive correlation was found in the amounts of cytokine binding to the amount available (eg, r^2=0.964 for the case illustrated). The K_d value for this example was 0.96μM.

bound against the amount that remained free (Figs.3.5.5 and 3.5.6). The calculations are described in Appendix C2. It is apparent that ^{125}-I TNFα binds α2Mm with a K_d approximately half that of ^{125}-I IL2 (ie $1x10^{-6}$M compared to $2x10^{-6}$M) indicating that the interaction with TNFα is the stronger of the two.

In the ^{125}-I TNFα study, the influence of the primary, secondary, or a non-related antibody on the binding levels observed were also investigated. It appeared that they had little effect on binding levels (Fig.3.5.7). For example, the bound/free value for these interactions was less than 0.1 whereas under normal assay conditions with α2Mm, rabbit anti-human α2M antibody and secondary antibody being present the value was greater than 0.2. However, in the absence of α2Mm the amount of ^{125}-I TNFα carried through to the final count was large.

3.5.7: Specificity studies

In competitive binding studies, the presence of unlabelled IL2 did not significantly affect the interaction of the labelled cytokine with α2Mm until the unlabelled ligand was present at a 1000 fold excess (Fig.3.5.8). This implies that the labelled ligand was binding to α2Mm by a different mechanism to the cold IL2. In contrast, cold TNFα was more effective at blocking the binding of labelled TNFα with α2Mm. Thus, in one instance, a significant decrease was seen with a 1:1 ratio of hot:cold cytokine, but in general the binding was blocked by at least 50% when a 1:100 ratio of hot:cold cytokine was achieved (Fig.3.5.9).

^{125}-I TNFα	Protein	Primary Antibody	Secondary Antibody
yes	α2Mm	~α2M	yes
yes	none	~α2M	yes
yes	none	none	none
yes	α2Mm	NRS	yes
yes	α2Mm	~α2Mm	none
yes	α2Mm	none	yes

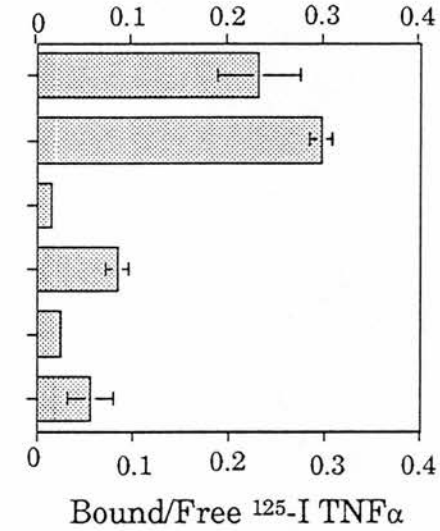


Figure 3.5.7: Controls for the examination of the binding of ^{125}-I TNFα with α2Mm by RIA.
The radioimmunoasay was carried out fundamentally as normal. However, some components were omitted as indicated in order to assess their contribution to the detected binding levels. It can be seen that the absence of primary or secondary antibody resulted in low binding levels when compared to the case of having the "normal" range of components. However, in the case of no α2Mm being present higher binding was seen.

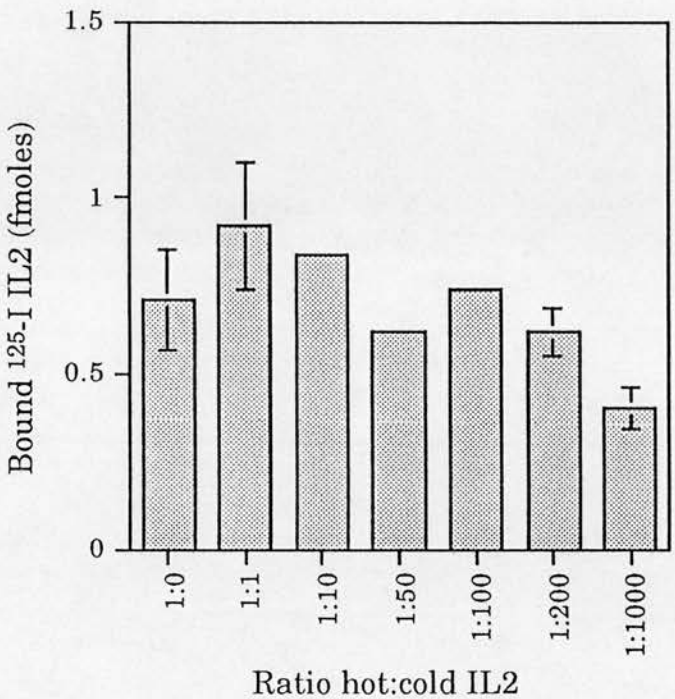


Figure 3.5.8: Competitive binding studies of IL2-α2Mm interactions

Radiolabelled (100ng) and unlabelled (range of amounts) of IL2 were mixed together in blocked RT30 tubes and incubated with α2Mm (10μg) for 2 hours at 37°C prior to the application of 100μl of rabbit anti-human α2Mm (1/100 dilution, Sigma). Following an overnight incubation at 4°C, 100μl of donkey anti-rabbit IgG bound to Sephacryl S1000 was added and the tubes incubated for 1 hour at room temperature with shaking (1000rpm+/-100). Material bound by the Sephacryl was separated from the free on a sucrose gradient and the radioactivity in the pellet assessed. The amount of ^{125}-I present was read as the quantity of labelled IL2 bound to the α2Mm. Values are mean +/- standard deviation.

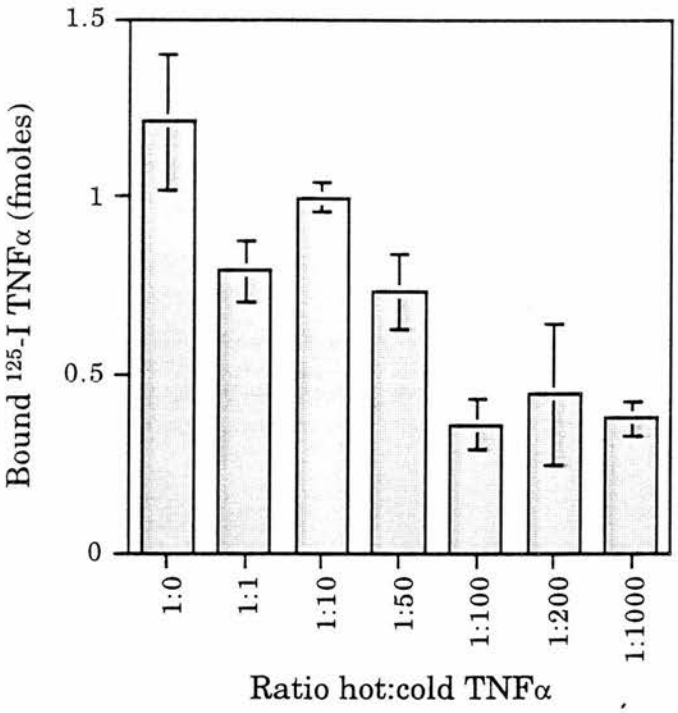


Figure 3.5.9: A study of the specificity of the binding of labelled TNFα with α2Mm

^{125}-I TNFα was mixed with the unlabelled cytokine (range of amounts) prior to incubation with α2Mm (10μg) for 2 hours at 37°C. Rabbit anti-human α2Mm (100μl, 1/100 dilution, Sigma) was applied followed by an overnight incubation at 4°C. Then, donkey anti-rabbit IgG bound to Sephacryl S-1000 was applied and the tubes were shaken (1 hour, room temperature). Material bound to Sephacryl was then separated from the free on a sucrose gradient and the cpm in the resultant pellet counted.

3.6: Quantitative studies of the binding of cytokines by α2M using zinc affinity chromatography on Sepharose

3.6.1: Adaptation of the zinc chromatography method

Affinity chromatography using zinc chelated Sepharose is the classical method employed for the separation of α2M from blood (Kurecki et al 1979; see Sec.2.1.1). In order to establish a quantitative technique for assaying cytokine-α2M interactions, the essential methodology of zinc based separation was exploited: the column system was adapted to enable analysis of 20-40 samples in 1.5ml Eppendorf tubes.

3.6.2: Assay development

To determine if this technique would be feasible for the investigation of α2M-cytokine binding, an initial study with IL2 was carried out: α2Mm (0.276M), BSA (2.98M), or PBS were incubated as normal with IL2 (~$1.4x10^5$cpm; see Sec.2.2.1). The reactants were then applied to prepared zinc chelated Sepharose for 1 hour, after which the Sepharose was washed three times in the presence of phosphate buffer pH6.5 and then three times in acetate buffer pH4.5. The supernatant recovered with each wash was decanted and counted for the presence of radiolabel. More radiolabelled IL2 was found in the acetate wash in the presence of α2Mm than with BSA or PBS (Fig.3.6.1). 24.4% of total recovered counts co-eluted with α2Mm, 3.3% with BSA, and 2.4% with PBS. In addition, it is apparent that two washes are necessary and sufficient for the removal of material from the gel: eg, with the phosphate buffer, one wash eluted 13.6% of the cytokine, a second wash increased the quantity of eluted material by 5% to 18.6%. The reading taken after a third wash with the phosphate buffer indicates a slightly lower recovery (17.2%) which would probably be due to fluctuations in the reading from the LKB gamma

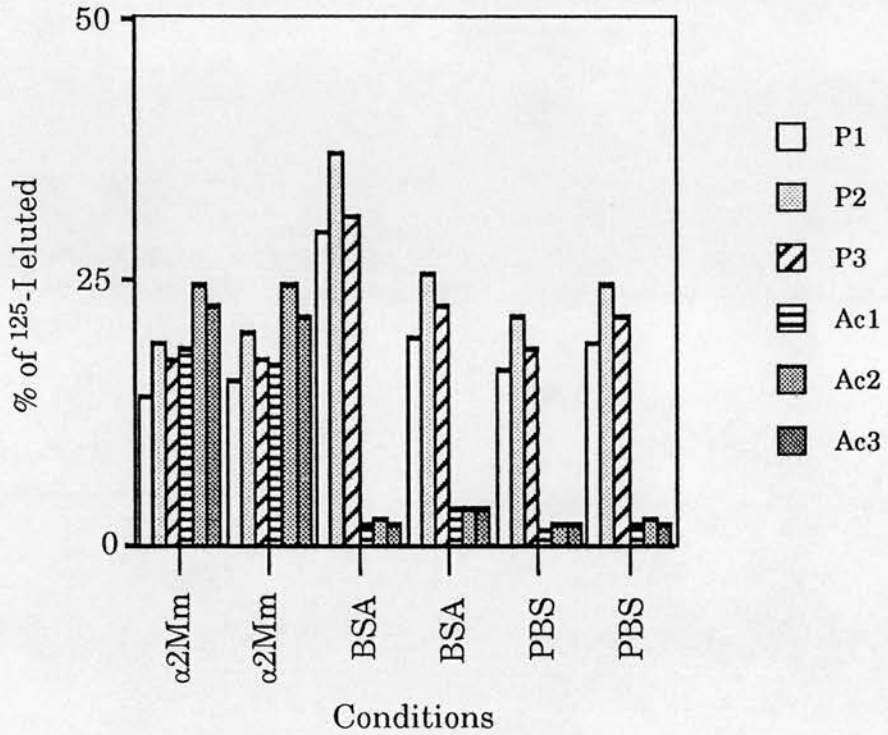


Figure 3.6.1: Establishing a protocol for quantitative analysis using zinc affinity chromatography
α2Mm (0.276 nmoles), BSA (2.98 nmoles), or an equivalent volume of PBS were incubated with ^{125}-I IL2 (138072cpm) for 2 hours at 37°C. Samples were then applied to prepared zinc chelated Sepharose with mixing for 1 hour in 1.5ml Eppendorf tubes. Tubes were then spun (200g, 10mins), the supernatant decanted and phosphate buffer pH6.5 applied with mixing for 1 hour. The tubes were centrifuged as before, the supernatant decanted and counted (P1). Washing was repeated twice, the supernatants being counted together (P2 and P3). This elution proceedure was carried out with acetate buffer pH4.5 to elute α2Mm ("Ac" fractions). It is apparent that only in the presence of α2Mm does IL2 elute in the acetate buffer, and that 2 washes with each buffer are necessary and sufficient for elution of material.

counter. However, this pattern of maximal recovery after two washes was seen in all instances and therefore subsequent work was conducted with two washes of the matrix with the phosphate and acetate buffers.

3.6.3: Binding studies

To assess the quantitative binding of α2Mm with IL2 and with TNFα, incubations were carried out as normal with 276pmoles of α2Mm and increasing amounts of cytokine. Figs.3.6.2 and 3.6.3 are representative of the results from these studies. As had been done with the data from the RIA, the gradient of the best fit line was calculated for each set of results and, knowing the concentration of α2Mm available, the K_d value was calculated (see Appendix C). For IL2 the K_d was found to be $3x10^{-5}$M, and for TNFα $2x10^{-5}$M.

3.6.4: Specificity studies

It was also of interest to investigate if cold cytokine could block the binding of ^{125}I IL2 and ^{125}I TNFα by α2Mm. There was a decrease in binding seen with IL2 and in one of the TNFα studies (Figs.3.6.4 and 3.6.5). It is evident that blocking was not possible with either cytokine.

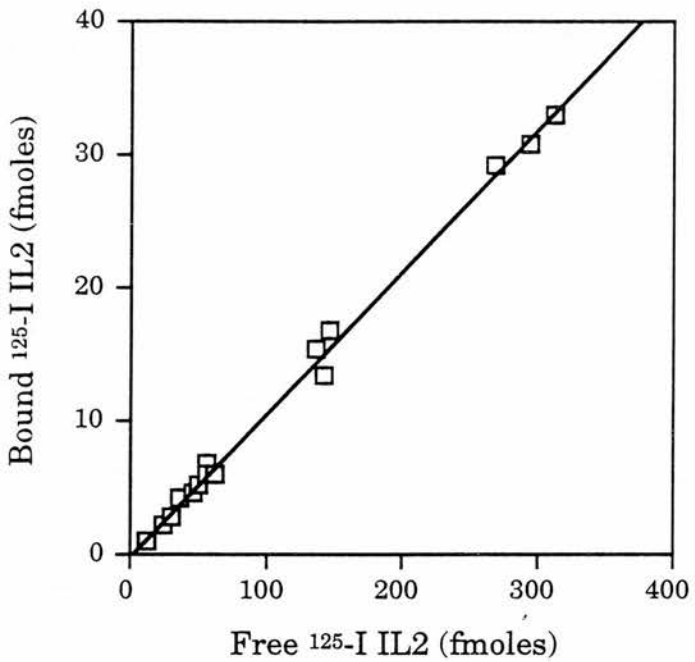


Figure 3.6.2: The interaction of ^{125}-I IL2 with α2Mm assessed by zinc affinity chromatography
α2Mm (276 pmoles) was incubated with ^{125}-I IL2 (range13.2-344.9fmoles) for 2 hours at 37°C and then applied to prepared zinc chelated Sepharose in 1.5ml Eppendorf tubes with mixing for 1 hour at room temperture. Tubes were spun (200g, 10 minutes) and the supernatant discarded. The matrix in each tube was washed twice with phosphate buffer pH6.5 and then twice with acetate buffer pH4.5, each time spinning as before. The supernatant from each wash was retained and the cpm counted. From the above plot a K_d value of $3x10^{-5}$M was calculated for the interaction of ^{125}-I IL2 with α2Mm, with an r^2 value of 0.996.

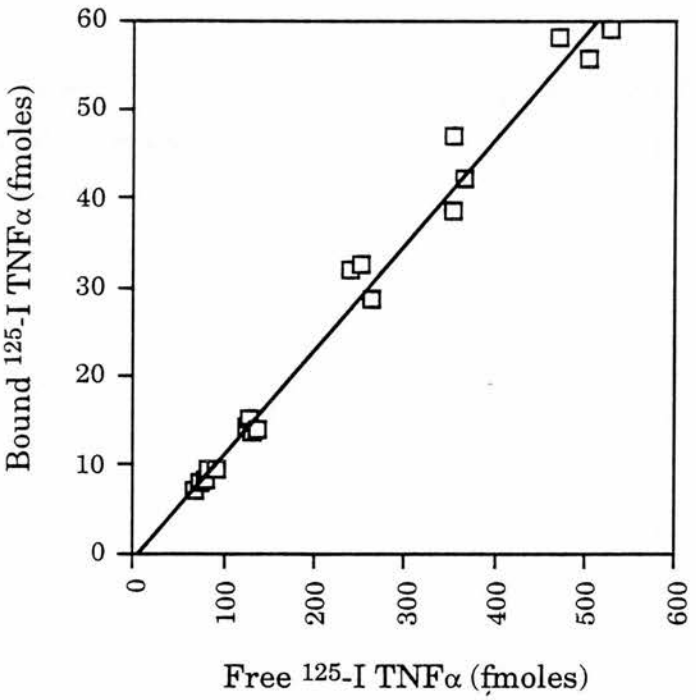


Figure 3.6.3: The interaction of ^{125}-I TNFα with α2Mm as examined by zinc affinity chromatography
α2Mm (276 pmoles) and TNFα (range76.1-585.1fmoles) were incubated for 2 hours at 37°C and then applied to prepared zinc chelated Sepharose with mixing for 1 hour in 1.5ml Eppendorf tubes. Tubes were centrifuged (200g, 10mins) and the supernatant discarded. The matrix was washed twice in phosphate buffer pH6.5 and twice in acetate buffer pH4.5, centrifuging as before and retaining the supernatants for counting radioactivity present. From this study a K_d value of $2x10^{-5}$M was calculated for the interaction of α2Mm with ^{125}-I TNFα, r^2 was 0.983.

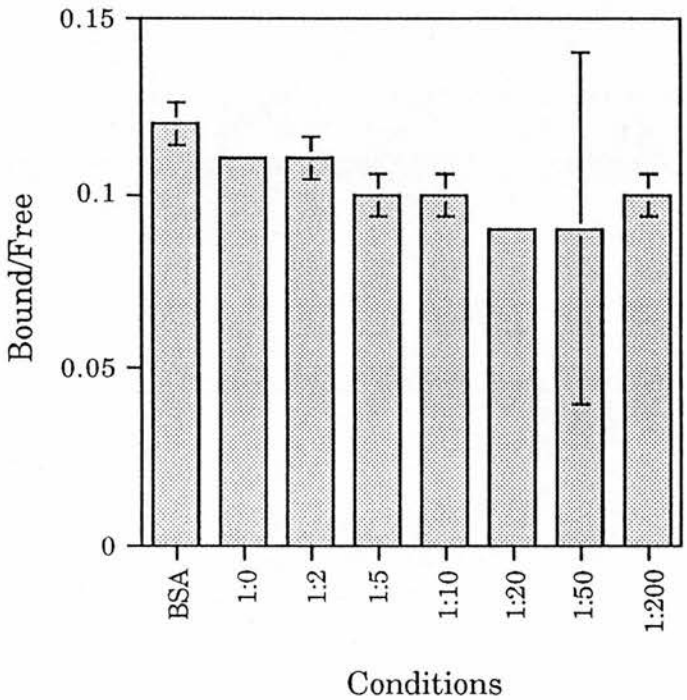


Figure 3.6.4: Competition of ^{125}-I IL2 and unlabelled IL2 for binding by α2Mm
α2Mm (267 pmoles) was incubated with ^{125}-I IL2 (52.8 fmoles) and increasing amounts of unlabelled IL2 (ratio to ^{125}-I IL2 as indicated) for 2 hours at 37°C. Free and bound cytokine were separated on zinc chelated Sepharose in 1.5ml Eppendorf tubes. It is apparent that the binding of ^{125}-I IL2 cannot be blocked with unlabelled IL2 even at 200 fold excess.

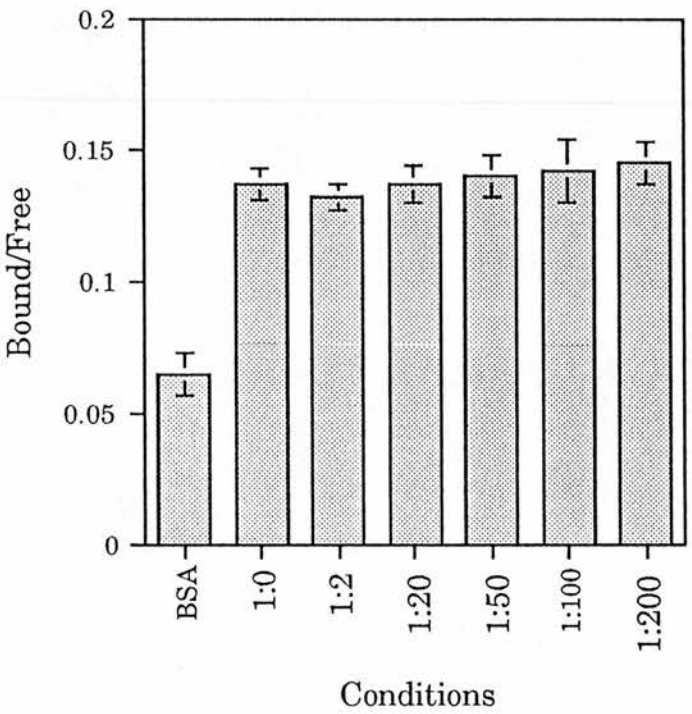


Figure 3.6.5: Competition of ^{125}-I TNFα and unlabelled TNFα for binding with α2Mm

α2Mm (276 pmoles), ^{125}-I TNFα (70.4 fmoles) and unlabelled TNFα (ratio as shown) were incubated for 2 hours at 37°C. Bound and free cytokine was then separated on zinc chelated Sepharose in Eppendorf tubes. It is apparent that the presence of cold cytokine did not block the binding of hot cytokine to α2Mm. BSA was included as a control protein to indicate non-specific binding.

3.7: The effect of α2M on cytokine immunoassays

3.7.1: Strategy for cytokine immunoassay studies

The interaction of α2M with a cytokine in biological fluid or in tissue culture supernatant could alter the detection of the cytokine in immunoassay systems. In order to investigate this, α2M was preincubated with the cytokine standards in various commercial immunoassay kits which were then used in the immunoassay according to the manufacturers instructions. The cytokine standards were incubated alone and assayed as controls.

Preincubation of cytokine standards with α2M had three possible effects on the cytokine standard curves, as reported below.

3.7.2: Effects of α2M and albumin on immunoassays

(i) α2Mm can specifically inhibit certain assays. For example, in the Amersham IL2 kit, the presence of α2Mm caused a down shift in the standard curve, the decrease was of the order of 26-40% below that observed with cytokine standards alone (Fig.3.7.1a). A similar shift in the presence of α2Mm was also seen with the Amersham IL4, Amersham TNFα, and Medgenix TNFα systems (Figs.3.7.1b, c, and d). In the Medgenix TNFα system the reduction of the slope was particularly pronounced when α2Mm (5mg/ml) was present (Fig.3.7.1d). The presence of albumin caused less of a downshift in each of the standard curves.

(ii) A general protein effect was observed in a number of the kits examined. Albumin was tested in conjunction with α2M and α2Mm in all kits. In the Medgenix IL1β, IL2, IL6 and IL8 kits the standard curve was shifted in the presence of all the proteins tested (Figs.3.7.2 a-d). For the first three kits the shift was upward, and for the Medgenix IL8 kit it was downwards. The size of the shift appears to be independent of the protein tested. A protein

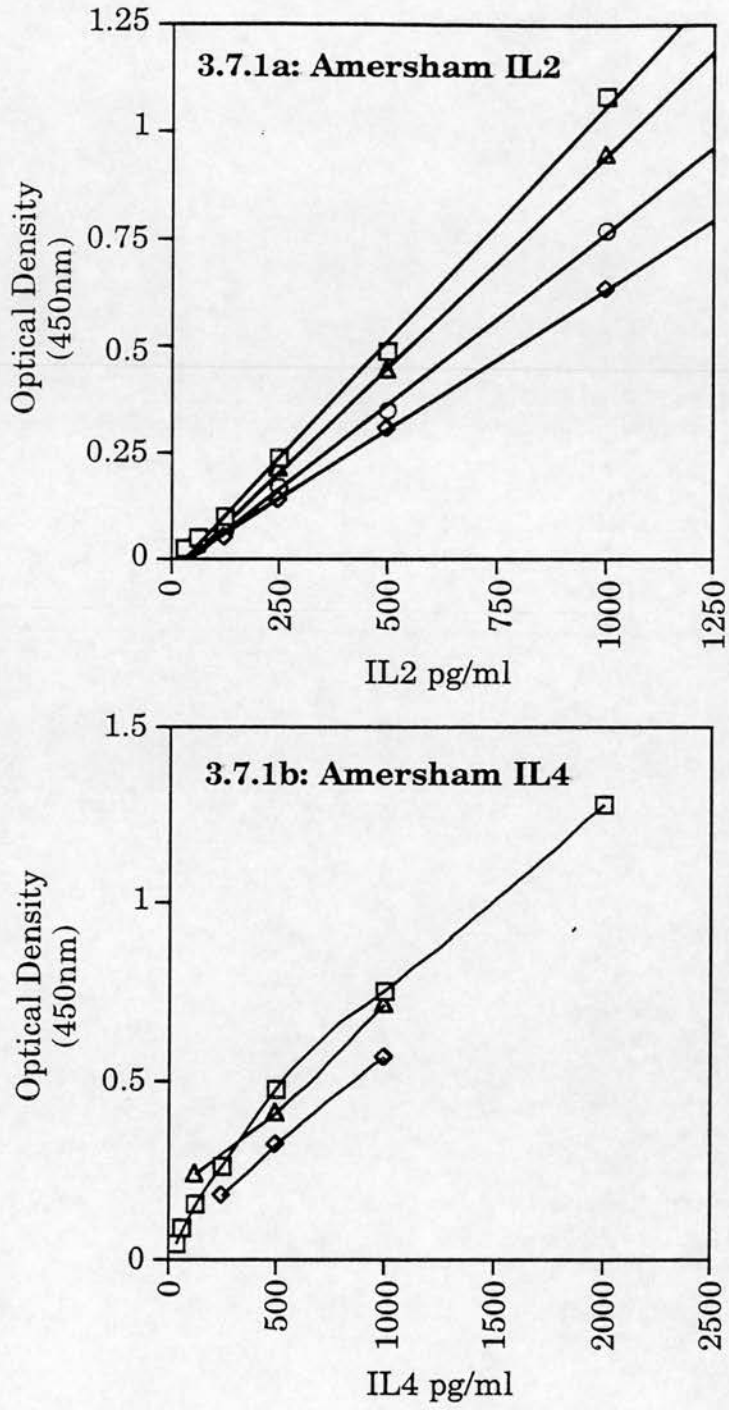


Figure 3.7.1 a and b: Cytokine immunoassays specifically affected by α2Mm

α2Mm (◇), α2M (○), and albumin (△), all at 5mg/ml, were incubated for 2 hours at 37°C with prepared cytokine immunoassay standards prior to application of the standard to the assay. In addition the standard was applied alone to act as a control (□). In a number of assays examined, it was found that α2Mm induced a more pronounced downward shift in the standard curve compared to α2M and albumin.

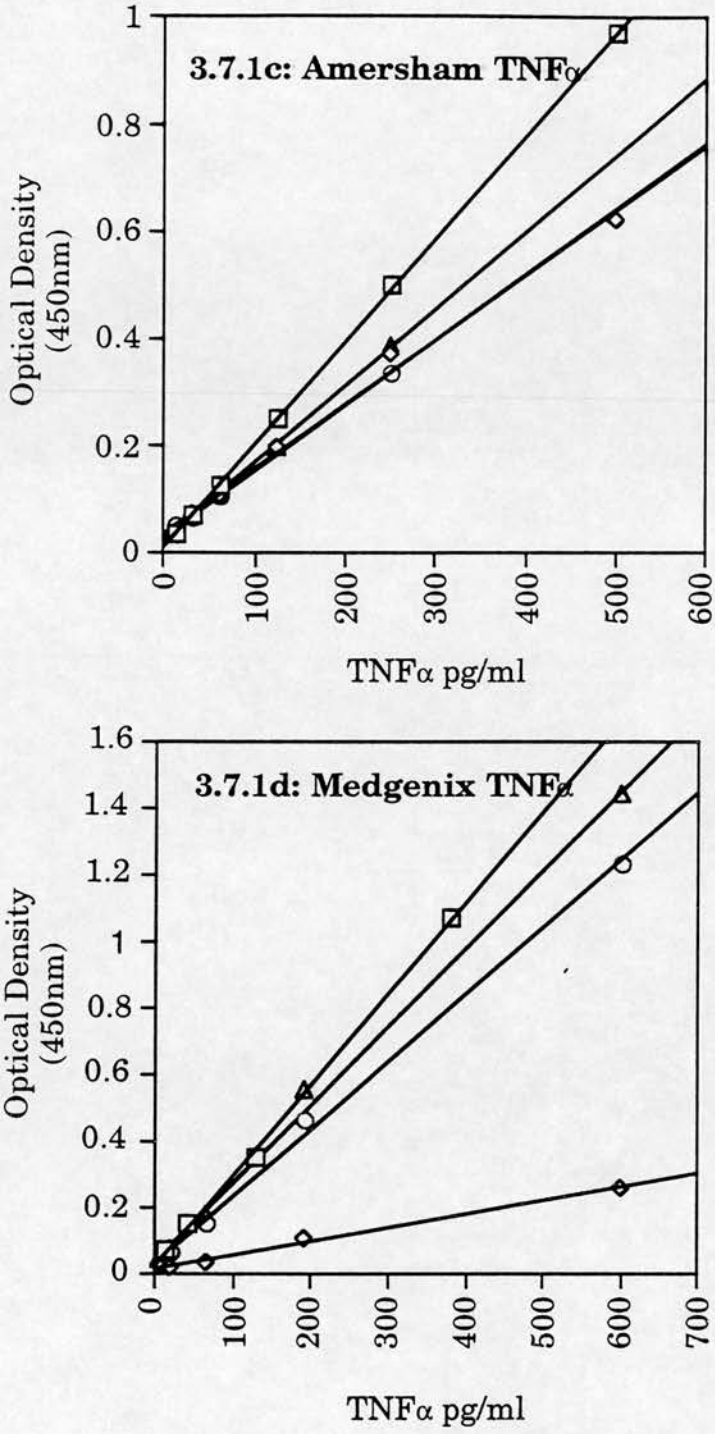


Figure 3.7.1 c and d: Cytokine immunoassays specifically affected by α2Mm

α2Mm (◇), α2M (○), and albumin (△), all at 5mg/ml, were incubated for 2 hours at 37°C with prepared cytokine immunoassay standards prior to application of the standard to the assay. In addition the standard was applied alone to act as a control (□). In a number of assays examined, it was found that α2Mm induced a more pronounced downward shift in the standard curve compared to α2M and albumin.

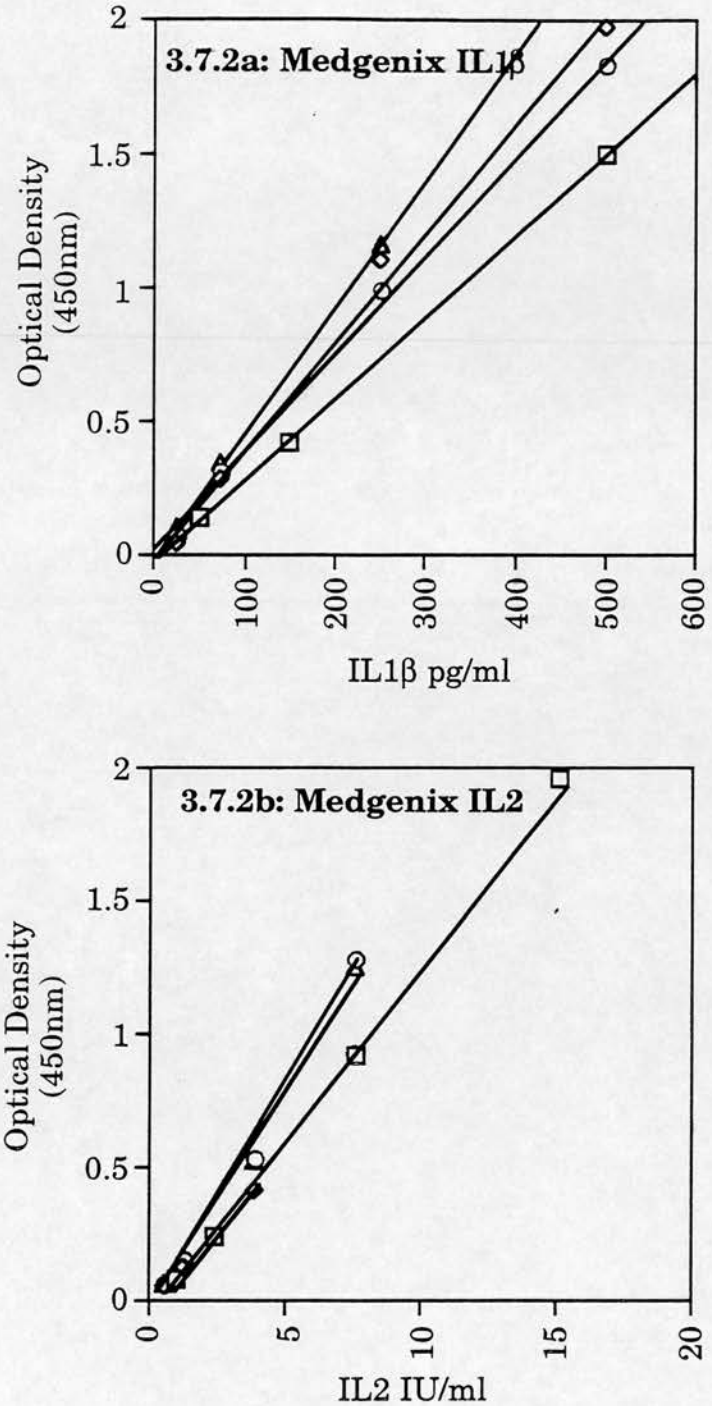
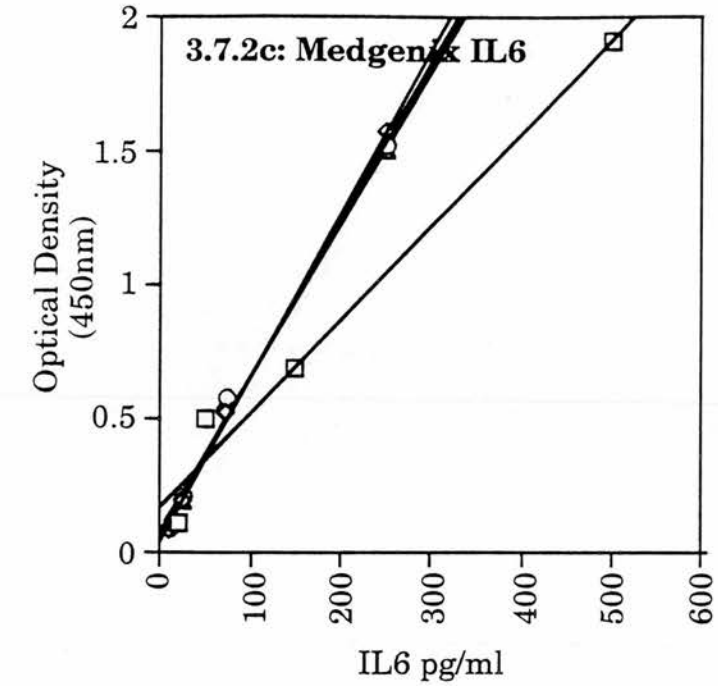


Figure 3.7.2 a and b: Commercial cytokine immunoassays affected by the presence of protein.
α2Mm (◇), α2M (○) and albumin (△), all at 5mg/ml, were preincubated for 2 hours at 37°C with immunoassay kit standards prior to applying them to the immunoassay. The standards alone were also applied to act as controls (□). In a number of kits examined it was found that there was a general protein effect upon the standard curve as illustrated.



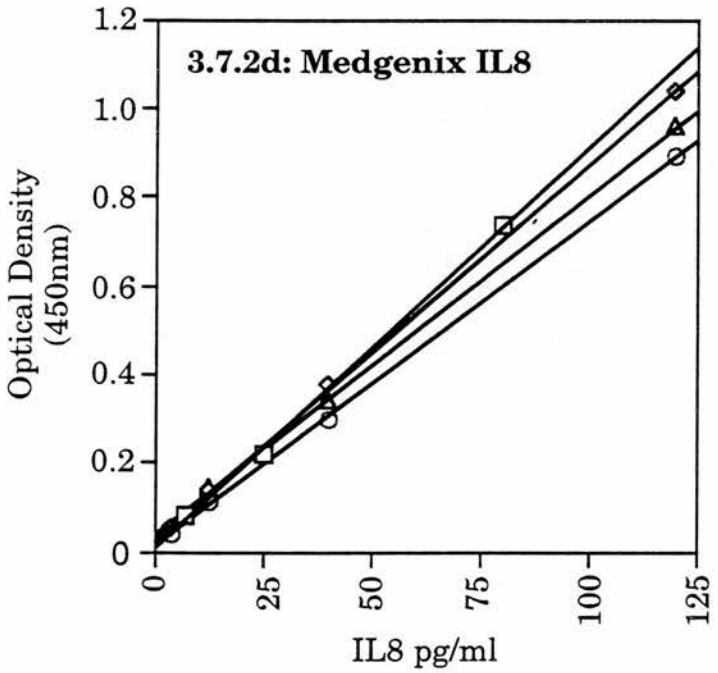


Figure 3.7.2 c and d: Commercial cytokine immunoassays affected by the presence of protein

α2Mm (◇), α2M (○) and albumin (△), all at 5mg/ml, were preincubated for 2 hours at 37°C with immunoassay kit standards prior to applying them to the immunoassay. The standards alone were also applied to act as controls (□). In a number of kits examined it was found that there was a general protein effect upon the standard curve as illustrated.

effect was also seen when the Endogen kit for IFNγ was examined (not shown): the standard curve was moved downwards, the effect being slightly greater in the presence of albumin.

(iii) No effect on the standard curve was seen in some cases. None of the proteins tested significantly altered the standard curves in the Amersham kits for IL6 and for IL8, and in the Medgenix kits for IL4 and for IFNγ (Figs.3.7.3 a- d). This result was also found with the Boehringer-Mannheim kit for TNFα (not shown).

3.7.3: Effect of α2M concentration on immunoassays

In addition to testing the effect of proteins on the standard curves, the effect of a concentration range of α2M was examined. Figs.3.7.4 a-d illustrates four examples of assays showing differing responses according to the concentration of α2M incubated with the cytokine standard. In the Amersham IL4, Amersham TNFα, and Medgenix TNFα kits there was a decrease in the shift of the standard curve seen in the presence of α2Mm at 1mg/ml as compared to at 5mg/ml (Figs.3.7.4a-c). These three systems had shown specific responses to α2Mm (Fig.3.7.1b, c and d). In the Medgenix IL2 system, it was found that the curve shifted upwards more in the presence of α2Mm at 1mg/ml than at 5mg/ml. Intermediate concentrations of protein showed a concentration dependent response.

3.7.4: Standard deviation in assay results

It is apparent from Table 3.7.1 that the standard deviation for most assays was low. In some cases, there was a shift from the overall trend for that assay at low concentrations of cytokine causing a greater standard deviation for the results as a whole.

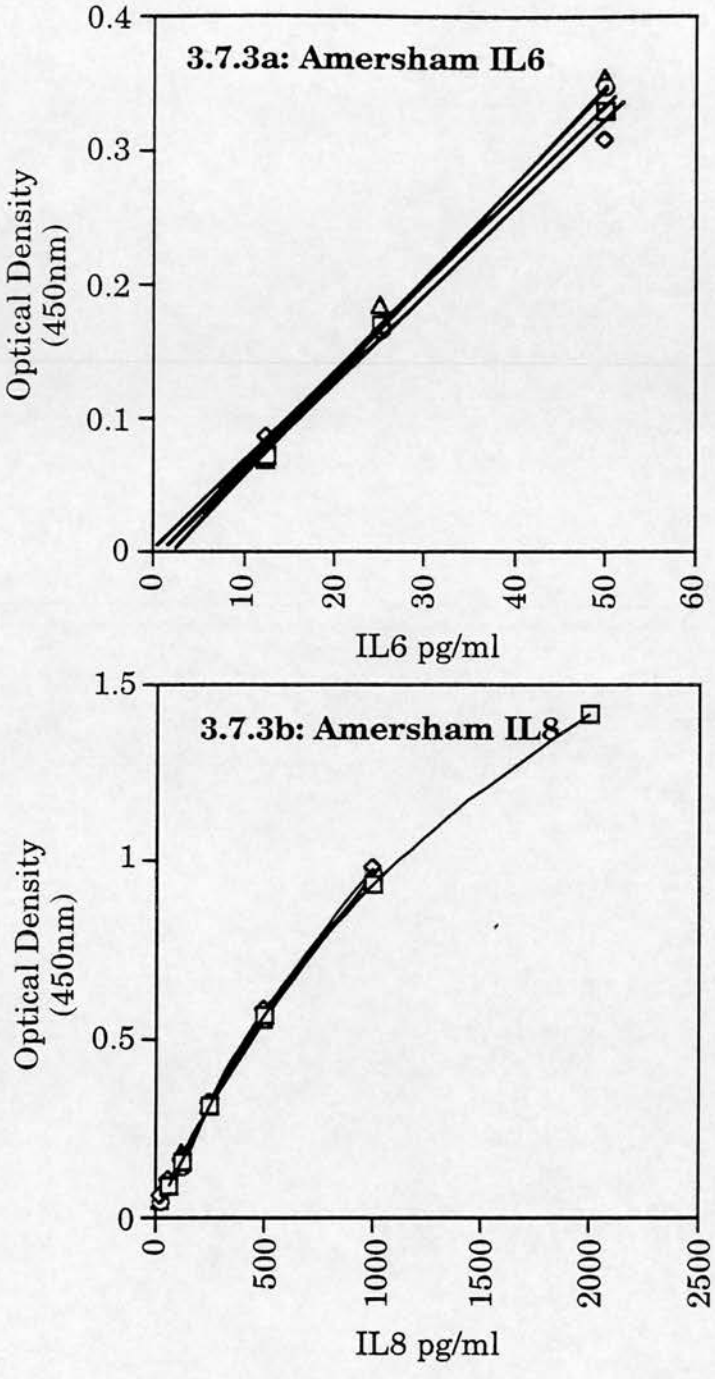


Figure 3.7.3a and b: Commercial immunoassays not affected by the presence of protein

α2Mm (◇), α2M (○), and albumin (△) (all 5mg/ml) were incubated for 2 hours at 37°C with immunoassay kit standards prior to applying them to the immunoassay. The standards alone were also applied to act as controls (□). In a number of the kits examined it was found that none of the proteins had an effect upon the slope of the standard curve.

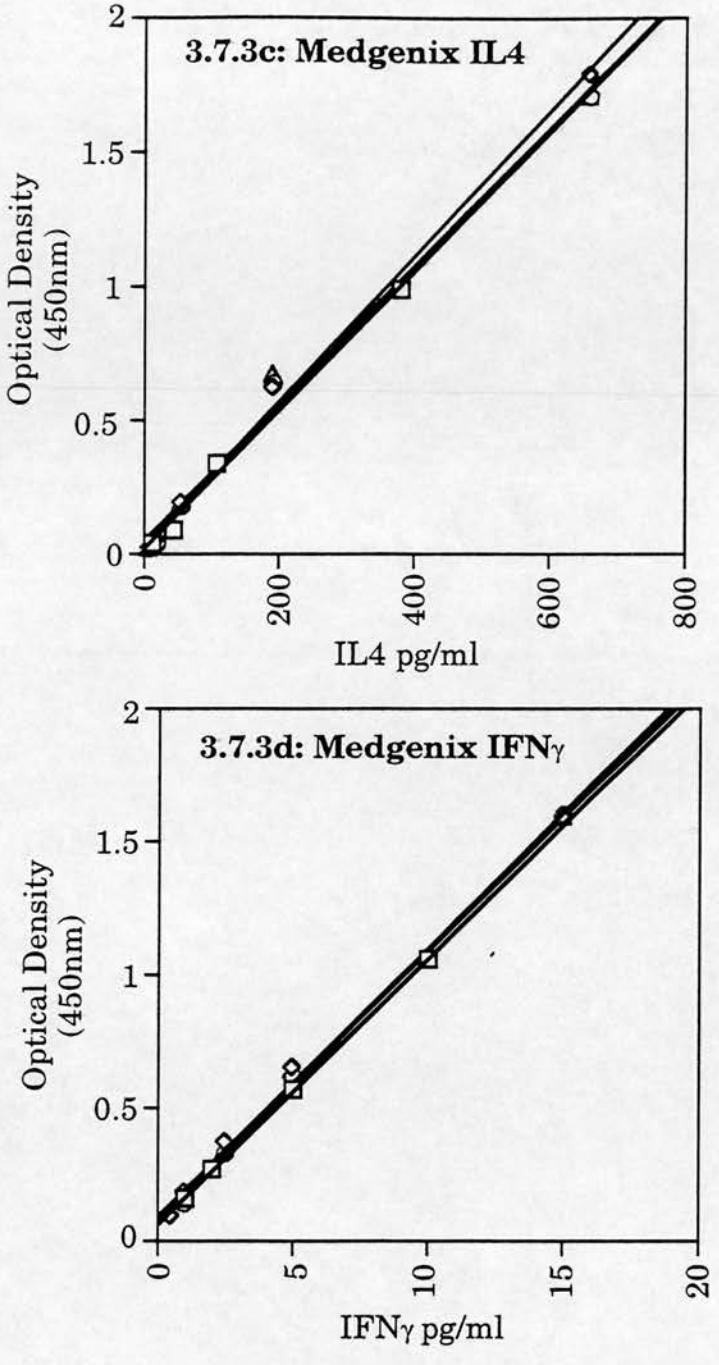


Figure 3.7.3c and d: Commercial immunoassays not affected by the presence of protein

α2Mm (◇), α2M (○), and albumin (△) (all 5mg/ml) were incubated for 2 hours at 37°C with immunoassay kit standards prior to applying them to the immunoassay. The standards alone were also applied to act as controls (□). In a number of the kits examined it was found that none of the proteins had an effect upon the slope of the standard curve.

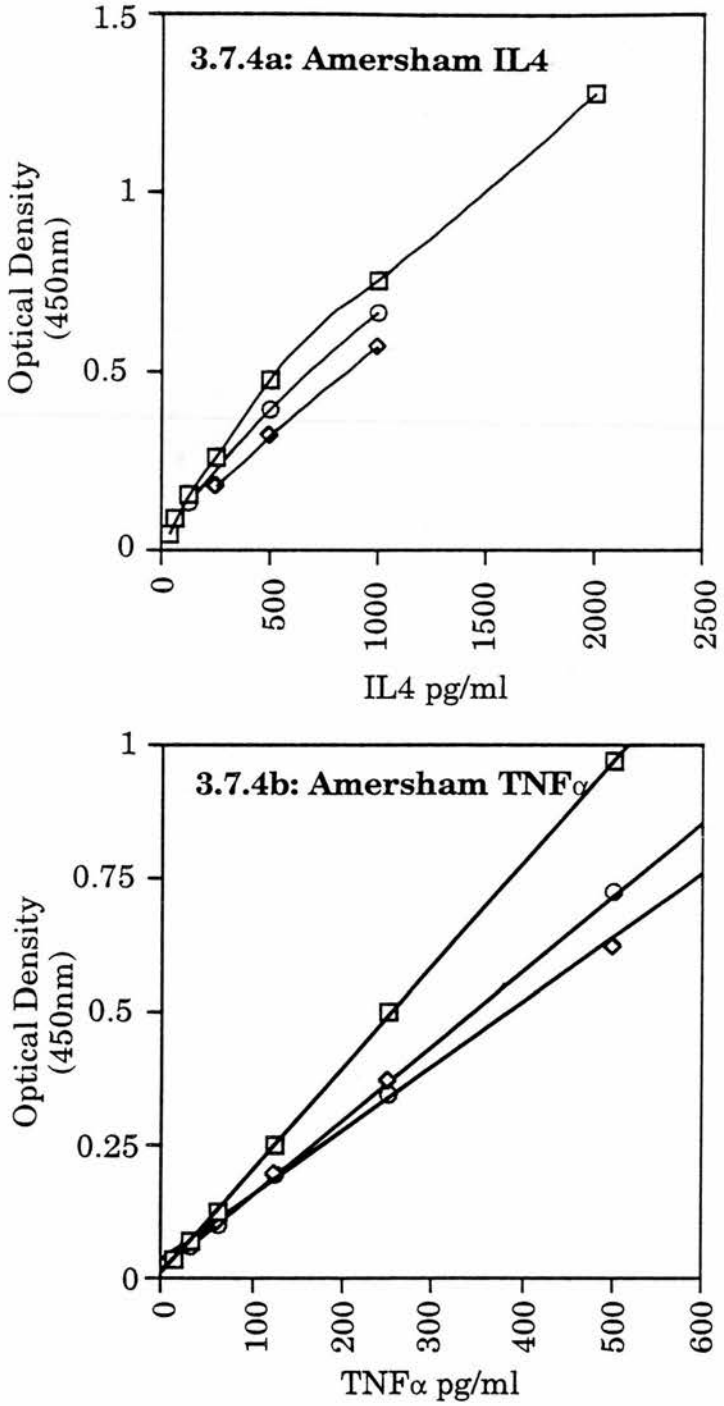
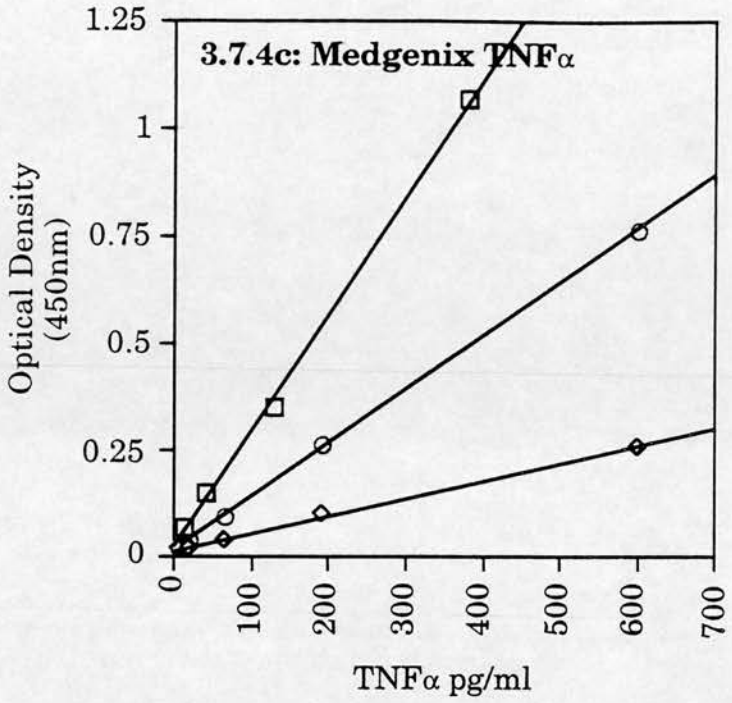


Figure 3.7.4 a and b: The affect of increasing α2Mm concentration on immunoassay standard curves

α2Mm at 5mg/ml (◇) and at 1mg/ml (○) were preincubated with cytokine immunoassay standards for 2 hours at 37°C prior to applying them to the assay. Cytokine standards alone were used as controls (□). Note that the inhibitory effects increase with increasing α2Mm concentration in 3.7.4a and b, and in 4c overleaf.



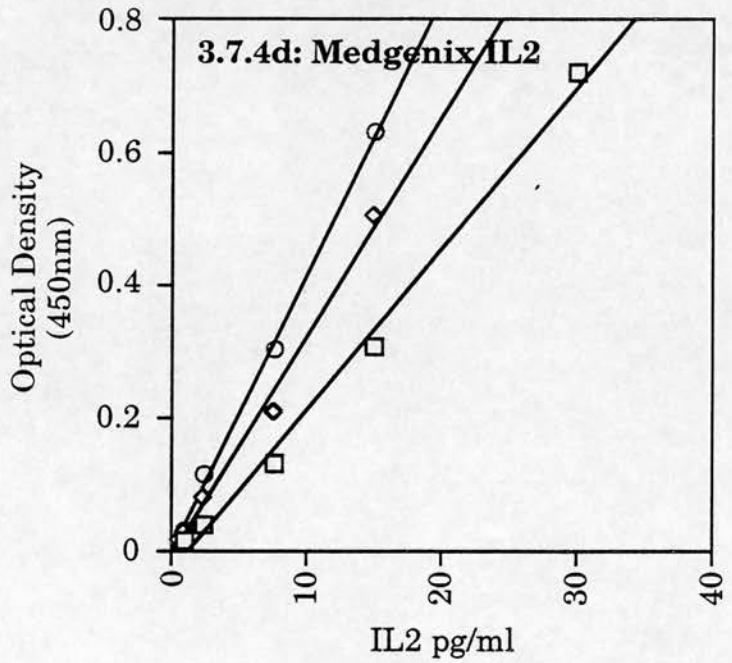


Figure 3.7.4 c and d: The affect of increasing α2Mm concentration on immunoassay standard curves

α2Mm at 5mg/ml (◇) and at 1mg/ml (○) were preincubated with cytokine immunoassay standards for 2 hours at 37°C prior to applying them to the assay. Cytokine standards alone were used as controls (□). Note that the inhibitory effects increase with increasing α2Mm concentration in Figure 3.7.4c, and in Figures 3.7.4a and b overleaf.

Kit	No.repeats of assay	Effect	Effect of Protein on OD Reading	
			α2Mm 5mg/ml	Albumin 5mg/ml
Amersham IL2	10	α2Mm	-35.6 (+/-16.7)	-14.25 (+/-6.0)
Amersham IL4	2	α2Mm	-29.5 (+/-6.4)	-9 (+/-2.8)
Amersham TNFα	3	α2Mm	-18 (+/-12.3)	-5 (+/-21.3)
Medgenix TNFα	1	α2Mm	-74.4 (+/-16.6)	-3.6 (+/-7.3)
Medgenix IL2	3	Protein	+12.4 (+/-14.6)	+41.25 (+/-11.1)
Medgenix IL6	1	Protein	+39.5 (+/-13.5)	+37.7 (+/-19.1)
Medgenix IL1β	2	Protein	+38 (+/-8.2)	+49.25 (+/-12.9)
Medgenix IL8	1	Protein	+18.75 (+/-29.4)	+0.3 (+/-18.1)
Endogen IFNγ	1	Protein	-19.8 (+/-15.1)	-17.75 (+/-10.7)
Amersham IL6	4	None	+0.5 (+/-17.7)	-51 (+/-8.2)
Amersham IL8	1	None	+18.5 (+/-19.6)	-3.4 (+/-5.4)
Medgenix IL4	1	None	+8.8 (+/-26.6)	+8.8 (+/-26.6)
Medgenix IFNγ	2	None	+10.6 (+/-6.0)	+5.4 (+/-6.2)
Bǫehringer-Mannheim TNFα	1	None	-6.9 (+/-8.3)	-5 (+/-14.2)

Table 3.7.1: Study of the effect of α2Mm and albumin on the standard curve in commercial cytokine immunoassays

α2Mm and albumin (5mg/ml) were incubated with cytokine immunoassay standards for 2 hours at 37°C prior to their application to the immunoassay. It was apparent that the cytokine standard curve was in some instances shifted due to the presence of the α2Mm, or that the shift was due to a general protein effect. The number of times an assay was repeated is also indicated. Calculation of the mean deviations is indicated in Appendix C3.

3.7.5: Reproducibility of results

Due to financial constraints, not all assays could be tested several times to ensure that on an interassay basis reproducible results were obtained. However, for those that were, the data obtained in independent tests with different batches of protein were consistent. Table 3.7.2 illustrates this for the Amersham IL2 assay: it is apparent that α2Mm consistently inhibited the standard curve by 26-40%.

IL2 standard (pg/ml)	Percentage inhibition of standard curve			
	Kit 1	Kit 2	Kit 3	Kit 4
62	30.0	40.1	37.0	42.6
125	32.0	38.7	38.7	30.9
250	34.5	40.9	42.2	34.8
500	32.2	32.2	35.2	31.2
1000	30.5	37.8	34.7	26.4

Table 3.7.2: Percentage inhibition observed in Amersham IL2 assays
The percentage inhibition observed when α2Mm was applied to a range of cytokine standards in 4 identical Amersham IL2 ELISA kits. α2Mm (5mg/ml) was incubated with standards from the kits for 2 hours at 37°C prior to application to the kit system. It is evident that the decrease in the standard curve was consistent at various concentrations of IL2 and between kits.

3.8: Investigations into the bioactivity of IL2 and of TNFα when bound to α2Mm

3.8.1: Study of the bioactivity of α2Mm-IL2

Complexes of α2Mm-IL2 were prepared by incubation as normal followed by separation of free and bound IL2 on a G200 Sephadex column (see Sec.2.2.5). Eluted fractions were tested in a lymphoproliferative assay for IL2 bioactivity. These studies revealed that IL2 maintained its bioactivity when bound to α2Mm (Fig.3.8.1). The high molecular weight peak fractions containing α2Mm-IL2 complexes (Fig.3.8.1a) induced higher lymphoproliferation than did addition of comparable fractions obtained by separation of IL2 alone (Fig.3.8.1b). It should be noted that α2Mm alone showed no bioactivity above background levels.

The principal fractions investigated in the bioassay were also analysed for their IL2 content using an Amersham IL2 immunoassay. Comparison of Figs.3.8.1 a and b indicates that Fractions 31-48 contain α2M-IL2, and Fractions 37-48 also contain free IL2. Thus, only Fractions 31-36 appear to conatin the a2M-IL2 complexes of interest. Fig.3.8.2 indicates that when using the immunoassay, the levels of complexed IL2 detected in fractions 31-36 are less than when the bioassay is employed. This confirms the results from the immunoassay studies showing α2M interference in the Amersham IL2 immunoassay system (Sec.3.7). Transformation of the bioactivity data from radioactivity units to pg/ml of IL2 revealed that the level of binding of IL2 by α2Mm was in the range of 362.5 - 2500pg/ml, whereas the range seen in the immunoassay was 50-2300pg/ml. Conversely, more IL2 was recorded in the bioassay than in the immunoassay when the lower molecular weight fractions composed of IL2 alone (79-96) were examined.

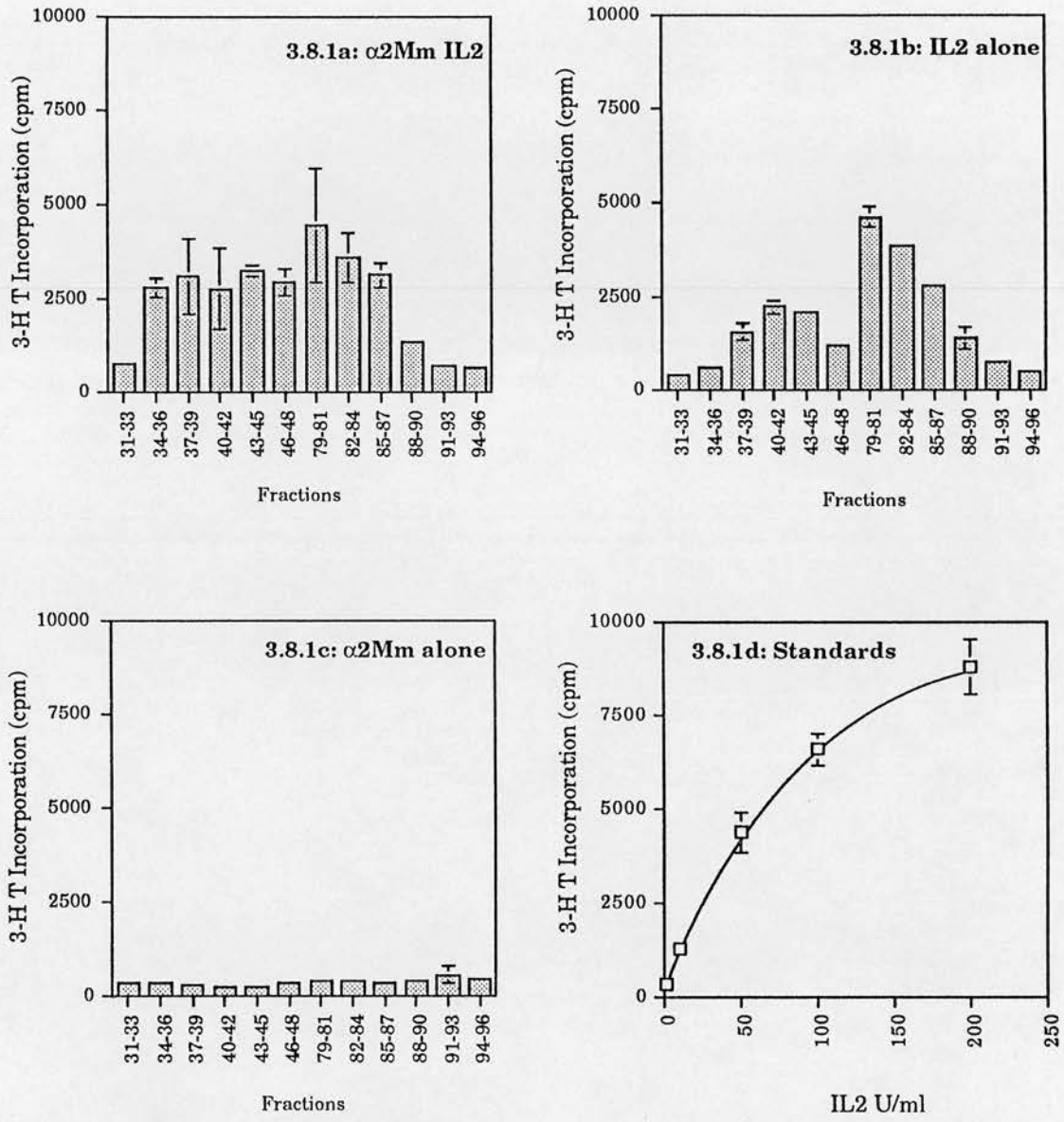


Figure 3.8.1: Investigation of the bioactivity of IL2 when associated with α2Mm

α2Mm, IL2 or a mixture of the two was preincubated for 2 hours at 37°C then passed through a G200 Sephadex column. Eluate was collected as fractions which were then applied to PBL cells in 96 well plates and grown for 4 days with a ^{3}H thymidine pulse 18 hours prior to harvesting. Cell cpm content was assessed in a scintillation counter. Known concentrations of IL2 were applied in a similar manner to PBL cells to form a standard curve. It is apparent that when eluate from the α2Mm-IL2 study was applied to cells there was an increased proliferation seen in the high molecular weight α2Mm peak (fractions 31-48, panel a) as compared to that observed on G200 gel filtration of IL2 alone. Note also the presence of bioactivity in the free cytokine containing fractions, especially fractions 79-81, and the absence of activity in fractions obtained on the separation of α2Mm alone (Fig.3.8.1c).

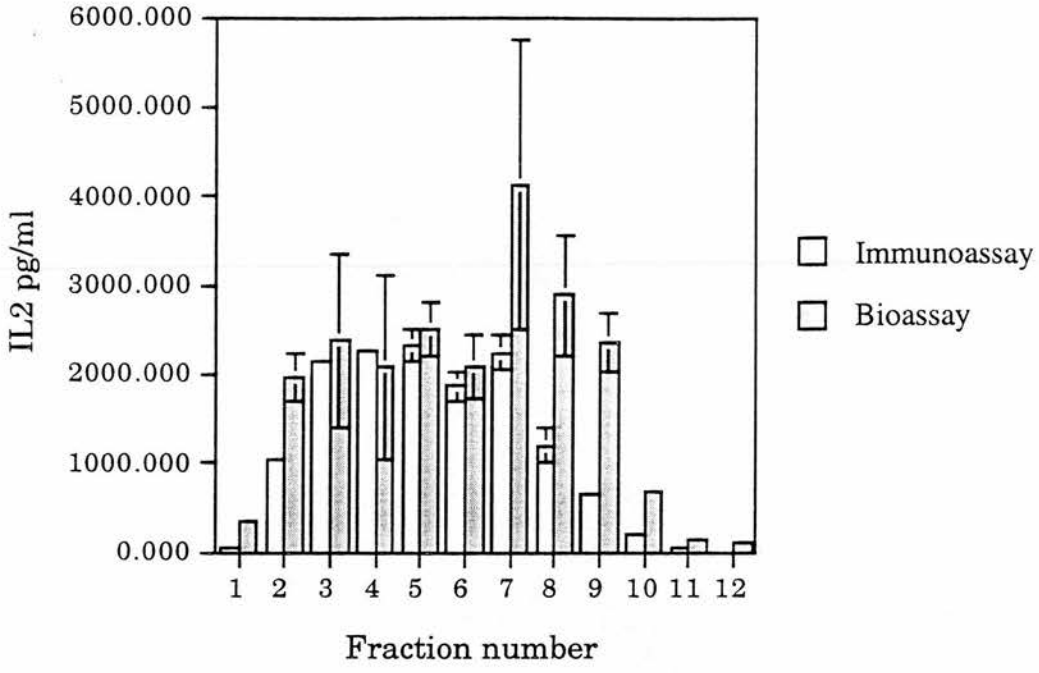


Fig.3.8.2: Comparison of the concentration of IL2 recorded by immunoassay and bioassay
α2M-IL2 complexes were formed by incubation at 37°C for 2 hours prior to separation of unbound cytokine on a G200 column. A lymphoprolifeative bioassay indicated that IL2 activity was present in the α2M fractions (31-48) and in the free IL2 fractions (79-96). Subsequent immunoassay of the fractions indicated that the material present could be distinguished using the Amersham IL2 immunoassay.

3.8.2: Study of the bioactivity of α2Mm-TNFα

α2Mm-TNFα complexes were prepared by incubation as normal followed in this case by separation of bound and free cytokine on a zinc affinity Sepharose column (see Sec.2.2.5). Free TNFα was eluted by phosphate buffer pH6.5, and α2Mm with bound TNFα was recovered in acetate buffer pH4.5. The bioactivity of individual fractions was tested by determining their cytotoxic activity on the TNFα sensitive cell line L929.

Essentially the fractions recovered in phosphate buffer had TNFα-like cytotoxicity (ie α2Mm–TNFα and TNFα alone, Figs.3.8.3a and 3.8.3b). In contrast, those recovered in acetate exhibited comparatively low levels of cytotoxicity. There was one fraction in the α2Mm alone incubation and several from the buffer incubation that showed cytotoxicity but this probably arose due to infection and was not found in those particular fractions when the bioassay was repeated.

Selected fractions from the α2Mm-TNFα incubation, based upon the bioassay results (Fig.3.8.3a), were examined for the presence of TNFα by immunoassay. It was evident that fractions from the phosphate wash contained a large amount of TNFα, eg in the range 4375-31250pg/ml. The acetate wash fractions contained much less TNFα, ie 263-963pg/ml (Fig.3.8.4).

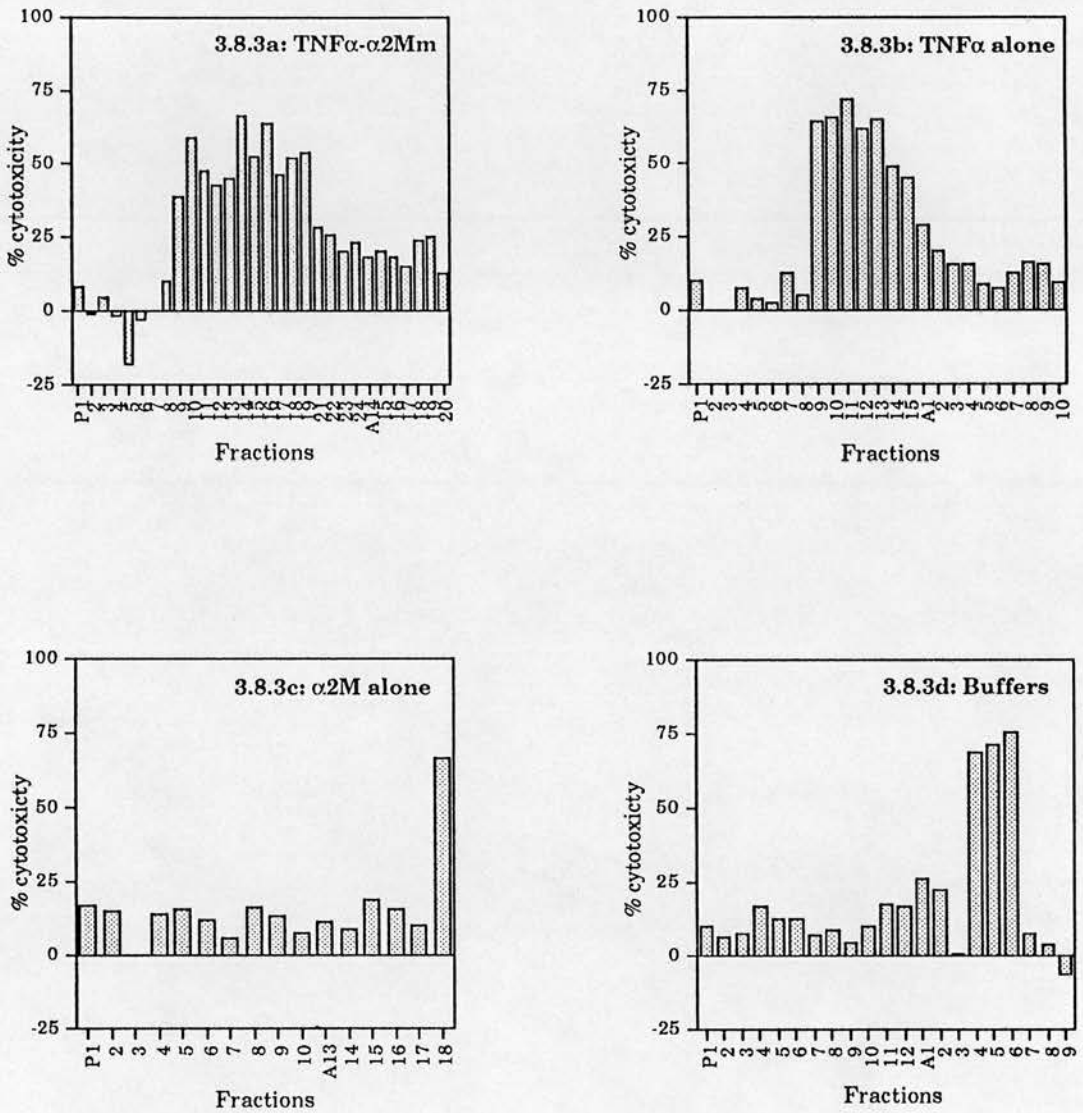


Figure 3.8.3: The effect of the interaction of α2Mm with TNFα on the cytokine's bioactivity

α2Mm (80.1mg) and TNFα (4μg) were preincubated for 2 hours at 37°C and then passed through a zinc affinity Sephacryl column to separate free cytokine from that bound to α2Mm. Controls were prepared and run under similar conditions. Fractions were collected from the column using phosphate pH6.5 to elute TNFα ("P" fractions), or with acetate to elute α2Mm and bound TNFα ("Ac" fractions). Fractions were applied to prepared L929 cells and their cytotoxicity assessed after 18 hours by use of MTT, levels of cytoxicity being compared to known standards. Note that the cytotoxicity associated with Ac fractions α2Mm-TNFα is similar to that with TNFα alone.

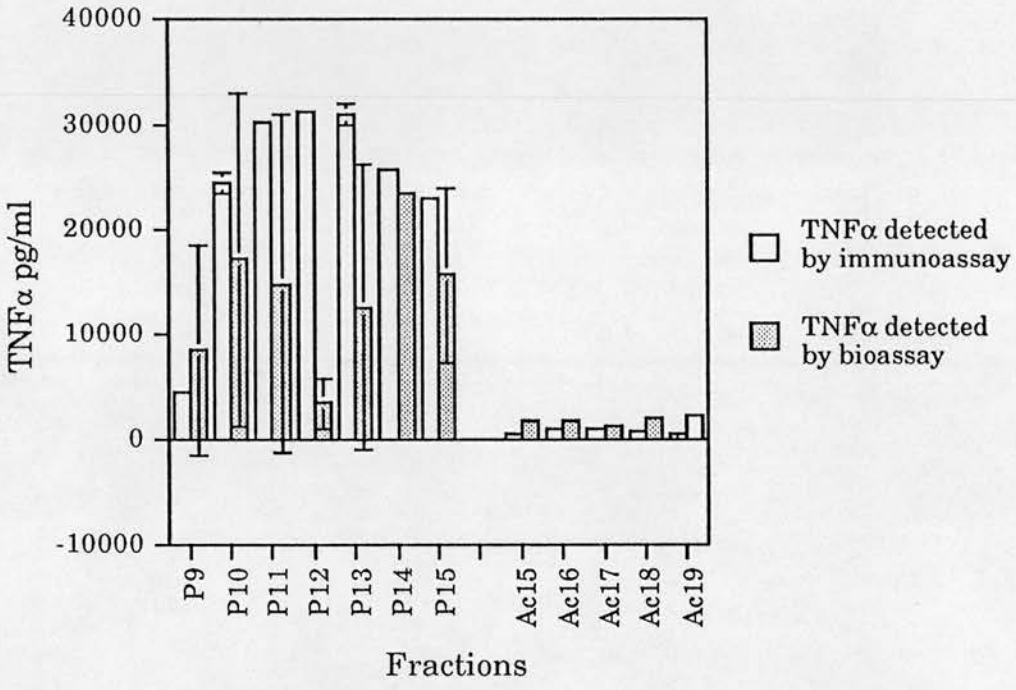


Figure 3.8.4: Detection of α2Mm complexed TNFα using an immunoassay and a bioassay

α2Mm (80.1μg) and TNFα (4μg) were incubated for 2 hours at 37°C and then separated on a zinc affinity Sepharose column. TNFα was eluted using phosphate buffer pH6.5 ("P" fractions), and α2Mm with associated TNFα using acetate buffer pH4.5 ("Ac" fractions). Selected fractions were tested for their TNFα content using an Amersham TNFα immunoassay. All fractions required dilution to the low range of the kit (1-1000pg/ml). Note the variation in the detection of TNFa comparing "P" and "Ac" fractions, and in the two assay systems.

3.9: TCA precipitation studies of radiolabelled cytokines

3.9.1: TCA precipitation studies on cytokine stocks

TCA precipitation studies were conducted on ^{125}-iodine labelled cytokines on arrival, and subsequently during usage. The aim of these studies was to establish how much of the label remained attached to intact cytokine. Degradation of the cytokines may occur during storage due to proteolysis or radiation induced damage. Such degradation may effect the interpretation of binding study results.

Table 3.9.1 illustrates the results of studies carried out on a number of batches of ^{125}-I IL2 and of ^{125}-I IFNγ. The amount of radiolabel that could be precipitated decreased during the period of storage, implying that the amount of ^{125}-iodine that was bound to whole cytokine decreased during that time.

Only one time course TCA precipitation study was carried out with TNFα. Over a 28 day period the amount of radiolabel that could be precipitated decreased from 97.7% to 96.5%, a change of 1.2%. This decrease is much smaller than that seen in the IL2 and IFNγ studies suggesting that this TNFα sample was more stable.

3.9.2: TCA precipitation studies on column radioactivity peaks

On the basis of the above observations, peaks eluted from G200 Sephadex and zinc affinity Sepharose columns were tested for the precipitation of the radiolabel therein.

When radiolabelled cytokine alone was applied to G200, radioactivity eluted in 3 peaks (Fig.3.9.1). Peak 1 eluted in the void volume; it was very small compared to that seen in the presence of α2M. The second peak of radioactivity eluted in the region of 20kDa particles, and the third peak was composed of low molecular weight material. TCA precipitation of the

3.9.1a: IL2

Batch	Day (x-y)	% Change in Precipitation
BO26	1	2.4
BO23	13	1.6
BO22	14	1.9
BO25	16	1.1
BO30	25	4.5
BO22	39	6.0
BO21	41	5.0
BO21	55	6.5
BO28	64	24.9
BO26	127	13.7

3.9.1b: IFNγ

Batch	Day (x-y)	% Change in Precipitation
BO81	15	4.7
BO86	10	7.6
BO82	106	36.3
BO81	141	21.6

Table 3.9.1: TCA precipitation studies with batches of ^{125}-I IL2 and ^{125}-I IFNγ

The number of counts associated with intact cytokine were assessed by precipitation with 20% (v/v) TCA in the presence of carrier protein on days x and y , at an interval of days (as x-y). The decrease in the percentage of TCA precipitable counts is recorded here. Assay of radiolabelled cytokine was repeated at intervals as indicated ("days between assessments"). It is apparent that during storage there is a decrease in the amount of radioactivity that can be precipitated and therefore by implication the amount of ^{125}-iodine bound to the protein.

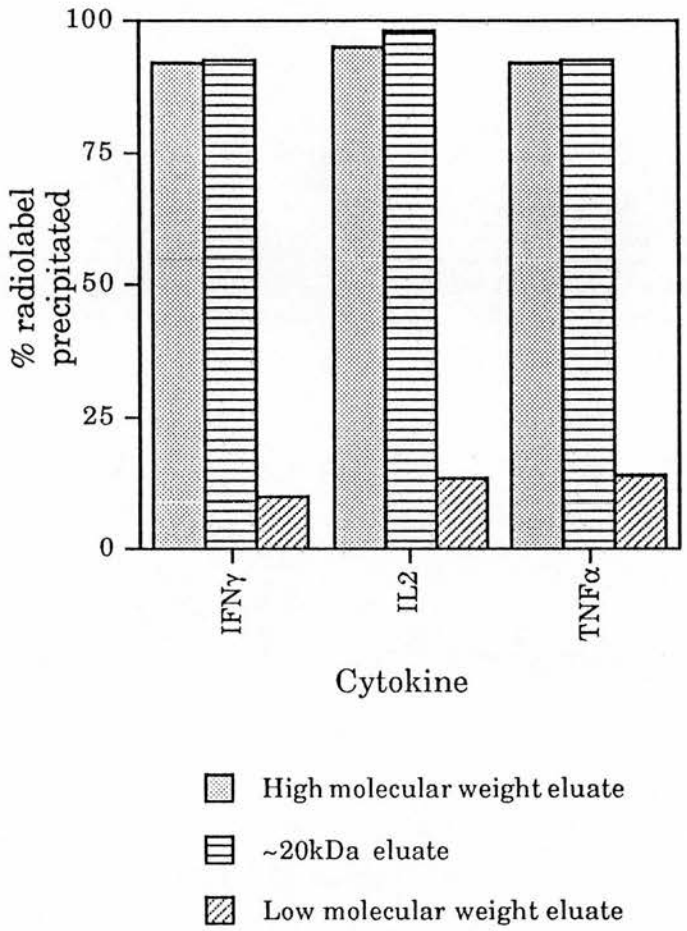


Figure 3.9.1: TCA precipitation studies with cytokine eluted from G200 columns
Cytokines were applied to G200 columns and the eluate collected. Radioactivity present in peaks from the column were subjected to TCA precipitation. It was evident that ^{125}-I in the HMW and 20kDa fractions could be precipitated but not that in the LMW fractions suggesting that counts in the latter were due to free iodine or iodine associated with cytokine degradation fragments.

eluate indicated that the first two peaks could be precipitated, the third peak could not.

The peaks from zinc affinity Sepharose columns were analysised by TCA precipitation. The first phosphate peak and the single acetate peak were precipitated, the second phosphate peak was not (Fig.3.9.2). In addition, fractions eluted from zinc affinity columns were passed through G200 Sephadex columns to analyse their size: the first peak from the phosphate wash was ~20kDa, ie cytokine, and the second phosphate peak was of molecular weight in the region of peptides or free iodine. The ^{125}-I in the acetate peak was associated with a substance of a molecular weight in the void volume of the G200 column, ie greater than 200kDa, presumably α2M or its derivatives.

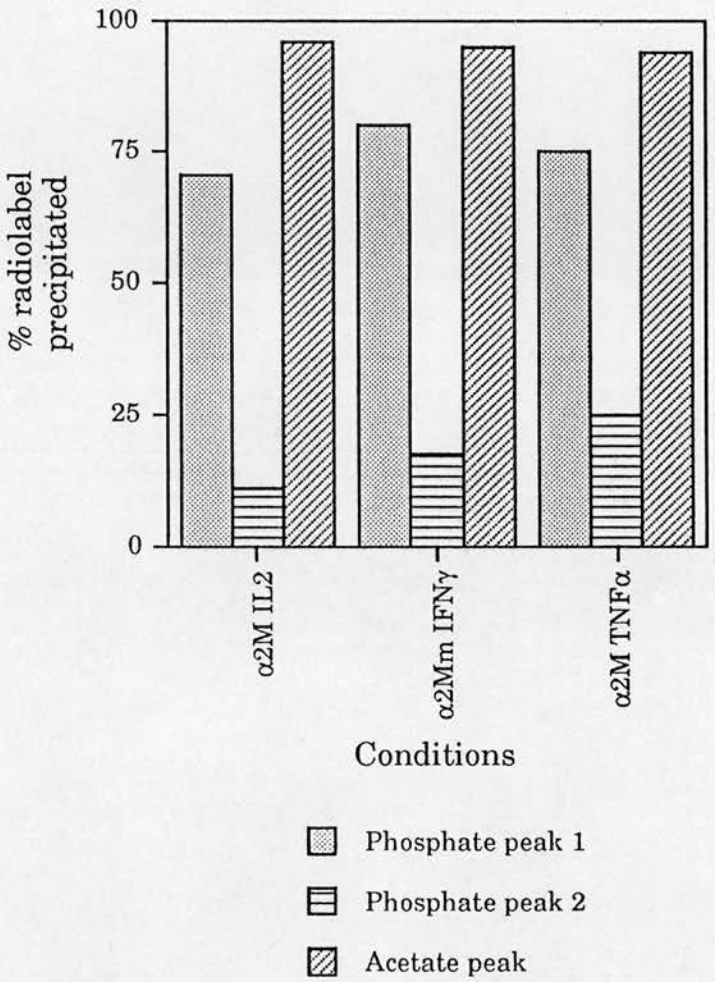


Figure 3.9.2: Study of the precipitation of ^{125}-iodine in radiolabel peaks from zinc affinity Sepharose columns α2M and cytokine, incubated at 37°C for 2 hours, were applied to a zinc chelated Sepharose column and the cytokine eluted in phosphate buffer pH6.5, the α2M in acetate buffer pH4.5. There were two peaks of radioactivity in the phosphate wash. In the first, the counts were precipitated by 20% TCA indicating that they were bound to protein. Conversely, in the second phosphate peak only a small proportion of the counts were precipitated indicating that it contained free ^{125}-iodine or degraded cytokine. The counts in the acetate peak were almost entirely precipitated with 20% TCA indicating that they were probably associated with α2M which is eluted with that buffer.

Section 4: Discussion

4.1: A summary of the results of α2M-cytokine interactions

The aim of this thesis has been to study the qualitative and quantitative interaction of cytokines with α2M, and to examine the possible effects of binding on cytokine bioactivity and immunoassay. Table 4.1.1 summarises the key findings.

4.1.1: Qualitative binding studies

In the initial investigations, binding was examined on a qualitative level (see Sec.3.3 and 3.4). Column studies revealed binding of ^{125}-I IL2, ^{125}-I TNFα and ^{125}-I IFNγ with native and fast α2M. Binding was greatest with the fast forms, such as serum derived or methylamine converted α2M. This was also seen in PAGE studies.

The column work enabled semi-quantitative analysis of the relative binding of cytokines with α2M. In G200 studies, ^{125}-I IL2 was bound at a higher level than IFNγ. FPLC studies were carried out with ^{125}-I IL2, and also with ^{125}-I IFNγ. The FPLC IL2 data confirmed the binding patterns observed with the G200 system, ie more cytokine was bound by the fast forms of α2M. The results from the IFNγ column experiments have not been included: they revealed the heterogeneity of the labelled cytokine and could not be interpreted to provide binding information. It appeared that during storage radiolabelled IFNγ, and to a lesser extent IL2 and TNFα were degrading to form labelled peptides, and probably free iodine was also being released. The heterogeneity of the material is discussed below (see Sec.4.6, and Sec.3.9).

A "novel" zinc affinity column system was established as an alternative to the G200 and FPLC columns. It is not entirely a novel system since it uses the column material and methodology required for separation of α2M

^{125}-I IL2, ^{125}-I TNFα, ^{125}-I IFNγ are bound by α2M, preferably to the fast form

Bonds used are covalent for IL2 and IFNγ, TNFα is bound by non-covalent bonds

^{125}-I IL2 is bound non-specifically by α2Mm with a K_d of $2x10^{-6}M$

^{125}-I TNFα is bound specifically by α2Mm with a K_d of $10^{-6}M$

α2Mm can depress or enhance detection of cytokines in a number of immunoassay systems

α2M, α2Mm and albumin induce a "protein" effect upon certain cytokine immunoassays

α2Mm-IL2 complex retains IL2-like bioactivity

α2Mm-TNFα complex does not retain TNFα-like bioactivity

Table 4.1.1: Principle observations from this study of cytokine and α2M interactions
The interactions of α2M with IL2, TNFα and IFNγ were examined at a qualitative and quantitative level using a number of techniques to reveal binding to α2M, the nature of the bonds involved, K_d values and specificity. The effect of α2M-cytokine interactions on detection of cytokine in immunoassays and in bioassays was also examined.

from plasma or serum (Kurecki et al 1979). The very clean peaks of cytokine and of α2M eluted by this column make it preferable to the G200 and FPLC systems which tend to retain cytokine giving rise to broad peaks of radioactivity that were sometimes difficult to interpret (see Figs.3.3.1-3.3.5). The results obtained with the zinc affinity system corroborate with those from G200 and FPLC for IL2 with respect to the levels of binding to the various forms of α2M, and distinguish IL2 and TNFα binding. In zinc affinity columns, more IL2 than TNFα was bound by α2Mm. This result was subsequently contradicted by the quantitative assay studies, ie RIA and zinc affinity tube system, and highlights the problems of such semi-quantitative analysis using columns (see Sec.3.5 and Sec.3.6).

4.1.2: Bonds employed in α2M-cytokine interactions

The bonds used by α2Mm to interact with IL2, TNFα or IFNγ were examined in SDS-PAGE in the presence or absence of mercaptoethanol (see Sec.3.4.2). TNFα uses non-covalent bonds to interact with α2Mm, whereas IL2 and IFNγ use covalent bonds. It was interesting to see the unusual pattern of protein and radioactivity revealed in the presence of mercaptoethanol: it seems that the reductant negates all interactions between protein and IL2 or TNFα, and that the freed cytokine migrates as oligomers (see Figs.3.4.4 and 3.4.6). However, IL2 remained bound in the presence of SDS alone (Fig.3.4.5). Thus, IL2 binds with α2Mm by covalent bonds. IFNγ binding was maintained in the presence of mercaptoethanol, ie binding was nondisulphide covalent. However, the appearance of the bands on the gel were very weak (not illustrated).

4.1.3: Quantitative binding studies

The zinc affinity column methodology was subsequently adapted to a tube based assay (see Sec.2.4.3 and Sec.3.6). It was employed, in corroboration with a novel RIA, to examine the binding of ^{125}-I IL2 and ^{125}-I TNFα by α2Mm at a quantitative level. Table 4.1.2 summarises the results of these studies. It was found in both assay systems that TNFα was bound with a higher K_d than was IL2, although the difference is small. For both cytokines, the RIA detected higher K_d's by a factor of 10 compared to the zinc affinity system. The zinc system did not show specific binding for TNFα, the RIA did. In both assays, binding of ^{125}-I IL2 was found to be non-specific. The relationship of the K_d values for binding with α2Mm to those for the receptors for the cytokines will be discussed in Sec.4.4.

4.1.4: Influence of α2M on cytokine immunoassays

α2Mm, previously shown to bind a number of cytokines, may influence the immunoassay of certain cytokines (see Sec.1.9, Table 1.9.1, Sec.3.7). Table 4.1.3 illustrates the effects observed and summarises possible mechanisms of interference. These are discussed briefly below.

(i) <u>α2Mm effect on immunoassays:</u> In immunoassay kits from a number of sources it was found that α2Mm can depress the detection of a range of cytokines by as much as 75% when present at physiological concentrations, ie 1-5mg/ml (see Sec.3.7 Fig.3.7.1). Although levels of fast form α2M are normally low *in vivo*, they do increase in some disease states as described, and they may then influence the immunoassay of cytokines (see Sec.1.2, 1.7.2, 1.7.3, and 4.5).

(ii) <u>The effects of protein on cytokine immunoassays:</u> The presence of

Cytokine / Binder	IL2	TNFα
α2Mm (RIA)	$2x10^{-6}M$	$10^{-6}M$
α2Mm (Zn2+)	$3x10^{-5}M$	$2x10^{-5}M$
cytokine receptor	$10^{-11}M$	$10^{-10}M$

Table 4.1.2: Dissociation constants for the interaction of IL2 and TNFα with α2Mm and with their receptors
Dissociation constants for the interaction of ^{125}-I IL2 and ^{125}-I TNFα with α2Mm were calculated by use of novel RIA and zinc affinity systems. They are compared here with previously published values for cytokine binding to membrane receptors. Receptor data from Ringheim et al 1991, Smith et al 1990, and Aggarwal et al 1985.

Observation	Possible explanation
Depression by α2Mm of cytokine detected	-Shields cytokine from antibody by binding in the interior of α2M, or to neoantigens on its outer surface
Protein effect	-Protein increases stability of cytokine -Excess protein mops up enzymes protecting cytokine from degradation
No effect	-May have protein-cytokine interaction, but no interference results

Table 4.1.3: The effects of α2M, α2Mm, and albumin on cytokine immunoassays

The presence of α2M, α2Mm, and albumin in cytokine immunoassays resulted in a depression of the detection of cytokine by α2Mm, or a more general protein effect observed for all three proteins. In some assays, no change in the detection of cytokine was seen. There are a number of possible explanations for these effects as proposed here.

protein, ie α2M, α2Mm or albumin resulted in similar changes in cytokine detection in a number of immunoassays, indicating a general protein effect (see Sec.3.7, Fig.3.7.2 and Table 3.7.1). Thus, in addition to α2M influencing cytokine immunoassay due to its role as a cytokine binder, there is the potential of it acting simply in a protein capacity to stabilise the cytokine, or it may interfere with the cytokine-antibody interaction. Interpretation and consequences of the immunoassay results will be further discussed in Sec.4.5.

4.1.5: The bioactivity of α2Mm-cytokine complexes

The influence of cytokine binding to α2M was examined in bioassays for IL2 and for TNFα. It was found that IL2 bioactivity was maintained, whereas that of TNFα was negated. Complexes of cytokine and α2Mm free of unbound cytokine were prepared by G200 Sephadex (IL2) or on zinc affinity Sepharose (TNFα). These different procedures may have given rise to the different results. However, the evidence for the binding of IL2 and TNFα by α2Mm presented here would suggest that the cytokines are bound differently by α2Mm, and it is therefore plausible that their bioactivity is differently affected. The effects upon cytokine bioactivity are interesting with respect to the physiological consequences of binding, and will be discussed further below (see Sec.4.4).

4.2: Confirmation and extension of α2M-cytokine investigations

Investigations of the interaction of α2M with molecules of the immune system has a history dating back to the 1960's and earlier (see Sec.1.9; reviewed in James 1990, and in Borth 1992). The work herein serves to confirm and extend previous studies as illustrated in Table 4.2.1.

4.2.1: Confirmation

The results presented within this thesis confirm the concept of α2M as a cytokine binder *in vitro* (reviewed in James 1990, and in Borth 1992). As found with other cytokines and growth factors, there is a variation in the binding with native and fast form protein (see Table 1.9.1; Sec.1.9). In addition, the investigation of the bonds involved in the binding of cytokines by α2M has indicated once more that the macromolecule is capable of using a range of bonds in such interactions. The results presented also serve to confirm that TNFα can be bound by α2M, with a preference for the fast form (Wollenberg et al 1991; see Sec.1.9).

Previous reports in the literature have indicated that an interaction of cytokine with α2M could result in a loss of the cytokines antigenicity (see Sec.1.9; Table 1.9.1). Within this present work, cytokine standards from immunoassays were incubated with α2M prior to application to the assay (see Sec.3.7). In some instances a depression in the level of detected cytokine was observed in the presence of α2Mm. This may have occurred due to steric hindrance of antibody-cytokine contact upon binding with α2M confirming the earlier findings concerning antigenicity.

4.2.2: Extension of previous findings

As well as confirming accounts of α2M as a cytokine binder, these studies

Confirmation
α2M binds cytokines
Variability of binding according to conformation of α2M
Variability of bonds used for different molecules
Loss of cytokine antigenicity on binding
Extension
First reports of IL2 and IFNγ binding by α2M
Time/temperature studies
Development of quantitative assays and presentation of K_d values
Assessment of the influence of α2M on cytokine immunoassays
Bioassay of isolated α2Mm-cytokine complex

Table 4.2.1: Observations to confirm and extend α2M-cytokine studies

The present thesis has served to confirm previous reports of α2M as a cytokine binder. It has also served to extend the field of research on a number of fronts relating to the nature and effects of the interactions. These are here presented in brief.

have served to extend this field of research on a number of fronts. TNFα has previously been examined with respect to its binding by α2M and for its hepatic clearance from murine circulation (Wollenberg et al 1991; see Sec.1.10). There have been accounts of IL2, TNFα and IFNγ activity being influenced by α2M, purporting an indirect influence on cytokine activity (Borth and Teodorescu 1986; Scuderi et al 1989; Roche et al 1990; see Sec.1.9 and 1.10). Herein however is the first report of IL2 and IFNγ binding by α2M. In addition, these studies extend knowledge of α2M-TNFα interactions, specifically with respect to quantitative analysis of binding and the bioactivity of the complexes.

It is surprising to note that there have been few reports of the influence of time or temperature on α2M-cytokine interactions (Borth et al 1990a). The length of incubations reported vary from 1.5 to 24 hours, and temperatures employed from 4-37°C (LaMarre et al 1991; Teodorescu et al 1991; Bonner et al 1992). Herein, time and temperature studies were carried out for ^{125}-I IL2 and ^{125}-I IFNγ binding to α2Mm, in each case revealing 37°C as the optimal temperature, and 2 hours as necessary and sufficient for effective interactions to occur (see Sec.3.2). Many previous α2M studies have used these conditions which suggests that other groups may have examined the influence of time and temperature on the interaction of cytokines and α2M, but not reported their findings in the literature.

The development of 2 assay systems to determine K_d values has produced the first fully quantitative study of cytokine-α2M interactions. The RIA and zinc affinity studies were developed to provide a means of rapidly producing sufficient data for quantitative analysis of binding. A recent study in the literature has examined binding affinities for the interaction of α2M with a number of cytokines (Crookston et al 1994). Radioactivity

levels were counted in protein bands from PAGE gels of incubated α2M-cytokine samples. The binding affinity of the α2M-TNFα interaction, with a K_d of $7.5x10^{-7}$M, is in the range of that calculated with the RIA system, ie 10^{-6}M (see Sec.4.1; Table 4.1.1). The RIA and zinc affinity systems utilize properties unique to α2M to achieve separation of free and bound cytokine. Therefore these assays may be employed for subsequent examination of labelled molecules, cytokine or otherwise, to α2M.

Reports in the literature have indicated that binding of cytokines by α2M can decrease their antigenicity. Herein the first full scale study of the consequences of the α2M-cytokine interactions on the immunoassay have been presented. They are discussed in Section 4.5.

The influence of binding to α2M on the bioactivity of cytokines has been investigated prior to this study (see Sec.1.9; Table 1.9.1). However, many of the reports did not employ α2M-cytokine complexes separated from unbound cytokine. Therefore, where reports have been made relating a maintenance of bioactivity, it may be due to unbound cytokine that would simultaneously have been applied to the cells. Thus, in this study cytokine-α2M complexes were incubated as normal, then separated from unbound material by a column procedure. The column eluate consisting of α2M-cytokine complexes alone was then tested in the appropriate bioassay. This result for TNFα is at odds with reports in the literture (Wollenberg et al 1991; James et al 1992; see Sec.1.9, 3.8, and 4.3). The application of bound only cytokine to the L929 cells in the present study could account for the differences between these and previously published results.

4.3 Points of controversy

4.3.1: The bioactivity of TNFα bound to α2Mm

There is one principle point of controversy raised by the results presented herein, namely the *in vitro* bioactivity of TNFα bound to α2M. As indicated above, a study on the bioactivity of TNFα has previously been made (Wollenberg et al 1991). TNFα activity was reported to be maintained when the cytokine was bound to α2M. However, α2M-TNFα freed from unbound cytokine was not used, with the consequence that the bioactivity detected cannot be unequivocally attributed to α2M-TNFα complex since it may have arisen from unbound TNFα which would have been present. The results presented here have shown that the complexes did not have TNFα-like cytotoxicity, even though the cytokine was present as subsequently detected by immunoassay (See Sec.3.8).

4.3.2: Examination of binding affinities

There are many reports of cytokine-α2M interactions. However, qualitative techniques have previously been employed (Huang et al 1984; O'Connor-McCourt and Wakefield 1987; Dennis et al 1989; Borth and Luger 1989; see Sec.1.9.1). Nevertheless, many investigators have assumed a physiological role for such interactions. The use herein of a RIA and a zinc affinity tube system to examine binding at a quantitative level permits discussion of the physiological relevance of interactions observed *in vitro*. These will be addressed below (see Sec.4.4). It is suggested that other cytokines may also be investigated by these methods.

4.3.3: Evidence for the instability of radiolabelled cytokine

There is evidence that radiolabelled cytokines degrade during storage (Sec.3.9). This is revealed by TCA precipitation of the original labelled cytokine preparations, and by G200 Sephadex studies where TCA analysis of the eluted radioactive peaks revealed that there were three peaks, namely: a high molecular weight peak greater than 200kDa and a 20kDa peak both of which could be precipitated by TCA, and a low molecular weight peak that was principally composed of material that could not be TCA precipitated.

These results were observed with all three cytokines examined (Fig.3.9.1) and have generally been assumed by ourselves and others to represent the precipitation of intact cytokine, either bound to α2M as in the high molecular weight peak or free cytokine itself. However, on the basis of the studies presented it cannot be excluded that some of the counts associated with α2M might be attributable to bound degraded products. The existence of such low molecular weight products is not only evident from the gel filtration studies referred to above but has also been confirmed by chromatography and PAGE studies (Sec.3.9, 3.3 and 3.4). Possible variations in the amounts and composition of cytokine degradation products in labelled cytokine preparations may have clouded interpretation of the PAGE and blocking studies reported in this thesis.

4.3.4: Non-alignment of protein and radioactivity bands on SDS-PAGE in the presence of mercaptoethanol

It was found that in the presence of mercaptoethanol, the binding of radiolabelled cytokine by α2M was negated (Figs.3.4.4 a and b, and Figs.3.4.6 a and b). Nevertheless, bands of radioactivity did appear in the 200-250kDa region of the autoradiographs (Figs.3.4.4 b and 3.4.6b). One reason for the appearance of these bands may be the binding of radiolabelled cytokine to proteins of the 200-250kDa mass, present in the cytokine or α2M preparations,

that were not detected by Coomassie blue staining. The introduction of silver staining or an alternative sensitive technique into the gel protocol may reveal the presence of such bands. Such proteins have been reported in the literature as naturally occurring cytokine binding proteins including soluble receptors and autoantibodies; these could be present in low concentrations in the materials used and therefore interfere with the assay. However, it should be stated that to date we have no other evidence for these proteins. Alternatively, aggregation of the radiolabelled cytokine, or its degradation products may have occurred leading to the detection of higher molecular weight bands not associated with α2M or its reduction products.

4.3.5: The absence of blocking by cold cytokine of the binding of radiolabelled cytokine by α2M

In the quantitative zinc binding studies, it was not possible to block the binding of radiolabelled cytokine by α2M using autologous cold cytokine (Figs.3.6.4 and 3.6.5). However, using the RIA system there was some evidence of blocking of ^{125}-I TNFα binding in the presence of the cold cytokine, which decreased to 50% in the absence of the cold protein. These results seem to suggest that there may be two binding sites on α2M for TNFα both of which can bind ^{125}-I TNFα, while only one binds cold TNFα. This may suggest that ^{125}-I TNFα can bind to α2M via the iodine atom, or by some conformational structure resulting from the iodination process itself. Cold IL2 did not block the binding of ^{125}-I IL2 in either system (Figs.3.5.8 and 3.6.4).

These results have significant consequences with respect to the binding of the radiolabelled proteins and interpretation of such results. As noted above for TNFα, on the basis of these studies, it is feasible to speculate that the binding of ^{125}-I cytokine may rely upon iodine interacting with the protein, possibly in a non-specific manner. Furthermore, the identification of breakdown products in radiolabelled cytokine preparations, as noted previously, may imply that

radiolabelled cytokine fragments, rather than the whole protein are bound. It is interesting to compare these studies, which employ cold cytokine up to 100 fold excess over labelled cytokine, to those in the literature where up to 10^5 excess of cold cytokine was used to block the binding of the radiolabelled protein which authors subsequently claimed demonstrated specific binding (Borth and Luger 1989; Huang et al 1984). Obviously further studies in the area are warranted.

4.4: Physiological relevance of α2M-cytokine interactions

4.4.1: Binding affinity of α2M-cytokine interactions

The calculation of binding affinities is a relatively novel concept in α2M-cytokine studies. Previous reports have simply looked for binding, and a number of papers do not even go so far as to differentiate the form of α2M bound (Remold and Rosenberg 1975; O'Connor-McCourt and Wakefield 1987; Koo and Stach 1989; see Sec.1.9 Table 1.9.1). As discussed above, there has been one recent publication that has attempted to address this problem (Crookston et al 1994; see Sec.4.2.2).

Within the literature on α2M-cytokine interactions, many authors have discussed the physiological relevance of the binding they detected. However, their qualitative analysis has used arbitrary concentrations of protein and cytokine, that may have no physiological equivalent. Herein, quantitative studies have been presented on the binding of cytokine by fast form α2M as detected by RIA or zinc affinity tube assays, and these may be compared to K_d values established for cytokines and their known cell surface receptors, as illustrated in Table 4.1.2. From Table 4.1.2, it is apparent that the dissociation constant for binding of IL2 or TNFα to α2M is much less than that of the receptors for IL2 and TNFα. The disparity in the K_d values implies that under normal circumstances α2M will not compete with the membrane receptor for binding. However, in disease states competition may result as receptor levels fall and the fast form α2M concentration rises.

The occurrence of α2M-cytokine interactions *in vivo* and the fate of the complexes is illustrated in Fig.4.4.1, and is discussed below.

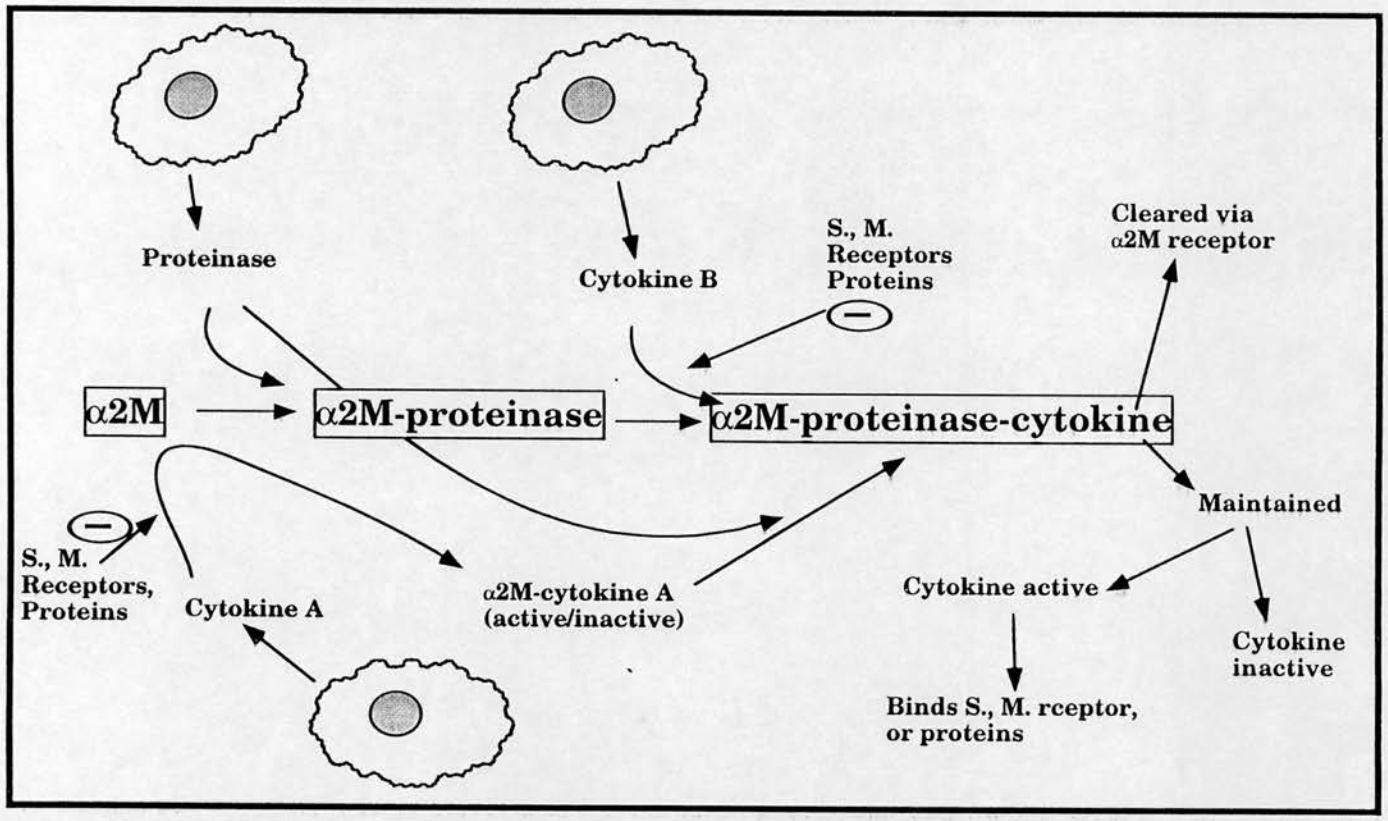


Figure 4.4.1: Scheme of interactions of α2M with proteinases and cytokines
α2M interacts with proteinases to form α2M-proteinase complexes. It can bind cytokines as native α2M and as α2M-proteinase, depending on the cytokine. The interactions with cytokines can be inhibited by soluble (S.) or membrane bound (M.) receptors, or by other proteins capable of binding cytokines. The α2M (or α2M-proteinase) complexed cytokine may be active or latent. In the proteinase form, the complexes can be cleared by the α2M receptor, or maintained in an active or inactive state.

4.4.2: α2M-cytokine interactions in vivo

As shown herein, IL2 and TNFα bind *in vitro* to α2M, principally to the proteinase or methylamine converted fast form of the protein (see Sec.3.3 and 3.5). *In vivo*, such interactions could result in clearance of the cytokine via α2M receptors. However, the highest concentrations of α2M-proteinase are recorded in disease states such as in the rheumatoid joint where the clearance system for fast form α2M is overloaded (Abbink et al 1991; see Sec.1.2.6). Therefore, under such circumstances if cytokines are bound by α2M-proteinase their bioactivity and bioavailability would be affected. This would be expressed as an increase in the half life of the cytokine if bound to native α2M, or to fast form if clearance was restricted. The cytokine may or may not still be active (see Sec.1.9 Table 1.9.1). If clearance of fast form α2M was not restricted the result would be a decrease in the half life of the cytokine. The evidence presented here suggests that such binding would result in a loss of TNFα activity, whereas IL2 would remain active (see Sec.3.8). Such binding may explain reports of α2M as an immunomodulator in that it may direct its effects through the cytokines (see Sec.1.8).

In vivo the effects may be different than those observed *in vitro*. For example, although α2M-IL2 has been demonstrated to have PBL proliferative activity *in vitro*, binding *in vivo* to the fast form of α2M may result in clearance. This would result in inhibition of T cell activation. Furthermore, it has previously been noted that α2M-trypsin can inhibit IL2 activity due to the retention of activity by the bound enzyme (Borth and Teodorescu 1986). This highlights that the present findings are for *in vitro* circumstances, and *in vivo* there are additional effects that may be of importance. Thus, maintenance or negation of bioactivity when bound by α2M may occur for IL2, TNFα or other cytokines *in vivo*. As previously

argued, in a number of disease states such α2M-cytokine interactions may assume a physiological significance, for example in rheumatoid arthritis, in adult respiratory distress syndrome, or in venous ulceration (Abbink et al 1991; Wewers et al 1988; Falanga and Eaglstein 1993; see Sec.1.2).

4.4.3: α2M's role in relation to cytokine receptors

As previously noted, α2M is the "backup" inhibitor of plasmin assuming a more significant role when levels of plasmin's primary inhibitor, antiplasmin drop (see Sec.1.2.8; Harpel 1977; Burtin et al 1987). α2M is the back-up inhibitor for other proteinase inhibitors. Its activity is primarily due to its availability, and to the broad spectrum of proteinases bound by it. Thus, as inhibitor supplies become exhausted α2M takes over. In a similar fashion to the inhibitor scenario, α2M may have the opportunity to bind cytokine if the receptor levels are low, or if cytokine levels are especially high as may occur in disease states. Binding of cytokines could result in inhibition of contact between the cytokine and its receptor, and of cytokine clearance via the local or hepatic α2M receptor. Alternatively, binding may enhance cytokine presentation to the receptor. In addition, α2M may be in competition or collaboration with soluble receptors. Indeed, α2M may be one of a range of proteins present *in vivo* that have a capacity to retain cytokines, and inhibit or promote their activities.

Such a role has been proposed for α2M, fibrin and other macromolecules in venous ulceration (Falanga and Eaglstein 1993; see Sec.1.2.9). The concept of α2M as a cytokine binder may be considered in terms of its effects upon the sequence of growth factor availability. As noted before, Falanga and Eaglstein proposed that in venous ulceration α2M and other

macromolecules "leak" into the dermis due to hypertension, therein binding TNFα and TGFβ, and inhibiting their activity (see Sec.1.2.9). They proposed that alterations of cytokine levels at certain points in the repair mechanism could occur. Thus, in venous ulceration and under other circumstances involving cytokine responses it would not be necessary to clear all of a particular cytokine but by altering its half-life or bioavailability the function of the cytokine could be dysregulated.

Further evidence for α2M and general protein interference with cytokines is presented in the immunoassay studies herein (see Sec.3.7, Sec.4.1.4, and Sec.4.5).

4.4.4: Cytokine-α2M bonds

The binding sites for IL2 and for TNFα on α2M are unclear, as will be discussed in Sec.4.5. TNFα binds with noncovalent bonds which may be hydrophobic or ionic in nature. The covalent bonds that IL2 interacts with may be through the -SH group of the cleaved thiol ester, or they could be through a histidine residue, ie similar to IL1β (Teodorsecu et al 1991; see Sec.1.9).

For both cytokines, "in situ" binding in the presence of converter, ie proteinase or primary amine, would be an interesting study, and may reveal more data that will be of physiological relevance. Recent reports have examined the nascent state of α2M that forms upon activation with proteinases or small nucleophiles (Chu et al 1991; Chu and Pizzo 1993; see Sec.1.9). As discussed earlier, nucleophiles can bind the cleaved glutamyl residue of the thiol ester bond in the nascent state. Cysteinyl residues are also available after cleavage and could be bound by a wide range of proteins. Thus, incubation of cytokine "in situ" with α2M during conformational change may reveal a higher level of binding that reflects

the *in vivo* activity of α2M towards cytokines. Nevertheless, it is possible that small cytokines may gain access to the closed trap: IL2 is active as a 15.5kDa protein and at that size may be able to enter the sprung "trap" and bind with free cysteinyl residues.

4.5: Implications of the immunoassay study

Investigation of the effects of α2M on the detection of cytokines by immunoassay was a logical extension of reports in the literature relating loss of cytokine antigenicity upon α2M binding (see Sec.1.9 Table1.9.1). As illustrated in Table 4.1.3, there were principally three effects observed when α2M, α2Mm or albumin were incubated with cytokine standards and applied to immunoassays as discussed below (see also Sec.3.7).

4.5.1: The α2Mm effect

In the presence of α2Mm cytokine detection was depressed in a number of assays (see Sec.3.7, Fig.3.7.1 and Table 3.7.1). This effect, as was noted for the Amersham IL2 system, was found to be reproducible at intra and interassay levels (see Sec.3.7, Table 3.7.2). The decreased detection may have been due to binding of cytokine to the interior of α2Mm. This is feasible for small cytokines: when α2M is conformationally altered molecules in the region of 20kDa or less can still gain access to the cylinder interior (Travis and Salvesen 1983; Chu and Pizzo 1993; see Sec.1.7.5). Alternatively, cytokines may bind to a "pocket" on the outer surface of the cylinder within which they are partially or completely shielded from antibodies. This concept of an exterior binding site/sites is backed up by the finding that IL4 and TNFα detection were depressed in some assay systems but in others no effect upon detection was found (see Sec.3.7, Table 3.7.1). Detection of IL2 was depressed in the presence of α2Mm in the Amersham assay, but in the Medgenix system detection was enhanced. By partial shielding of the cytokines in an external pocket on α2Mm the epitopes required for some antibodies may be exposed even after binding, whereas those of others are not. However, it is also known that the Fab fragment can contact molecules enclosed within

conformationally altered α2M close to the trap "mouth" (Gonias et al 1988; Delain et al 1992). Thus, an interaction with α2Mm may be inferred from an observed change in cytokine detection, but it is difficult to predict from these results the location of the binding sites.

In addition, it is apparent that native α2M does not alter cytokine detection as much as α2Mm in the illustrated assays (see Sec.3.7, Fig.3.7.1). During the conformational change of the native protein neoantigens are revealed (Barrett and Starkey 1973). Therefore, it could be proposed that neoantigens present after α2M converts to the fast form bind cytokines and affects their detection.

4.5.2: Protein effect on cytokine detection

Native and fast form α2M, and albumin were found to influence the detection of cytokine in some assays (see Sec.3.7, Table 3.7.1 and Fig.3.7.2). The increased concentration of cytokine detected in some assays may be due to a better presentation of cytokine to antibodies. This is understandable for α2M/α2Mm in their capacity as cytokine binders. The presence of protein, be it α2M, α2Mm or albumin, could increase the stability of the cytokine being assayed. This would result in detection of a larger portion of the cytokine present, ie not only that part stable under normal assay conditions. In addition, excess protein could serve as substrate which would mop up proteolytic enzymes which could otherwise digest the cytokine.

4.5.3: No observed effect on cytokine detection

In some immunoassays, no effect by any protein upon the detection of cytokine was found (see Sec.3.7, Table 3.7.1 and Fig.3.7.3). This does not rule out an interaction with those cytokines, but it does indicate that if an

interaction has occurred it does not obstruct cytokine-antibody interactions, ie that any interaction does not mask the cytokine epitopes recognised by the antibodies used in the assay.

4.5.4: Conformation of α2M examined in the immunoassays

It was shown within the qualitative studies of this thesis, that IL2, TNFα and IFNγ bind to fast form α2M better than they do to native α2M. Nevertheless, both conformations were examined in the immunoassay study: previous reports indicated that some cytokines bind equally well or better to native α2M (see Sec.1.9, Table 1.9.1). However, in the analysis of results it is apparent that for the range examined only in the Medgenix IL8 assay did native α2M appear to have a more pronounced effect than the fast form (see Sec.3.7, Table 3.7.1). It may be that an antigen available in the native form is required for the interaction of IL8 and α2M, and that it disappears during the conformational change. There are no reports in the literature of α2M-IL8 interactions.

4.5.5: Practical consequences of the immunoassay results

The immunoassay data has consequences in the laboratory with respect to the diagnostic and research appraisals of cytokine levels in biological fluids, and also in cell culture supernatants which may mimic the *in vivo* state. As discussed previously, α2M and α2Mm levels may rise in a number of diseases, and a range of cells have been identified that synthesize α2M including lymphocytes, macrophages and fibroblasts (see Sec.1.2 and 1.3). In addition, foetal calf serum contains α2M which has been observed to interfere in some assays (Danielpour and Sporn 1990). Thus, in practice the cytokine concentration detected by immunoassay could be altered by α2M or α2Mm at normal or at elevated concentrations.

The specific effects of α2Mm, and the more general protein influence observed do raise the question of the reliability of cytokine kits. As referred to above, soluble receptors of cytokines, and a plethora of other proteins present *in vivo* and *in vitro* bind cytokines and therefore have the potential to interfere with these assays. Manufacturers do test for cross-reactivity, but when approached with the present data were reticent to assume responsibility of problems with their own assays. Medgenix are attempting to address this problem.

4.6: Limitations of these investigations

As with all scientific study, limitations are placed upon the interpretation of the observations due to the techniques employed, and as a consequence of the quality and quantity of materials available. The limitations of this study are discussed below.

4.6.1: Techniques

(i) Column studies: The principle difficulty with the G200 studies was timescale. Following the normal 2 hour incubation of protein and cytokine, samples were loaded onto a G200 column and the final radioactivity eluted approximately 20 hours later. Thus, to permit throughput of an increased number of samples work was carried out on an FPLC system: this reduced the elution time to 1 hour. However, the recovery from the column of loaded material was often only 50%. To circumvent the disparity between applied and recovered counts, calculations relating to all 3 column systems were based upon total recovered radioactivity (see Appendix C.1).
As indicated above, it was often difficult to distinguish exact boundaries for peaks of radioactivity eluting from the G200 and FPLC columns (see Sec.4.1). In this respect the final system that was employed, that is the zinc affinity column, provided very clean peaks and would be the author's system of choice for subsequent studies.

(ii) PAGE studies: These provided a guide to binding and indicated the conformation of preference. However, in some cases the bands of slow and fast α2M were not sufficiently distinct to determine to which form binding was occurring (see Sec.3.4). Nevertheless, the column studies did provide such information, and where available PAGE corroborated the findings of those systems.

(iii) RIA/Zinc affinity studies: in establishing the optimal conditions for the RIA, myosin was employed as a control protein (see Sec.3.5, Fig.3.5.4). In the presence of myosin with anti-human α2M antibody and solid-phase bound secondary antibody one would expect a high level of binding of ^{125}-I α2Mm to the primary antibody and that the primary antibody should bind to the secondary. It appears that myosin inhibits formation or detection of one or both of those interactions. This may have been due to a direct interaction of myosin with the primary antibody inhibiting the interaction with ^{125}-I α2Mm or with the secondary antibody, or due to binding to the secondary antibody so interfering with the interaction of primary and secondary antibodies. However, it appears that in the final assay with cytokine present, and using BSA as a control, such interference does not occur, thus this problem was peculiar to myosin and does not limit the interpretation of the final data and calculation of K_d values.

In addition, the "stickiness" of radioactive material was a constant feature of all of these studies, and was overcome very effectively in the RIA system by precoating the tubes with BSA and transferring reactants from this tube before the final count (see Sec.3.5, Table 3.5.1).

4.6.2: Materials

(i) Sources of α2M and α2Mm: age conversion was noted for α2M stored for long periods of time, ie for 6 months or more (see Sec.3.1, Fig.3.1.2; Sec.3.3, Fig.3.3.3d). This phenomenon has been documented previously: the thiol ester bond undergoes autolytic cleavage resulting in conformational change of the whole molecule (Barrett et al 1979; see Sec.1.6.6). In all quantitative binding studies, the immunoassays and bioassays were carried out with slow α2M or with α2Mm prepared from the slow form.

It was not possible within this study to prepare α2M converted with proteinases: plasmin was tested, however incubation resulted in degradation of the α2M molecule (see Sec.3.3, Fig.3.3.2f). α2M-proteinase and α2Mm are structurally very similar, and it was therefore feasible to make this substitution (see Sec.1.6).

(ii) Heterogeneity of radiolabelled cytokine: As discussed above, it was noted during FPLC studies that ^{125}-I IFNγ purchased from Amersham was very heterogeneous (see Sec.4.1.1). Subsequent examination by TCA precipitation of ^{125}-I IL2 and ^{125}-I IFNγ upon arrival and during storage indicated that heterogeneity increased during storage (see Sec.3.9, Table 3.9.1). This effect arises because of primary and secondary decomposition of the labelled molecule (Guide to the self-decomposition of radiochemicals, Amersham International plc). In addition to TCA precipitation of samples from cytokine batches, peak fractions of IL2, TNFα, and IFNγ from G200 and zinc affinity columns were examined for the precipitation of radioactivity (see Sec.3.9.2, Figs.3.9.1 and 3.9.2). It was found that 2 out of 3 peaks of radioactivity eluted from the columns could be precipitated. Size analysis indicated that one peak was greater than 200kDa, ie in the range of α2Mm, and the other in the weight range of whole free cytokines. The third peak could not be precipitated: it consisted of low molecular weight fragments and free ^{125}-iodine. Once this problem was identified, radiolabelled cytokine was ordered at its production date and used as rapidly as possible after arrival, ie within a matter of days, in order to minimise the contamination of the batch with decomposition products. TNFα was also tested and showed very little degradation.

(iii) Immmunoassays: Commercial assays for cytokines range in price

from £300-£400 for a 96 well kit. In most kits, 16 wells are required for the standard curve. This leaves 80 wells for analysis of samples. Therefore, in examining the effect of α2M on immunoassay standard curves, consisting of 4-8 points, there was a distinct limitation as regards the number of conditions that could be tested and the number of repetitions that could be made for each one. In addition, though many kits were tested at least twice, financial restrictions meant that a few were examined once only (see Sec.3.7, Table 3.7.1).

(iv) Bioassays: In the IL2 and TNFα bioassay studies, the presence of cytokine in the α2Mm fractions was measured using the appropriate Amersham immunoassays (see Sec.3.8, Figs.3.8.2 and 3.8.4). The levels of IL2 detected in Fractions 37-48 were similar in both the bioassay and immunoassay. Immunoassay of the α2M-IL2 and of the cytokine alone fractions detected less IL2 than did the bioassay (Fractions 31-36 and 79-96 respectively). This implies that the immunoassay does not detect all of the cytokine that was bioactive. With respect to the α2M-IL2 fractions, this may be due to the interference of the serum protein on the immunoassay of the cytokine.

In the assay using L929 cells, the acetate fractions containing α2Mm-TNFα complex showed slight variations between cytokine levels. However, in the phosphate wash fractions, where TNFα was present alone, the level of cytokine recorded by immunoassay was much greater than that in the bioassay. This result implies that a portion of the TNFα applied was not bioactive.

4.6.3: Limitations of Observations

The limitations of techniques and materials discussed above means that,

as with all scientific study, there are limits as to the interpretation of the results and conclusions thereof. Corroboration between studies does help, for example PAGE and a number of column techniques were employed to investigate binding at a qualitative level, the RIA and zinc affinity tube assay were used to examine the quantitative nature of binding and its specificity. All assays were carried out with inter and intra assay repetition. Thus, by corroboration between techniques, and by repetition of conditions the limitations of the interpretation of the data presented is minimalised.

4.7: Future work

There are seven key areas of study that can be highlighted for future investigations:

4.7.1: Is binding increased in the nascent state?

Apart from studies of EGF and insulin, all work with α2M and cytokines has involved native or fast form protein (see Sec.1.9, Table 1.9.1). It would be interesting to see if the binding investigated here or elsewhere is increased if α2M, cytokine and an "activator", for example proteinase or methylamine, are present together. In addition, the bonds involved could be identified, ie do cysteinyl or glutamyl residues interact with the cytokine?

Furthermore, Chu and Pizzo proposed that α2M may be able to non-specifically interact with any available nucleophile during the conformational change (Chu and Pizzo 1993). Their theory could be tested by competition studies with various cytokines.

4.7.2: What are the K_d values for the binding of cytokines with α2M?

As noted above, the RIA and the zinc affinity tube assay use α2M, not cytokine, as their crucial component for separation. Thus, binding affinity and specificity for the interaction of α2M, be it native, nascent, or fast form, with other cytokines could be investigated using either system. It would be especially interesting to investigate PDGF and TGFβ both of which have been reported to associate with α2M *in vivo* (Crookston et al 1993; see Sec.1.9). Information from such studies would also permit further discussion on the physiological relevance of cytokine-α2M interactions.

4.7.3: Do bound cytokines retain their bioactivity?

α2M-cytokine complexes previously reported to have altered or maintained bioactivity *in vitro* could be reexamined to see if the same result is found when α2M-cytokine complexes, free of unbound cytokine, are applied to cells.

The bioactivity studies with IL2 and TNFα could be continued by examining complexes prepared by the alternative column method to that presented herein. Results may also be affected if the assay is carried out in a serum free environment. However, the contribution of α2M from foetal calf serum in the bioassays herein was in the region of 0.2mg/ml and it is therefore unlikely to have made a substantial difference to the results.

4.7.4: Do other proteins influence cytokine immunoassays?

The immunoassay studies revealed the actions of 2 proteins in cytokine immunoassays. As discussed above, biological fluids and cell culture supernatants contain a wide range of molecules that may also influence cytokine immunoassay (see Sec.4.5). It may be in the researcher's interest, and in those of the manufacturer, that such matters are investigated further.

4.7.5: Can α2M-cytokine complexes be identified in disease states?

There is much interest in studies that have indicated hepatic clearance of cytokines in association with α2M, and the identification of links between α2M, IL1β and rheumatoid arthritis (LaMarre et al 1991; Borth et al 1990; Teodorescu et al 1991; see Sec.1.2, 1.9 and 4.4). However, the majority of studies concerning α2M-cytokine interactions have been carried out *in vitro*. In order to clarify the physiological link it is necessary that α2M-cytokine complexes formed *in vivo* be identified. This has been done for

TNFα in plasma (Wollenberg et al 1991; see Sec.1.9).

4.7.6: Is the interaction reversible?

Under what conditions can the interaction be reversed, eg by decreasing pH such as in the endocytic vesicle (see Sec.1.7.4). This could be of importance with respect to the *in vivo* significance of α2M-cytokine interactions.

4.7.7: Does PZP interact with cytokines?

As outlined in Sec.1.5, humans have other thiol ester plasma proteins apart from α2M. It may be interesting to study their interaction with cytokines. This may be especially important for PZP which shares the tetramer form of α2M and they have definite sequence homology (Sottrup-Jensen et al 1984). PZP appears in the serum at pregnancy, and may have a specific role in binding growth factors and cytokines required at that time (Sand et al 1985).

4.8: Summary

The present study has confirmed evidence in the literature that α2M is a cytokine binder. It has extended this field of research on a number of fronts: prinicipally to investigate such binding at a quantitative level, and to provide novel information on the effects of α2M on cytokine assays. There is a growing list of factors that can bind and interfere with cytokine activity *in vivo*, which includes soluble cytokine receptor anatgonists and cytokine auto-antibodies (Klein and Brailly 1995, Bendtzen, Svenson, Jonsson and Hippe 1990). The findings presented herewith serve to confirm the place of α2M in that list of circulating factors.

Section 5: References

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Section 6: Appendices

Appendix A: Buffers

A.1: For general use:

Phosphate buffered saline pH7.2 - 60mM with 150mM NaCl

32.2g $Na_2HPO_4.12H_2O$
4.68g $NaH_2PO_4.2H_2O$
17.5g NaCl

Made up to 1.9litres with distilled water, pH tested as 7.2, then filled to 2 litre level.

A.2: For use in Zinc affinity studies

Stock phosphate buffers

0.2M $Na_2HPO_4.12H_2O$ - 71.64g/litre distilled water
0.2M $NaH_2PO_4.2H_2O$ - 31.21g/litre distilled water

0.1M Phosphate pH8.0, 0.8M NaCl

473.5ml 0.2M $Na_2HPO_4.12H_2O$
26.5ml 0.2M $NaH_2PO_4.2H_2O$
0.87g NaCl
Check pH, make up to 1 litre with distilled water.

0.1M Phosphate buffer pH6.5 (0.8M NaCl)

155ml 0.2M $Na_2HPO_4.12H_2O$
345ml 0.2M $NaH_2PO_4.2H_2O$
Check pH, add 46.75g NaCl if required, and make up to 1 litre with distilled water.

0.1M Acetate buffer pH4.5, 0.8M NaCl

380ml 0.1M sodium acetate (13.61g $C_2H_3O_2Na.3H_2O$/litre water)
620ml 0.1N acetic acid (6.05g/litre distilled water)
Check pH, add 46.75g NaCl.

0.05M EDTA pH7.0, 0.8M NaCl

18.612g EDTA/litre distilled water

46.75g NaCl
Check pH.

A.3: For use in the preparation of α2Mm

0.2M Tris stock
24.23g Tris
Made up to 1 litre with distilled water.

0.1N HCl
8.75ml concentrated HCl
Made up to 1 litre with distilled water.

50mM Tris-HCl with 200mM methylamine
250ml 0.2M Tris
229ml 0.1N HCl
Made up to 900ml with distilled water, adjusted pH to 8.2, added 6.2g methylamine, and filled up to 1 litre with distilled water.

A.4: For use in the iodination of α2Mm

1M $Na_2HPO_4.12H_2O$
358.2g $Na_2HPO_4.12H_2O$
Made up to 1 litre with distilled water. Made 0.1M from this by diluting 10ml to 100ml with distilled water.

1M NaH2PO4.2H2O
156.05g $NaH_2PO_4.2H_2O$
Made up to 1 litre with distilled water. Made 0.1M from this by diluting 10ml to 100ml with distilled water.

0.05M phosphate, pH7.5
42ml 0.1M Na_2HPO_4 (17.799g $Na_2HPO_4.2H_2O$ made up to 1 litre)
8ml 0.1M NaH_2PO_4 (15.6g $NaH_2PO_4.2H_2O$ made up to 1 litre)
Made up to 90ml, tested pH as 7.5 and filled up to 100ml.

0.25M phosphate buffer, pH7.5

42ml 1M $Na_2HPO_4.12H_2O$
8ml 1M $NaH_2PO_4.2H_2O$
Made up to 190ml with distilled water, adjusted pH and filled to 200ml.

12.5mg cysteine-HCl in 20ml 0.05M phosphate buffer, pH7.5
50mg potassium iodide in 10ml 0.05M phosphate buffer, pH7.5
25mg chloramine T in 10ml 0.25M phosphate buffer, pH7.5

A.5: For use in PAGE studies

Tris-glycine electrode buffer stock solution, pH8.3
6g Tris
28.8g glycine
Made up to 900ml with distilled water, adjusted pH to 8.3, and filled up to 1 litre. for use, this stock soution was diluted with distilled water in a ratio of 1:9, stock:water.

Tris-chloride buffer, pH8.9
48ml 1N HCl
36.6g Tris
Made up to 90ml with distilled water, adjusted pH to 8.9, added 0.23ml of Temed then made up to 100ml.

Tris-chloride buffer, pH6.7
48ml 1N HCl
5.98g Tris
Made up to 90ml with distilled water, adjusted pH to 6.7, added 0.46ml of Temed, and filled up to 100ml.

Resolving acrylamide stock solution
28g acrylamide
0.74g NN'-methylenebis acrylamide
Made up to 100ml with distilled water. Stored in dark bottle at 4 ^{o}C for up to 2 weeks.

Resolving gel at 5%

5ml Tris-chloride buffer pH8.9
6.6ml resolving gel acrylamide solution
28.4ml deionised water
Mix and degas
300ul ammonium persulphate (10%w/v)
Pour immediately

Stacking gel
2.5ml Tris-chloride buffer pH6.7
3.1ml resolving gel acrylamide
14.1ml deionised water
Mix and degas
300ul ammonium persulphate (10%w/v)
Pour immediately

Sample Buffer
0.1ml 1%(v/v) bromophenol blue
10ml glycerol
25ml TRIS stock pH6.7
Mix and fill to 100ml
Use in 1:4 ratio with samples

Fixative
500ml methanol
200ml glacial acetic acid
Made up to 2 litres with distilled water. Used to fix proteins to acrylamide gels after electrophoresis - required ~500ml per gel - left for 1 hour in a sandwich box on a shaking plate, poured off and replaced fluid with Coomassie blue stain.

Coomassie Blue Stain
0.5g Coomassie Blue
200ml methanol
Made up to 2 litres with distilled water in a glass measuring cylinder. Used for staining proteins on acrylamide gels after electrophoresis - left for 1 hour in a sandwich box on a shaking plate, poured off and replaced with

destain.

Destain
200ml methanol
200ml glacial acetic acid
Made up to 2 litres with distilled water. Replaced fluid 3 times each time incubating the gel for ~2 hours in a sandwich box on a shaking plate - carried this out at 37°C and in the presence of a small piece of sponge - these two practices improved the removal of background stain on the gels.

A.6: For use in SDS-PAGE

TRIS-glycine electrode solution, pH8.3
15.15g TRIS
72g glycine
5gSDS
Make up to 4.5litres with deionised water, check pH. Fill to 5 litres.

TRIS-SDS stock solution, pH6.8
30.29g TRIS
2g SDS
Make up to 950ml with deionised water, check pH. Fill to 1 litre.

TRIS-SDS stock solution, pH8.8
90.86g TRIS
2g SDS
Make up to 950 ml with deionised water, check pH. Fill to 1 litre.

Resolving gel solution
9.9ml acrylamide solution
30ml TRIS-SDS stock solution, pH8.3
Make up to 60ml with deionised water
Degas
15ul Temed
1.5ml 1%(w/v) ammonium persulphate
Mix and pour immediately.

Stacking gel solution
2ml acrylamide solution
10ml TRIS-SDS stock solution pH6.8
Make up to 20ml
Degas
10ul Temed
1ml 1%(w/v) ammonium persulphate
Mix and pour immediately.

Sample buffer
25ml TRIS-SDS stock, pH 6.8
2g SDS
10ml glycerol
5ml 2 mercaptoethanol
0.1ml 1% (v/v) bromophenol blue
Make up to 100ml with deionised water

A.7: For use in development of the RIA

0.1M Sodium Acetate
13.608g made up to 1 litre.

0.1M Acetic Acid
5.72ml glacial acetic acid made up to 1 litre

0.1M Acetate, pH5.0, with 5mM sodium m-periodate
70ml 0.1M sodium acetate
30ml 0.1M acetic acid
Mixed and tested pH as 5.0. Added 5ml of sodium m-periodate prepared as 214mg in 10ml of distilled water.

0.25M Citrate, pH4.0
62g citric acid
60.3g sodium citrate
Made up to 1.9 litres, tested pH as 4.0, then filled up to 2 litres.

0.1M Sodium Bicarbonate, pH9.0
42g sodium bicarbonate
1.7g sodium hydroxide
Made up to 4.9litres, tested pH as 9.0 and filled up to 5 litres.

0.25M Tris
151.4g of Tris
Made up to 5 litres with distilled water.

A.8: For use in the RIA

Prewash solution

0.06M PBS pH7.2, 0.15M NaCl
1% (v/v) Tween 20

Wash buffer
0.06M PBS pH7.2, 0.15M NaCl
1% Tween 20
10% sucrose

Appendix B: Addresses

The following is a list of addresses for reagent and equipment supliers used in this study.

Amersham International PLC
PO Box 929
Slough
Berks.
England
SL1 4YW

Amicon Ltd
Upper Mill
Stonehouse
Gloucestershire GL10 2BJ
England

Appligene
Pinetree Centre
Durham Road
Birtley
Chester-Le-Street
Co.Durham DH3 2TD
England

Bayer UK Ltd.
Pharmaceutical Business Group
Bayer House
Strawberry Hill
Newbury
Berkshire
England
RG13 1JA

Behringwerke Ag
Marburg
Germany

Bibby Sterilin Ltd
Stone
Staffordshire
ST15 0SA
England

Bio-rad Laboratories Ltd
Bio-rad House
Maylands Avenue
Hemel Hempstead
Hertfordshire HP2 7TD

England

Boehringer Mannheim UK
(Diagnostics and Biochemicals) Ltd
Bell Lane, Lewes
East Sussex BN7 1LG
England

Canberra Packard Ltd
Brook House
14 Station Road
Pangbourne
Berkshire RG8 7DT
England

Costar Corporation
Cambridge MA
USA

Du Pont (U.K.) Ltd.
Diagnostic and Biotechnology Systems
New England Nuclear Research Products
Wedgwood Way
Stevenage
Herts.
England
SG1 4QN

Dynatech
Daux Road
Billingshurst
West Sussex RH14 9SJ
England

Endogen
AMS Biotechnologies Ltd
Unit 15 Earn Avenue
Rigshead Industrial Estate
Bellshill ML4 3JQ
Scotland

Fisons Scientific Equipment
Bishop Meadow Road
Loughborough
Leicestershire LE11 0RG
England

Genzyme Corporation
50 Gibson Drive
Kings Hill

West Malling
Kent
England
ME19 6HG

Gibco BRL
PO Box 35
Trident House
Renfrew Road
Paisley
Scotland
PA3 4EF

Hoefer Scientific Instruments U.K.
PO Box 351
Newcaslte-Under-Lyne
Staffs.
England
ST5 0BR

Kabi Diagnostica
Quadratech
PO Box 167
Epsom
Surrey
England
KT17 2SB

Mackay and Lynn Ltd.
2 West Bryson Road
Edinburgh
Scotland
EH11 1EM

Medgenix U.K. Ltd.
Unit 8
Warren Yard
Warren Farm Office Village
Wolverton Mill
Milton Keynes
England
MK12 5NW

Pharmacia Biotech Ltd
Davy Avenue
PO Box 100
Knowlhill
Milton Keynes MK5 8PB
England

Scottish Antibody Production Unit (SAPU)
Law Hospital
Carluke
Lanarkshire
Scotland
ML8 5ES

Sigma Chemical Company Ltd.
Fancy Road
Poole
Dorset
BH17 7BR

Skatron Instruments
Unit 11
Studlands Park Avenue
Newmarket
Suffolk CB8 7DB
England

Vickers Laboratories Ltd
Burley-Wharfedale
West Yorkshire
England

Wallac UK
EG and G Instruments Ltd
20 Vincent Avenue
Crownhill Business Centre
Crownhill
Milton Keynes MK8 0AB
England

Appendix C1: Calculations appertaining to the column studies

1) The quantity of protein eluting from G200 Sepahdex, FPLC Superose 6B or zinc affinity columns was calculated thus:

$$\frac{\text{Absorbance total of protein peak}}{\text{Extinction coefficent}} = \text{Protein (g/100ml)}$$

where the extinction coefficent is 12.8

$$\text{Protein (g/100ml)} \times \frac{1000}{100} = \text{Protein (mg/ml)}$$

$$\text{Protein (mg/ml)} \times \text{Total volume of fractions in peak (ml)} = \text{Total protein in peak (mg)}$$

2) The amount of ^{125}-I cytokine eluting from the columns was calculated thus:

$$\frac{\text{cpm}}{\text{specific activity (cpm/nmole)}} = \text{nmoles } ^{125}\text{-I cytokine}$$

3) The percentage of ^{125}-iodine eluted in any peak from the columns was calculated thus:

$$\frac{\text{cpm in peak} \times 100}{\text{total cpm eluted}} = \%\text{ eluted } ^{125}\text{-iodine}$$

4) The relation ship of K_{av} to the log of molecular weight was used to establish the molecular weight of fractions eluting from the columns used in Section 2.3.

K_{av} was calculated thus:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where

V_e = elution volume, the volume at which an individual fraction has eluted

V_o = void volume, the volume in which blue dextran elutes from the column

V_t = total volume of the column

K_{av} is a constant value for each molecular weight provided that the column and elution conditions remain the same

Appendix C2: Calculation of K_d values for RIA and zinc affinity studies

In an equilibrium situation of receptor and ligand interactions, the following state exists:

$$R+L \rightleftarrows RL$$

$$\text{where } K_d = \frac{[R][L]}{[RL]}$$

where [RL]= concentration of complex = bound material = B
[R] = concentration of free receptor = F
[L] = concentration of free ligand

The usual assumption is:

$$[R] = [\text{Total R}] - [RL]$$

ie, that the total receptor concentration (T) minus that complexed to ligand is the concentration of available receptor.

Thus,

$$[R] = T-B$$

Thus,

$$\frac{(T-B)F}{B} = K_d \quad \text{(Equation A)}$$

Which implies: $(T-B)F = BK_d$

Which implies: $TF - BF = BK_d$

Which implies: $TF = B(K_d+F)$

Which implies: $B = \frac{TF}{K_d+F}$

This equation forms the usual Scatchard plot.

However, in the present study the concentration of α2M (R) is much greater than that of cytokine (L), and therefore:

$$T >> L$$

Therefore, the decrease in T (the total concentration of R) due to the interaction with ligand is imperceptible, and hence:

T (total α2M concentration) - B (amount of α2M bound to cytokine) = T

This simplifies Equation A thus:

$$\frac{(T-B)F}{B} = K_d$$

$$T-B = T, \text{ therefore } \frac{TF}{B} = K_d$$

and therefore,

$$B = \frac{K_d}{TF}$$

Plotted as B vs F, the slope is $m = T/K_d$

Therefore, $K_d = T/m$

where T = total concentration of α2M present
m = gradient of graph.

Appendix C3: Statistical analysis of immunoassay data

The mean value for each concentration of cytokine in the presence of additional protein (eg α2Mm at 5mg/ml) was calculated with respect to the control reading, and therefore awarded a value that related to the 100% value of the control. From these values the overall deviation around the control values was calculated. Thus, for Amersham IL6 in the presence of 1mg/ml α2Mm:

Control concentration (pg/ml)	% deviation in the presence of α2Mm
6.3	-8.8
12.5	12.7
25	0.6
50	6.2

Thus, the overall mean for the deviation in the absorbance read in the presence of 1mg/ml α2Mm was -1.75%+/- 13.49.

This gives the investigator an indication of the effect of the added reagent (ie α2Mm). It is prone to a large percentage error because of the wide variation observed in the presence of low concentrations of cytokine.

Section 7: Publications and Presentations

Publications and Presentations

Arising from these studies, there have been a number of presentations at national and international immunology conferences. In addition, the following publications have appeared:

James, K, Milne, I, Cunningham, A and Elliott, SF. The effect of α2 macroglobulin in commercial cytokine assays. J Immunol Meths 1994; 168: 33-37.

Cunningham, AJ, Elliott, SF, Black, JR and James, K. A simple method for isolating α2 macroglobulin-cytokine complexes. J Immunol Meths 1994; 169:287-292.

Copies of these papers are enclosed herewith.

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Journal of Immunological Methods 169 (1994) 287–292

JOURNAL OF IMMUNOLOGICAL METHODS

Short communication

A simple method for isolating α_2 macroglobulin-cytokine complexes

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(Received 9 September 1993; revised received 22 November 1993; accepted 15 December 1993)

Abstract

There is the need for a simple, effective procedure for separating α_2 macroglobulin-cytokine complexes from free cytokine in order that the nature and possible immunological significance of cytokine-binding by α_2 macroglobulin (α_2-M) might be further investigated. This presentation describes a method which exploits the presence of zinc-binding sites on α_2-M which permit the isolation of complexes from other proteins by zinc-affinity chromatography. Furthermore the method may be used in either a column or batch format.

Key words: α_2 Macroglobulin; Cytokine; Complex; Isolation

1. Introduction

α_2 macroglobulin (α_2-M) is a highly conserved major blood protein found at a concentration of 2–4 mg/ml in serum and extravascular fluid depending on age and sex (Coan and Roberts, 1989). No recorded cases of α_2-M gene deletion have been demonstrated to date, suggesting a key physiological importance. Human α_2-M has a molecular weight of 720 kDa and consists of four 180 kDa polypeptide sub-units held together by non-covalent and interchain disulphide bonds. α_2-M exhibits the propensity to bind a wide range of physiologically important molecules, including all known endopeptidases (Starkey and Barratt, 1977). This interaction results in important changes in both the α_2-M molecule and interacting enzyme (Sottrupp-Jensen 1989). Similar changes in the properties of α_2-M can also be achieved by treatment with primary amines such as methylamine (Gonias et al., 1982). This conformational change causes a corresponding shift in electrophoretic mobility from a 'slow' to 'fast' form and the subsequent exposure of a hydrophobic receptor-binding site on the α_2-M molecule which readily binds to receptors on the surface of macrophages, fibroblasts and hepatocytes resulting in its rapid removal from the circulation (Debanne et al., 1976). Recently it has become apparent that protease and methylamine-treated fast forms of α_2-M also bind a number of immunologically important cytokines including IL-1β, TNF-α and IL-2 (reviewed by James, 1990; Borth, 1992; James et al., 1992). These observations are relevant to both the assay of cytokines (James et al.,

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1994) and their therapeutic application. Furthermore as previously stressed, they may help explain some of the immunological effects previously attributed to α_2-M (James, 1990).

In order that the possible nature and immunological significance of cytokine binding might be further investigated there is the need for a simple, effective procedure for separation of α_2-M-cytokine complexes from free cytokine. This presentation describes one such method which exploits the presence of a large number of low affinity zinc-binding sites on α_2-M (Gettins and Cunningham, 1986) thus permitting the separation of this molecule from other plasma proteins by zinc affinity chromatography (Kurecki et al., 1979). This simple procedure should facilitate the study of the nature of cytokine interactions with α_2-M and permit investigations with isolated complexes themselves rather than the complicated mixtures hitherto studied.

2. Materials and methods

2.1. Source of α_2-M and cytokines

Human α_2-M was prepared from frozen normal human plasma by zinc affinity chromatography as previously described (Kurecki et al., 1979). It was converted to the electrophoretically 'fast' form by dialysis against 200 mM methylamine in 50 mM Tris-HCl buffer pH 8.2; this product is hereafter referred to as α_2-Mme. Unbound methylamine was removed by dialysis against 0.06 M PBS (phosphate-buffered saline pH 7.2, containing 0.15 M NaCl). The purity of the original α_2-M and its conversion to the 'fast' form were confirmed by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. The labelled cytokines used were commercially available recombinant human products; the ^{125}I-labelled IL-2 (specific activity > 600 Ci/mmol) and IFN-γ (specific activity approximately 675 Ci/mmol) were labelled by the chloramine-T procedure and were purchased from Amersham International, (Aylesbury, Bucks, UK), while ^{125}I-labelled TNF-α (labelled by the Bolton-Hunter method, specific activity > 30 μCi/μg) was purchased from DuPont (Stevenage, Herts, UK). It should be noted that according to the manufacturers' data sheet all the cytokines bind specifically to their receptors on appropriate target cells. After reconstitution in PBS containing 0.1% BSA the cytokines were aliquoted and stored at -70°C. The percentage of counts precipitated by 20% (w/v) tri-chloro acetic acid (TCA) was routinely assessed and always exceeded 85%. Complexes were prepared by incubating α_2-Mme and labelled cytokines for 2 h at 37°C. Experiments designed to assess the feasibility of the technique involved incubating 3 pmol of α_2-Mme with approximately 300 fmol of labelled cytokine. When larger amounts of complex were required for further investigation 150 pmol of α_2-Mme were incubated with 1 pmol of cytokine.

2.2. Isolation of α_2-M-cytokine complexes

Fast-flow chelating Sepharose 6B (Pharmacia, Milton Keynes, UK) was washed in 0.1 M phosphate buffer pH 6.5 to remove preservative and packed into a 180×15 mm column. After equilibration with 0.1 M phosphate buffer pH 8.0 (containing 0.8 M NaCl) the column was charged by pumping through 0.04 M zinc chloride until free zinc appeared in the effluent. Excess unbound zinc was then eluted from the column with 0.1 M phosphate buffer pH 6.5 (containing 0.8 M NaCl). The α_2-M-cytokine mixture was then applied to the column and eluted with 0.1 M phosphate buffer pH 6.5 (containing 0.8 M NaCl), followed by 0.1 M acetate buffer pH 4.5 (containing 0.8 M NaCl). The pump speed was maintained throughout at 12 ml/h. The protein concentration of the effluent was determined by measuring the optical density at 280 nm, whilst the presence of labelled cytokine was determined by γ scintillometry. On completion of each separation columns were regenerated for further use by eluting with 0.05 M EDTA (containing 0.8 M NaCl) followed by recharging with zinc as described above. The tube-based batch assay utilised the same procedure for equilibration and charging of the Sepharose as indicated for column preparation, except that the process was carried out in a 50 ml centrifuge tube and gel washing was achieved by centrifugation ($200 \times g$ for 10 min). Once charged

with zinc, 1 ml Sepharose was added to the prepared α_2-M-cytokine mixture (300 pmol α_2-M + 150 fmol cytokine) and mixed on a rotator for 1 h to ensure the α_2-Mme was thoroughly adsorbed to the Sepharose. The resultant slurry was washed and centrifuged, unbound cytokine was eluted in the phosphate buffer supernatant. The buffer was changed to 0.1 M acetate saline pH 4.5 and incubated for a further hour to elute the α_2-M complex in the supernatant and the Sepharose regenerated as per the schedule described above.

3. Results and discussion

In these experiments we have been able to demonstrate that α_2-M-cytokine complexes can be readily separated from free cytokine. It is apparent (Figs. 1*a*, 1*b*, 2*a* and 2*b*) that cytokine in the absence of α_2-M is almost completely recovered from the column in the equilibrating buffer (0.1 M phosphate buffer pH 6.5 containing 0.8 M NaCl), as was confirmed by enzyme immunoassay (data not shown). In contrast how-

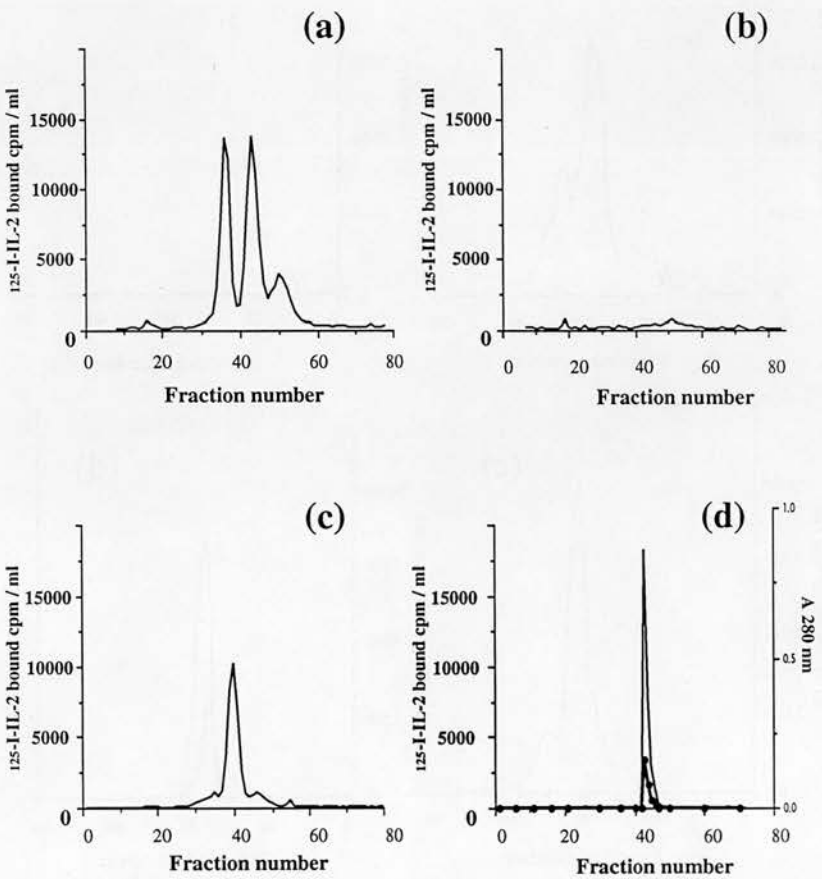

Fig. 1. The isolation of α_2-M/^{125}I-IL-2 complexes from α_2-M/^{125}I-I-2 mixtures by zinc affinity chromatography. This figure portrays the elution profiles of both free ^{125}-I-IL-2 (*a*, *b*) and ^{125}I-IL-2-α_2-M mixtures (*c*, *d*). Note that the free cytokine alone was recovered in the pH 6.5 phosphate buffer (*a*) while in the mixture an appreciable proportion of counts were eluted along with α_2-M by the pH 4.5 acetate buffer (*d*). In this experiment 3 pmol of α_2-M were incubated with 300 fmol of ^{125}I-IL-2 for 2 h at 37°C and applied to a 180 × 15 mm zinc affinity column. Elution was carried out at 12 ml/h and 1 ml fractions collected. (cpm (———); absorbance at 280 nm (●———●)).

ever, in the presence of α_2-Mme an appreciable proportion of the counts were eluted with the acetate buffer used to displace the α_2-M from the column (Figs. 1*c*, 1*d*, 2*c* and 2*d*). The presence of bound cytokine in the acetate recovered fraction was also confirmed by immunoprecipitation with specific antisera to α_2-M and by PAGE analysis followed by autoradiography (data not presented). On the basis of repeated separations of a number of α_2-M-cytokine mixtures by both the column and batch methods, it is apparent that the technique is highly reproducible (Table 1). Furthermore, studies have revealed as expected, that labelled cytokines bound more effec-

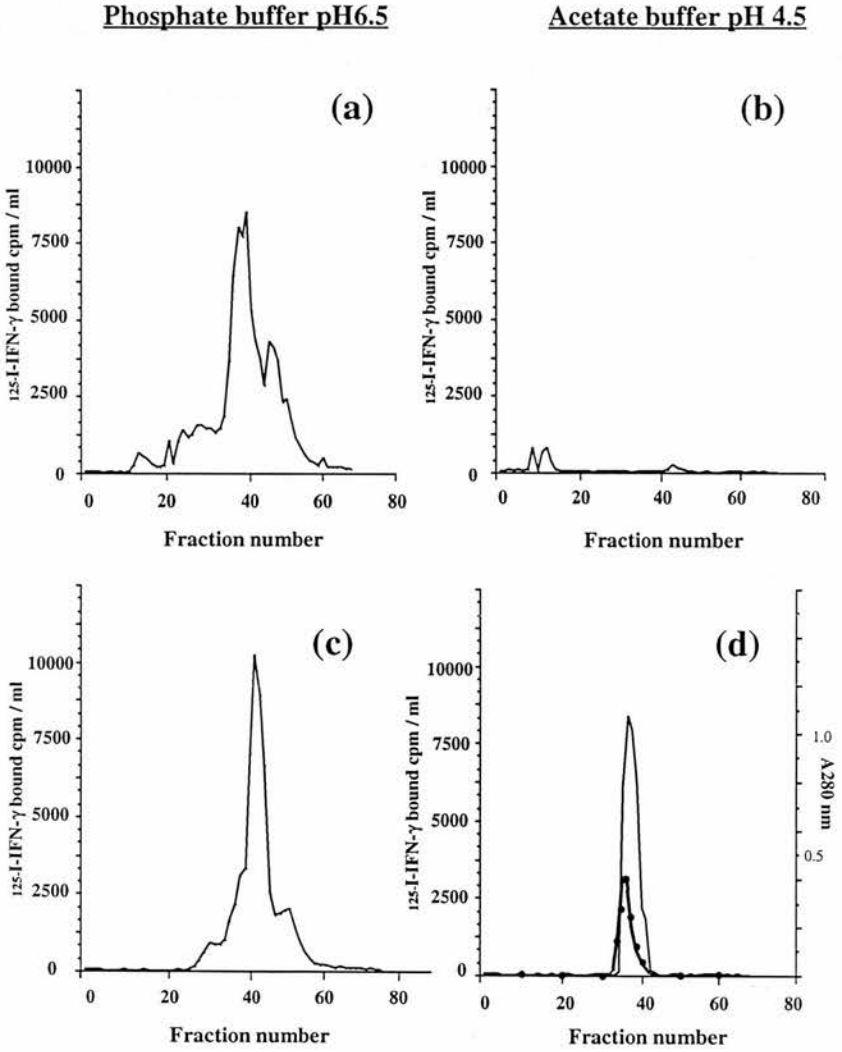

Fig. 2. The isolation of α_2-M/^{125}I-IFN-γ complexes from α_2-M/^{125}I-IFN-γ mixtures by zinc affinity chromatography. This figure portrays the elution profiles of both free ^{125}I-IFN-γ (*a*, *b*) and ^{125}I-IFN-γ/α_2-M mixtures (*c*, *d*). Note that the free cytokine alone was recovered in the pH 6.5 phosphate buffer (*a*) while in the mixture an appreciable proportion of counts were eluted along with α_2-M by the pH 4.5 acetate buffer (*d*). In this experiment 3 pmol of α_2-M were incubated with 300 fmol of ^{125}-I-IFN-γ for 2 h at 37°C and applied to a 180 × 15 mm zinc affinity column. Elution was carried out at 12 ml/h and 1 ml fractions collected. (cpm (———); absorbance at 280 nm (•———•)).

Table 1
The reproducibility of zinc affinity chromatography for isolating α_2-M cytokine complexes from α_2-M-cytokine mixtures

Cytokine [a]	Run	% of counts eluted in acetate buffer pH 4.5	
		Column	Batch
^{125}I-IL-2 + α_2-Mme	1	34.4	28.6
	2	35.9	29.8
	3	44.9	25.9
^{125}I-IFN-γ + α_2-Mme	1	14.5	11.3
	2	18.2	12.5
	3	18.4	13.3
^{125}I-TNF-α + α_2-Mme	1	28.3	18.4
	2	30.2	18.5
	3	–	19.1

[a] ^{125}I-labelled cytokine incubated with α_2-M at ratios indicated in the Materials and Methods section and separated either by the column or batch method.
Note: an appreciable and consistent number of counts are always eluted along with α_2-M by both column and batch methods, with more recovered by the column method due to more efficient washing.

tively to methylamine-converted 'fast' form of α_2-M than to the native preparation. Nevertheless some binding to the untreated form of α_2-M did occur, possibly indicating that these preparations contained small amounts of 'fast' α_2-M generated during the original isolation procedure.

This technique for α_2M-cytokine complex production is a logical adaptation of an existing α_2-M purification protocol and should be readily applicable to the separation of complexes between α_2-M and other biologically important molecules, providing that complex formation does not block zinc binding sites on the α_2-M molecule. It does not depend on specialised equipment or lengthy procedures for effectiveness and has also been successfully adapted by us to a centrifuge tube-based batch format, thus permitting the rapid processing of multiple samples. Furthermore it can be applied to more complex mixtures, e.g., serum and labelled cytokine. This technique should facilitate the study of cytokine α_2-M interactions. It should be stressed that in the column and batch procedures described an excess of solid phase was used to ensure maximum resolution. Studies undertaken by us indicate that columns containing 1 ml volume of the chelating gel are capable of binding 2 mg of isolated α_2-M under the conditions described. Furthermore columns of the dimensions used are routinely employed by us to completely remove the α_2-M from 25 ml of serum or plasma and have been shown to bind at least 150 mg of α_2-M. While it is possible that the binding of α_2-M may be inhibited by interaction with cytokine we believe that any such effects will not impair the usefulness of this procedure.

Of additional interest is the apparent heterogeneity of the labelled cytokine preparation (confirmed by gel permeation chromatography and PAGE analysis – data not shown). It is obviously important to determine how much binding is attributable to cytokine degradation products. In this connection it should be noted that our preliminary data suggests that α_2-M preferentially binds the intact cytokine although some interaction with degradation products may also occur. Furthermore others have shown that biologically active cytokines can be recovered by dissociation of α_2-M complexes (O'Connor-McCourt and Wakefield, 1987) and that complex formation itself is not an artefact of the iodination process (Gettins and Crews, 1993).

4. Acknowledgements

The authors would like to thank the Cancer Research Campaign (award no. sp2047/0201) and Tenovus for their generous financial support. S.-F. Elliott holds a University of Edinburgh Faculty of Medicine research studentship.

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The effect of α_2 macroglobulin in commercial cytokine assays

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(Received 2 August 1993, accepted 17 September 1993)

Abstract

α_2 macroglobulin (α_2M), a 725 kDa plasma protein, has been reported to bind a range of cytokines. We have therefore investigated its effect on a number of commercial cytokine assays. The methylamine converted fast form of the molecule was found to reproducibly depress, by 26% or more, the standard curves obtained with certain commercial assays for IL-2 and for tumor necrosis factor α, and had a small inhibitory effect on some IL-4 assays. In contrast it slightly enhanced or had no effect on ELISAs for IL-1β and IL-6. The inhibition observed was directly proportional to the concentration of α_2M used in the range 0.5–5 mg/ml. Studies in the IL-2 system also revealed that it was largely attributable to the fast form of α_2M. Preliminary evidence suggests that the effects observed may be dependent on the assay source. These findings may be relevant to the assay of biological fluids in which α_2M is present.

Key words: α_2 Macroglobulin; Cytokine; ELISA

1. Introduction

α_2 macroglobulin (α_2M) is a high molecular weight plasma protein (725 kDa) present at 2–4 mg/ml in the blood and also in relatively high concentrations in extravascular fluids including lymph. To date no individuals lacking this protein have been identified, suggesting that absence of the appropriate gene constitutes a lethal deletion. Furthermore, the protein is highly conserved, homologues having been identified in many lower species (Starkey and Barrett, 1982).

In plasma, it exists primarily, but not exclusively as an electrophoretically 'slow', native form, which can be converted to an electrophoretically 'fast' form by the action of proteases or primary amines (Barrett et al., 1979). The conformational changes which accompany conversion to the fast form expose a hydrophobic domain on the surface of the molecule which can bind to receptors on macrophages, hepatocytes and fibroblasts (Roche et al., 1988; Sottrup-Jensen et al., 1986; Van Leuven et al., 1986 a, b). Interaction of the fast form of α_2M with this receptor results in its rapid clearance from the circulation.

The primary physiological role of α_2M is that of a pan-protease inhibitor exhibiting the ability to bind all endopeptidases (Barrett and Starkey, 1973; Gonias et al., 1982). There have, however,

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Abbreviations: α_2M, α_2 macroglobulin; α_2Mm, α_2 macroglobulin methylamine.

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been many reports of α_2M impairing immune responses (reviewed in James, 1980), and recently it has been observed that α_2M may bind a number of cytokines, leading to the suggestion that the effects of α_2M on the immune system may be linked to its interaction with cytokines (James 1990; Borth, 1993). The cytokines reported to bind to α_2M include platelet derived growth factor (PDGF), IL-2, IL-1β, IL-6, tumor necrosis factor α (TNFα), and IFN-γ (Huang et al., 1984; Borth and Teodorescu, 1986; Borth and Luger, 1989; Matsuada et al., 1989; Wollenberg et al., 1991; James et al., 1992, respectively). In almost all cases it would appear that cytokine binding is predominantly to the electrophoretically 'fast' form of the molecule.

Within clinical and research environments there is an increasing interest in the assay of cytokines in biological fluids and tissue culture supernatants. In view of the high concentration of α_2M in many body fluids and tissue-culture supernatants, and the α_2M-cytokine interactions referred to above, it seemed desirable to establish the effect of α_2M on a number of commercially available cytokine ELISAs. Our studies reveal that the 'fast' form of α_2M inhibits certain cytokine assays without seriously affecting others.

2. Materials and methods

Human α_2M was kindly donated by Behringwerke, Marburg, Germany, and was 82.6% pure as judged by electrophoresis, the principle impurities being albumin and immunoglobulins. In addition, highly purified electrophoretically 'slow' native α_2M was prepared from fresh plasma sources by zinc affinity chromatography (Kurecki, 1979). Electrophoretically 'fast' α_2M was generated from both α_2M sources by treatment with methylamine (Gonias et al., 1982). In brief, α_2M was dialysed against 200 mM methylamine in 50 mM Tris-HCl (pH 8.2) for 6 h. Unbound methylamine was then removed by dialysis against phosphate-buffered saline (pH 7.2, 0.06 M containing 0.15 M NaCl) (PBS). This product is referred to hereafter as α_2M methylamine (α_2Mm) or 'fast' α_2M. Conversion to the electrophoretically fast form was confirmed by polyacrylamide gel electrophoresis (PAGE) (King and Laemmli, 1973). Human serum albumin (98–99% purity, Sigma Chemical Company, Poole, England) was used as a control protein. All samples were diluted in and dialysed against PBS, aliquoted and then stored at −70°C prior to use.

The cytokine assay kits were purchased from a number of commercial sources as indicated in the results; all were two-site ELISAs, and the protocols followed were as specified by the manufacturer.

The effect of α_2M derivatives and albumin in the assays were determined as follows. 1 vol. of the protein under test was added to one volume of each standard to give a final protein concentration ranging from 0.5 to 5 mg/ml. Samples of these preparations were incubated for 2 h at 37°C prior to application to the ELISA plate. ELISA assays were simultaneously performed with standards not preincubated with these proteins. All assays were carried out in duplicate, and where pronounced inhibition was seen, assays were repeated 2–6 times.

Table 1
A summary of the effect of methylamine converted α_2M on ELISA assays for cytokines

Cytokine	Supplier	Number of kits assayed	Effect on ELISA assay of:	
			α_2Mm	Albumin
IL-2	Amersham	6	−36.5% (±3.8)	−5.5% (±6.0)
TNFα	Amersham	2	−23.4% (±3.4)	−10.4% (±4.5)
IL-6	Amersham	2	+13.8% (±0.0)	+3.75% (±2.6)
IL-1β	Medgenix	2	+18.0% (±12.3)	+31.5% (±4.3)
IL-4	Amersham	2	−22.6% (±11.4)	−11.4% (±3.0)

The methylamine treated α_2M inhibits the assays for IL-2, TNFα, and IL-4, and slightly enhanced those for IL-6 and IL-1β.

Unless otherwise stated, the results are presented either as the standard curves generated in the presence or absence of α_2M or as the percentage inhibition observed at the highest α_2M and standard concentrations.

3. Results

The results obtained are summarised in Tables 1 and 2, and are presented in Figs. 1–3.

It was found that α_2Mm reproducibly inhibited the detection of cytokine in the Amersham kits for IL-2, and for TNFα (Figs. 1A and 1B). This inhibition was observed with different batches of both ELISA kits, and was directly related to the concentration of α_2Mm added. In contrast, the inhibition of cytokine detection was independent of the concentration of the cytokine standard (Table 2), though the absolute level of inhibition did increase as the cytokine concentrations increased. Further studies in the IL-2 system revealed that the interference was primarily associated with the fast form of the molecule (Fig. 2). It should be noted that in these systems tested, a smaller inhibition was always observed with albumin.

The effect of α_2M on other assays studied were less marked. In brief, a small increase was seen in the Amersham ELISA for IL-6 and in the Medgenix assay for IL-1β (Table 1, and Figs. 3A and 3B), and a small variable inhibition was observed in an IL-4 assay (Table 1, and Fig. 3C). However, in most of these cases the presence of albumin yielded a similar effect to that observed with α_2Mm.

Table 2
The percentage inhibition observed when α_2 macroglobulin methylamine was applied to a range of cytokine standards in four similar IL-2 ELISA kits

Standard concentration (pg/ml)	Percentage inhibition			
	1	2	3	4
62	30.0	40.1	37.0	42.6
125	32.0	38.7	38.7	30.9
250	34.5	40.9	42.2	34.8
500	32.2	32.2	35.2	31.2
1,000	30.5	37.8	34.7	26.4

α_2M methylamine was pre-incubated with standards from the ELISA kits for 2 h at 37°C prior to application to the system. The decrease in the detection of IL-2 across the standard range is recorded. The inhibition observed is consistent throughout the concentrations of standard and between experiments.

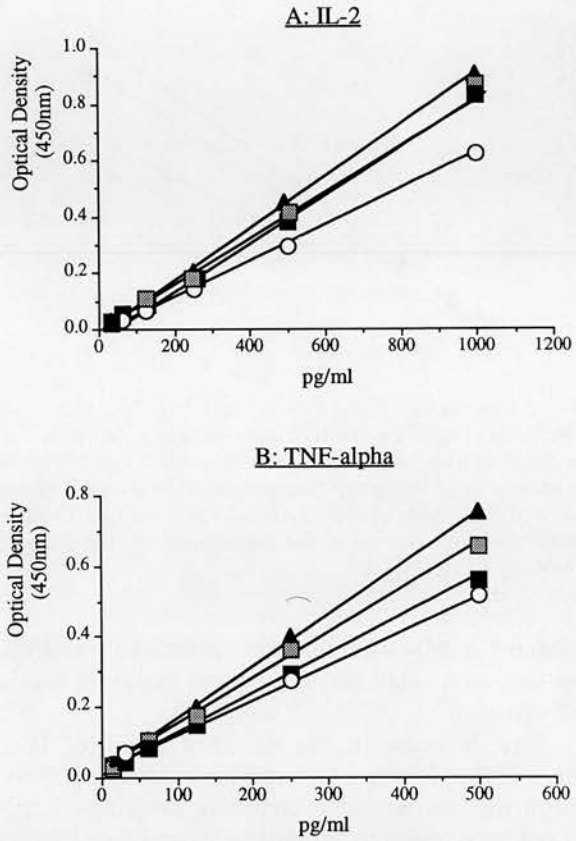

Fig. 1. The effect of methylamine treated α_2 macroglobulin at 5 mg/ml (○) or at 1 mg/ ml (■), or of albumin at 5 mg/ml (grey squares) on cytokine assays for IL-2 and TNFalpha was assessed by pre-incubating assay kit standards with these proteins for 2 h at 37°C and then applying the incubated mixtures to the assay systems. The figure compares the inhibitory effect found with the standards alone (▲). Note that α_2Mm at 5 mg/ml yielded the greatest inhibitory effect.

4. Discussion

In these studies we have shown that α_2Mm may influence cytokine ELISAs in different ways. Thus, with certain IL-2 and TNFα assays the presence of α_2Mm resulted in an inhibition of the detection of the cytokines, whereas in other

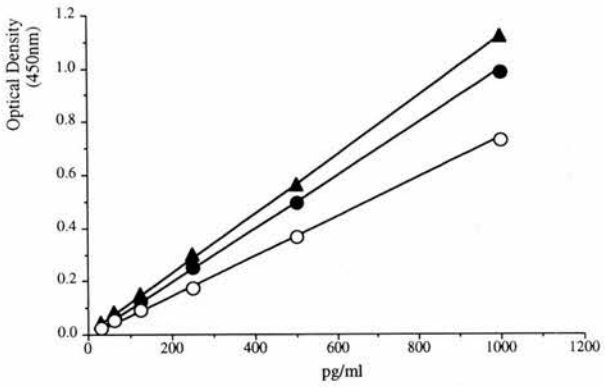

Fig. 2. Comparative study of the effects of 'fast' (○) and 'slow' (●) forms of α_2M on the IL-2 assay. Native α_2M ('slow') or α_2M methylamine ('fast') were incubated for 2 h at 37°C with standards in an IL-2 assay. Comparison of the standard curves derived were made against the standards alone (▲). Greater inhibition was achieved in the presence of the fast form of α_2M.

systems α_2Mm and albumin either had no effect or caused a small increase in the apparent levels of cytokine.

The decrease in the detected levels of IL-2 and TNFα in the presence of α_2Mm may have been due to partial or complete masking of epitopes recognised by either the 'capture' or 'detector' antibodies. The observation that the percentage inhibition is independent of the concentration of standard would seem to support this hypothesis. Such masking might result from the binding of cytokine to residues on the outer surface of the α_2M molecule or in the core of the protein in a similar manner to proteases. An alternative theory would be that endogenous proteases bound to α_2M degrade the cytokine standards (Borth and Teoderescu, 1986). Conversely, the enhancement seen in some instances may be due to improved exposure of particular epitopes resulting from α_2M-cytokine interactions, or improved stability resulting from the antiprotease activity of α_2M itself, or to a non-specific protein effect. These various hypotheses remain to be tested.

The limited studies that we have performed to date suggest that the effects of α_2M in cytokine assays may be dependent on the source of the kit used. For example, in preliminary studies our

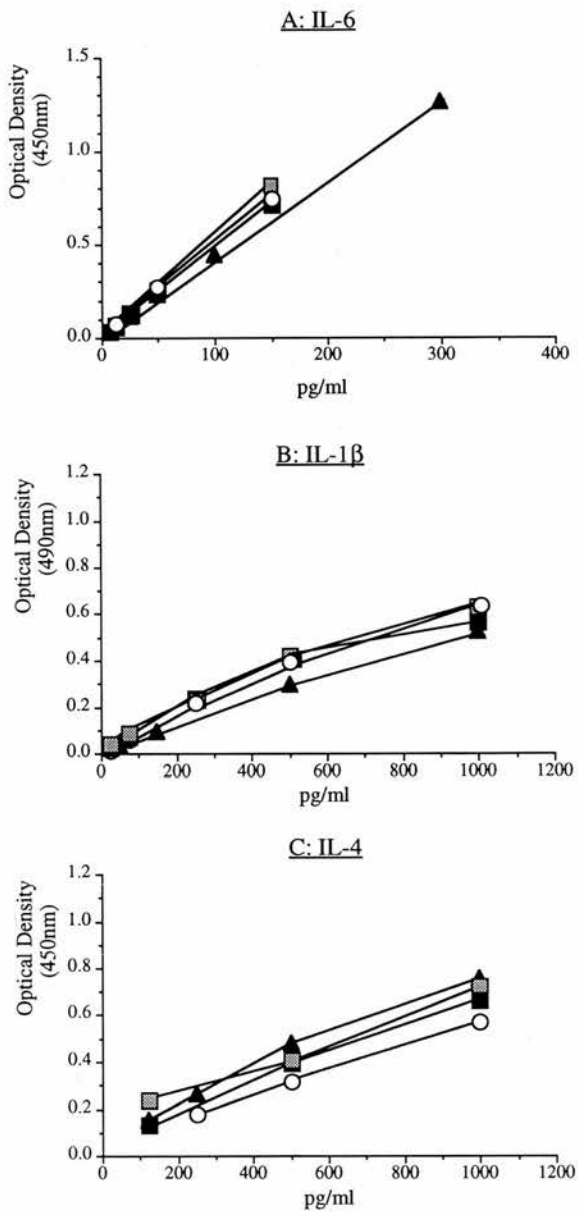

Fig. 3. The effect of α_2 macroglobulin methylamine at 5 mg/ml (○) and 1 mg/ml (■), or albumin at 5 mg/ml (grey squares) on cytokine assay kits after preincubation at 37°C for 2 h with kit standards. The incubated samples were applied to the assay kits as normal. The figures demonstrate the effect of the proteins on the detection of IL-6, IL-1β, and IL-4 in assay systems in comparison to controls, i.e., standards alone (▲). Note that the presence of α_2M and albumin at 5 mg/ml resulted in an increase in the apparent levels of IL-6 and IL-1β.

laboratory reported that α_2Mm did not impair an ELISA for TNFα (James et al., 1992). Further studies are necessary to establish whether the effects of fast α_2M on cytokine assays is independent of the source but such studies would be extremely expensive. We believe that the variation between kits and cytokines may possibly occur due to differences in the epitopes recognised by the antibodies in the two-site assays. In some cases the epitopes may be partially or completely masked following α_2M-cytokine interactions, whilst in others they remain exposed to both capture and detector antibodies. The possibility that α_2M preferentially binds a subpopulation of the cytokine, ie denatured protein or peptide fragments, is also worthy of consideration.

These studies confirm and extend the reported observations on cytokine-α_2M interactions (reviewed in James, 1990; Borth, 1993), and provide evidence that in certain cases these may interfere with cytokine assays. These results need to be considered when precise quantitation of cytokines is required and may be especially relevant in clinical situations where high levels of circulating α2M are known to exist (e.g., in diabetes), and where abnormally high levels of proteolytic activity may also be anticipated. Finally, it may be possible to circumvent the inhibitory effect observed in the current study by the use of combinations of antibodies recognising cytokine epitopes not masked by α_2M.

Acknowledgements

The authors wish to acknowledge the generous financial support of the Cancer Research Campaign (grant no. sp2047/0201) and Tenovus, Scotland. S.-F. Elliott was in receipt of a University of Edinburgh Faculty of Medicine research studentship. We are also indebted to Behringwerke AG for supplying α_2 macroglobulin.

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