STUDIES ON THE MODIFICATION OF THE ACUTE PHASE RESPONSE IN MAN

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DECLARATION

The work presented in this thesis was solely the work of the author, except where stated.

ABBREVIATIONS

ADP	adenosine diphosphate
BOC	t-butyloxycarbonyl
BW755C	3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride
CDI	1,1'-carbonyldiimidazole
CPS	C-polysaccharide
CRP	C-reactive protein
CSF	colony stimulating factor
Con A	concanavalin A
DCCD	dicyclohexylcarbodiimide
DIEA	diisopropylethanolamine
DMF	dimethylformamide
ELISA	enzyme linked immunosorbent assay
EMIT	enzyme mediated immunoassay technique
EP	endogenous pyrogen
ESR	erythrocyte sedimentation rate
ETAF	epidermal cell-derived thymocyte stimulating factor
HDL	high density lipoprotein
HSF	hepatocyte stimulating factor
IL	interleukin
iPrOH	isopropanol
IRMA	immunoradiometric assay
IV	intravenous
KLH	keyhole limpet haemocyanin
LAF	lymphocyte activating factor
LEM	leukocyte endogenous mediator
LPS	lipopolysaccharide
LT	leukotriene

MCF	mononuclear cell factor
PBMN	peripheral blood mononuclear cells
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PHA	phytohaemagglutinin
PIF	proteolysis inducing factor
PMA	phorbol myristate acetate
RBP	retinol binding protein
RIA	radioimmunoassay
RID	radial immunodiffusion
RLS	relative light scattering
SAA	serum amyloid protein A
SAP	serum amyloid protein P
TEMED	N,N,N′,N′-tetramethylethylenediamine
TNF	tumour necrosis factor
TSST-1	toxic shock toxin 1
TXA_2	thromboxane A_2

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SUMMARY

Methods for several positive and negative acute phase proteins were developed using a Hyland LAS-R nephelometer. The proteins measured were C-reactive protein, α_1-antichymotrypsin, α_1-antitrypsin, α_1-acid glycoprotein, haptoglobin, α_2-macroglobulin, albumin, and prealbumin. Using these methods, the effects were studied of an elevated environmental temperature (28°C) on the concentrations of proteins following elective upper gastrointestinal tract surgery. A control group of patients was nursed at a temperature of 20°C. Urine excretion of nitrogen, 3-methylhistidine, cortisol, 17-hydroxycorticosteroids, noradrenaline, and adrenaline was also measured.

All patients demonstrated an acute phase response which corresponded closely to that described in previous studies, but exposure to elevated temperatures did not affect the magnitude of the changes in serum proteins, nitrogen or 3-methylhistidine excretion, or the excretion of catecholamines. There were significant decreases in urine volume and in the excretion of cortisol and 17-hydroxycorticosteroids.

To improve the precision and sensitivity of the C-reactive protein assay, an immunoradiometric assay was developed. An antibody obtained from Seward Laboratories was coupled to microparticulate cellulose with 1,1′-carbonyldiimidazole. A second antibody obtained from Atlantic Antibodies was immunopurified using pure

C-reactive protein, also coupled to cellulose, and labelled with ^{125}I. Sample was incubated with the labelled antibody for two hours, after which the solid-phase antibody was added and incubation continued with agitation for a further two hours. The cellulose was washed, and bound iodine was counted. The assay correlated well with radial immunodiffusion, EMIT, and nephelometric methods, and the precision was good (between batch precision 11.9% at a level of 35.4 mg/l). The sensitivity of the method was 5 μg/l.

The effect of treating patients suffering from rheumatoid arthritis with either injectable gold (gold sodium thiomalate) or oral gold (auranofin) was compared with treatment with placebo over a period of 48 weeks. Concentrations of acute phase proteins were measured by immunonephelometry and C-reactive protein was measured by the immunoradiometric method. A number of clinical measurements were also carried out for comparison purposes (pain score, duration of morning stiffness, grip strength, and articular index). All of the acute phase proteins were decreased by active treatment, though oral gold was less effective than parenteral gold. C-reactive protein and haptoglobin were decreased to the greatest extent by treatment. The most effective proteins for the detection of active disease before treatment were C-reactive protein and α_1-antichymotrypsin.

A discriminant function was derived which was capable of separating the patient groups by treatment at the 24 week time point. Because of the substantial between-patient

variation, the acute phase proteins were less efficient at discriminating between groups of patients than they were at monitoring the progress of individual patients. The discriminant function depended mainly on the values of IgA and pain score. 74% of patients could be correctly classified by using this function.

To investigate the factors controlling the acute phase response more deeply, an assay for interleukin 1 was set up. The assay which was chosen was the lymphocyte activation assay. Murine thymocytes were incubated with samples containing interleukin 1 and a submitogenic concentration of phytohaemagglutinin, a plant mitogen. After three days, tritiated thymidine was added to assess the rate of cell proliferation, which was a function of the concentration of interleukin 1 in the sample. The effects of using different strains of mouse and different concentrations of mitogen were investigated.

The factors controlling the release of interleukin 1 from an immature monocyte cell line, the U937 line, were studied. It was found that the cells required a CO_2 atmosphere for rapid growth, and that interleukin 1 production was maximal in the presence of Ultroser G, a serum substitute, although the cells did not grow well in the absence of serum. Cell concentrations of greater than 10^6 /ml were required. Bacterial supernatants from strains of Staphylococcus aureus which caused toxic shock syndrome were found to be effective stimuli for interleukin 1 release.

Various drugs which affect the metabolism of arachidonic

acid were added to cultures of U937 cells in the presence of toxic shock toxin, and the production of interleukin 1 was measured. It was found that inhibitors of the cyclooxygenase and lipoxygenase enzymes (indomethacin and BW755C respectively) had no effect on interleukin 1 production, but that inhibition of thromboxane synthesis by Dazmegrel strongly inhibited interleukin 1 release. Low concentrations of prostacyclin and its stable analogue, Iloprost, augmented production, but higher concentrations inhibited it.

In a preliminary attempt to develop an immunoassay for interleukin 1, peptides chosen from the published amino acid sequence of interleukin 1β, the major form secreted by macrophages, were synthesised chemically. Polyclonal antisera were raised in rabbits against these peptides. It was found that high titre antisera could be produced against peptides corresponding to both the N terminus and the C terminus of interleukin 1. However, recombinant interleukin 1 did not bind avidly to the antisera directed against the N terminus, possibly because of modifications introduced during the synthesis of the recombinant material.

It is hoped that it will be possible to develop an immunoassay for interleukin 1 using these antisera, and that they will be used for its purification and for histochemical studies. These developments should lead to a greater understanding of the role of interleukin 1 in the acute phase reaction.

CHAPTER 1

INTRODUCTION

1.1. Historical Background

Inflammation has been described by Ebert & Grant (1974) as "a process which begins following sublethal injury to tissue and ends with permanent destruction of tissue or with complete healing." The importance of this definition is that it characterises inflammation as a dynamic process, which is capable of removing the threat to the organism. The features of inflammation may be caused by infection, mechanical injury, ischaemia, or immunologically mediated damage.

The principal signs of inflammation have been recognised since the time of the ancient Greeks. Hippocrates clearly distinguished the characteristics of several infectious diseases, including pleurisy, pneumonia, and tuberculosis, and made studies on fever. Celsus, a Roman medical writer who lived at around the time of Christ, noted that the cardinal signs of local inflammation were "rubor et tumor, cum calore et dolore" (redness and swelling, with heat and pain). Galen (c 130 - 200 A.D) added the sign of loss of function. Much of inflammation research has been devoted to explaining these features.

During the middle ages, there was a tendency to accept the work of Galen and the other ancient authorities without question, but the careful scientific study of the

body and its diseases was revived in the Renaissance. This new approach was typified by the work of John Hunter, an 18th century surgeon who wrote an extensive treatise on the nature and treatment of inflammation, and contrasted it with trauma. He noted that "There is a circumstance attending accidental injury which does not belong to disease - namely, that the injury has in all cases a tendency to produce both the disposition and the means of cure" (Hunter, 1794). In modern terminology, this implies that the physiological effects of injury are a response of the body designed to promote healing.

The work of Pasteur and the other great microbiologists of the 19th century culminated in the proof by Koch that many diseases could be caused by well-defined, isolable microorganisms. It became clear that much inflammation was caused by infection with bacteria, and that fever was a consequence of this inflammation, rather than a disease in its own right.

The mechanisms of local inflammation were studied by Cohenheim and Metchnikoff. Cohenheim emphasised the role of vascular endothelium in controlling the flow of fluid into inflamed tissue spaces. All the classical signs of inflammation could be explained as the consequences of the influx of blood and actively metabolising cells into the tissue, with the resultant increase in tissue pressure. Metchnikoff, on the other hand, pointed out that animals without a vascular system were also able to mount a defensive response against foreign bodies, mediated by the phagocytic cell. He regarded this cell type as the most

important in body defence, and thought that the vasculature merely controlled the access of these cells to tissues.

The primary role of the phagocyte now seems amply confirmed. As well as being important in the defence against bacteria and foreign bodies, it now appears that the macrophage, in particular, has evolved new functions in higher organisms to control the specific and non-specific immune responses (Unanue, 1981; Cohn, 1983). It secretes a huge variety of mediators, which affect most cells in the body (Takemura & Werb, 1984). It is likely, however, that the role of endothelial cells may not be simply to control the flow of cells and substrates from the blood into the tissues. They secrete mediators of their own, controlling processes such as blood clotting (Bevilacqua et al, 1984) and chemotaxis (Mercandetti et al, 1984) and they can express class 2 histocompatibility antigens, implying a possible role in antigen presentation or in immune regulation (Ashida et al, 1981).

1.2. Local Inflammation

The process of inflammation at the cellular level is complex, involving several major stages and the release of a large number of potentially important mediators (Smith, 1985). Haemostasis causes the release of platelet-derived factors, such as ADP, prostaglandins and thromboxane, and vasoactive amines. Contact activation of Hageman factor by damaged endothelium causes activation of the coagulation

and complement cascades, and release of kinins. Phagocytic cells, normally granulocytes first and then macrophages, are attracted into the area by complement components, bacterial products, and elements released from damaged tissue, and begin to debride the affected area. At this stage, uncontrolled release of proteases and active oxygen species can cause damage to normal surrounding tissue. Fibroblasts, endothelial cells, and smooth muscle cells are attracted into the wound by a variety of chemoattractants. Some have been well characterised, such as platelet-derived growth factor (PDGF). Lymphocytes are also attracted by mediators such as interleukin 1 (IL-1) which are secreted by the phagocytic cells. They may then mount an immune response under the influence of IL-1 to defend the body against bacterial invasion.

The connective tissue cells begin to proliferate to form a friable, highly vascular granulation tissue, stimulated by PDGF, IL-1, and various other fibroblast growth factors and angiogenic factors which are less well characterised, but may be released from mesenchymal cells, macrophages, and cells of the immune system. The fibroblasts secrete collagen and extracellular matrix to form a scar, obliterating many of the vascular cells in the process. If the initial removal of infection is not complete, acute inflammation may become chronic, possibly with the formation of granulomata. In such a complex series of events, it is hardly surprising that it is very difficult to disentangle the relative importance of the various mediators which are known to be present.

1.3. Systemic Signs of Inflammation

It has been obvious for many years that certain systemic signs are commonly exhibited during inflammation, regardless of the exact cause. Fever and increases in pulse rate are obvious and early recognised examples. Another sign common to many infectious and inflammatory states is leukocytosis, which is due mainly to an increase in neutrophils. Selye (1950) noticed that injection of various toxic substances produced a consistent triad of adrenal enlargement, involution of lymphoid tissue, and gastrointestinal ulceration, which he regarded as evidence for a syndrome of "being sick". He suggested that there was a "general adaptation syndrome" which was a protective reaction of the body to attack by any noxious agent. The response to any particular agent would be modified by the unique effects of that agent, and by the endogenous capabilities of the host. He regarded many common diseases as being caused by a defective response to stress.

Selye was too speculative for his ideas to achieve full acceptance. However, it is now well recognised that there are marked similarities between the biochemical responses to infection and to sterile inflammation. Many of the mediators involved will be stimulated by any tissue damage, whatever the cause. Neufeld & Pace (1982) carried out studies comparing the effects of injection of bacteria and turpentine in fasted rats, and concluded that there was a "stereotypic response to inflammation", consisting of rises in body temperature and plasma insulin and

decreases in plasma zinc and free fatty acids.

These findings provide some justification for the attempts to isolate a common pathway of systemic response to inflammation. In the context of injury, this constellation of biochemical changes has long been referred to as the "acute phase response to injury". It is to be expected, however, that the details of the metabolic response will vary depending on the exact nature of the stimulus. For instance, there is a greater increase in ketone body production after trauma than during infection (Neufeld et al, 1982).

1.3.1. Metabolic Rate

DuBois (1924) commented on the fact that there was an apparently regular increase of about 7% in metabolic rate with each degree Fahrenheit rise in body temperature. However, there was substantial variability about this figure. In later studies, Roe & Kinney (1965) found that many patients with severe injury or sepsis had a higher metabolic rate than expected from this relationship, implying a metabolic component in excess of that predicted from pyrexia per se. They also found that after moderate surgery, patients might follow the DuBois formula, have greater metabolic rates than predicted, or even show a decrease in metabolic rate when corrected for temperature (Kinney & Roe, 1962).

1.3.2. Nitrogen Metabolism

It was noticed at the turn of the century (reviewed in Lusk, 1928) that infected patients excreted an excessive amount of nitrogen in their urine. The increased nitrogen excretion bore a direct relationship to metabolic rate in many instances.

The studies initiated by Cuthbertson in the 1930s showed that the increase in nitrogen excretion was accompanied by excretion of several other elements, including potassium, sulphur, phosphorus, and zinc (Cuthbertson, 1931, 1936; Cuthbertson et al, 1972; Davies & Fell, 1974). The ratios between these elements and nitrogen were consistent with an origin from cells rather than from extracellular protein, and it was assumed that most of the excretion represented the breakdown of muscle protein, since this is the largest reservoir of protein in the body. Cuthbertson proved that the bulk of the nitrogen did not arise either from the direct effect of tissue damage or from breakdown of extravasated blood. Similar studies during infections have reached the same conclusions (Beisel, 1984). Most of the nitrogen is excreted in the form of urea, but increases have also been noted in metabolites more specifically derived from muscle, such as 3-methylhistidine (Gross et al, 1978; Long et al, 1981) and creatine (Cuthbertson et al, 1972). In infections there may also be an enhanced excretion of hydroxyproline, presumably derived from extracellular collagen (Beisel, 1984).

Cuthbertson (1942) divided the sequence of events after trauma into two main phases. Immediately after the injury, there was an "ebb" phase, in which body temperature fell, there was often a slight decrease in metabolic rate, and sodium and water were retained. After a few hours, the "flow" phase ensued. Temperature and metabolic rate rose again, often to levels greater than those expected in normal individuals, and nitrogen excretion also began to climb. The sodium which had been accumulated during the ebb phase was excreted during this time. Moore and Ball (1952) added a third stage which continued for weeks or even months after the signs of the catabolic flow phase had disappeared. This was the anabolic phase, in which full muscle strength was regained.

The increase in muscle breakdown was originally thought to represent the use of protein as a fuel, and it was considered that the heat produced by metabolism of amino acids could account for the increased metabolic rate, but it was later shown by Duke et al (1970) that the proportion of total energy needs provided by protein was not increased after injury. Current theories would suggest that the main uses of the amino acids which are released from the periphery and enter the liver are to provide gluconeogenic precursors and amino acid residues for plasma protein synthesis (Kinney, 1976). There may also be some local metabolism of amino acids to compensate for energy deficits in muscle (O'Donnell et al, 1976).

1.3.3. Intermediary Metabolism

It is now known that there are many other alterations in metabolism. An increased blood glucose is a common finding after injury and during infections. This is largely caused by an increased rate of glucose production by the liver (Gump et al, 1974; Long et al, 1971). The rate of disposal of glucose by peripheral tissues is not actually decreased compared to normal, but it fails to compensate for the increased plasma glucose, implying a degree of insulin resistance. If septicaemia becomes overwhelming, however, glucose levels can fall below normal because liver glucose production fails to keep pace with demand in peripheral tissues (Cerra et al, 1979; Wolfe et al, 1977).

It is known that a major contribution to metabolic fuel requirements is made by fat after trauma, but changes in serum concentrations of lipids are very variable. There is often an increase in the plasma concentration of free fatty acids after injury, which may reflect an increase in lipolysis (Barton, 1985), but levels found in infections are not consistent (Beisel, 1984). There may be increases in triglyceride concentrations, particularly during Gram-negative septicaemia, which are caused by a combination of increased triglyceride synthesis by the liver and a decrease in the activity of lipoprotein lipase in the periphery. There may be slight ketosis, but this is inappropriately mild for the degree of starvation commonly present, possibly because of suppression by insulin (Neufeld et al, 1982). This depression in the levels of

ketone bodies is not seen after leg fractures or other non-inflammatory stresses.

1.4. Control of Intermediary Metabolism during Inflammation

The mechanisms by which the metabolic response to injury or infection is regulated have been an active field of study for many years. Theories have been suggested involving wound hormones which might be released from damaged tissue, but none has ever been isolated. It seems more likely that the response is caused by a combination of the secretion of hormones from cells migrating to the site of injury and from endocrine glands, and of the action of afferent neural impulses running from the wound to the central nervous system.

The primary anabolic hormone of the body is insulin. Its functions, apart from regulating blood glucose, include stimulating the laying down of fat stores and the synthesis of protein. The three major catabolic hormones which oppose it are cortisol, glucagon, and adrenaline. Changes in the secretion of several other hormones have been reported, but it is likely that their contribution to the observed changes in intermediary metabolism immediately post trauma is small.

Plasma concentrations of corticosteroids increase within minutes of trauma (Mattingly & Tyler, 1965; Mohler et al, 1985). Peak plasma levels of cortisol in man occur about 6 hours after the start of a surgical procedure (Colley et

al, 1983), and the maximal excretion of cortisol in urine also takes place within the first 24 hours (Mohler et al, 1985). The concentrations in plasma then fall rapidly unless there is a continuing stimulus to production.

Catecholamine secretion also increases rapidly after trauma, though it has only recently been possible to confirm using specific radioenzymatic methods that there are increases in plasma concentrations of both noradrenaline and adrenaline (Nistrup Madsen et al, 1978; Davies et al, 1984). It is thought that the concentration of noradrenaline reflects the activity of the sympathetic nervous system, while adrenaline is secreted mainly from the adrenal medulla. The increases after moderate trauma are transient, and blood levels return to normal within 24 - 48 hours. Excretion in urine may continue for a few more days.

Since they are both elevated markedly immediately post-trauma, cortisol and catecholamines have each been suggested to be the primary hormone involved in the changes in intermediary metabolism after injury. Both are capable on their own of mimicking at least some of the changes noted. Cortisol is catabolic in peripheral tissues. It causes net breakdown of muscle protein, and a consequently increased uptake of amino acids by the liver. These are rapidly transaminated, and their carbon skeletons are eventually used to form glucose. The excess nitrogen is channelled to urea formation and excreted in urine.

The major metabolic effects of catecholamines which are

noticeable at physiological concentrations are mediated by adrenaline (MacDonald et al, 1985). This hormone is lipolytic, causes glycogen breakdown both in liver and muscle, and may also directly inhibit protein synthesis in muscle.

Alberti et al (1980) observed that the serum levels of alanine, lactate, free fatty acids, and urea were correlated with the increase in serum cortisol immediately after thermal injury. He therefore claimed that cortisol was of overriding importance immediately after burns, though there was more doubt about its role in the later stages. He was unable to demonstrate similar correlations after surgery, and suggested that this could have been due to the lesser degree of trauma.

On the other hand, Wilmore et al (1976) strongly advocated that the major effects of trauma could be best explained by a massive sympathoadrenal response. It has been claimed that there are correlations between the concentrations of catecholamines in blood and both the injury severity score (Davies et al, 1984) and the extent of burn injury (Wilmore et al, 1974a). A consistently elevated noradrenaline level in cases of septicaemic shock is a poor prognostic sign (Benedict & Grahame-Smith, 1978).

Alberti et al (1980) also observed increases in glucagon after burn injury, but peak levels were not reached until days 3 - 5 post injury. Increases were also observed by Meguid et al (1974) following major trauma but not after more minor injury. Glucagon may therefore have more

relevance to the continuing loss of body mass in catabolic patients during the flow phase, though these observations may be artefactual, because only systemic blood was sampled, and this may not reflect changes in portal vein glucagon.

Some, but not all stress-related stimuli require an intact afferent nerve supply from the periphery to the brain in order to produce metabolic effects. Spinal anaesthesia (Newsome & Rose, 1971; Kehlet et al, 1980) prevents the cortisol and adrenaline responses to abdominal surgery. However, surgically isolating the hypothalamus and pituitary from the rest of the brain abolished the glucocorticoid response to the fracture of a leg, but not the response to the ischaemic damage caused by a tourniquet (Greer et al, 1970). Endotoxin also does not seem to require intact neural pathways in order to exert its effects on the acute phase response (Makara et al, 1970).

If the response of adrenaline and cortisol is prevented by neurogenic blockade, the post-operative increase in nitrogen excretion is blunted, and the hyperglycaemic response is completely abolished (Kehlet et al, 1980). However, it has never been possible to mimic the response to injury by the infusion of either glucocorticoids or catecholamines alone. The increases in plasma glucose and urine nitrogen excretion are transient and smaller in magnitude than those following injury. It has now been shown that many of the metabolic consequences of trauma can be duplicated by a triple infusion of cortisol,

catecholamines, and glucagon, both in dogs and in humans (Eigler et al, 1979; Bessey et al, 1984; Gelfand et al, 1984). Glucose and nitrogen flux and nitrogen excretion increased to about the same extent as after moderate injury. There was also an increase in metabolic rate. It was noticeable, however, that there were minimal changes in body temperature and the plasma concentrations of metals and acute phase proteins, suggesting that these variables are controlled by other mediators apart from the catabolic hormones.

1.5. Acute Phase Proteins

One of the most striking aspects of the acute phase response is the marked increase in the serum concentrations of several proteins, known as acute phase proteins, some of which can increase as much as 1000 fold within a few days of an episode of trauma. Most are synthesised by the liver, though there is evidence for synthesis by other cells, such as monocytes and lymphocytes (Lipsky et al, 1979; Andersen, 1983). Certain other proteins, such as albumin, prealbumin, and retinol binding protein, decrease after injury. Some authors refer to these as negative acute phase proteins. There are several comprehensive reviews on the nature and significance of the acute phase proteins (Owen, 1967; Koj, 1974; Fischer & Gill, 1975; Laurell, 1985).

A great deal is now known about the physicochemical properties of these proteins and about their behaviour

during episodes of inflammation. Most of them are glycoproteins, and run as alpha globulins under standard electrophoresis conditions. It has been known for many years that a wide variety of stimuli, such as trauma, infection, and neoplastic conditions, can cause increases in their concentrations in blood. Much less is known about the mechanisms controlling the increases in their concentrations, or about their functions.

1.5.1. Classification of Acute Phase Proteins

The main features of the human response to surgery were described by several groups of workers in the 1960s and early 1970s (Aronsen et al, 1972; Werner & Cohnen, 1969; Werner & Odenthal, 1967; Crockson et al, 1966; Clarke et al, 1971). It was found that the acute phase proteins could be divided into three main groups according to the extent to which they increased (table 1.1).

C-reactive protein (CRP) and serum amyloid protein A (SAA) increase rapidly after injury to several hundred times their original values and usually attain their maximal values on the second or third day after surgery. A second group of proteins, including haptoglobin, α_1-antitrypsin, and α_1-acid glycoprotein, increase to levels of only two or three times their original values, and may take a week or more to reach their maximal concentration. The third group, including C3 and caeruloplasmin, only increase by 50 - 100% of their original values. The relative ranking of these proteins is

Table 1.1

Classification of Human Acute Phase Proteins

Group 1. Marked Response

C-reactive Protein

Serum Amyloid Protein A

Group 2. Moderate Response

α_1-antichymotrypsin

α_1-antitrypsin

α_1-acid glycoprotein (orosomucoid)

Haptoglobin

Fibrinogen

Group 3. Weak Response

Caeruloplasmin

C3

preserved in general in infections and chronic inflammatory states, though the exact pattern depends on the disease state.

1.5.2. C-Reactive Protein

C-reactive protein was one of the first proteins to be described as an "acute phase protein". Its biochemical nature and clinical significance are reviewed in Pepys & Baltz (1983). Tillett & Francis (1930) originally described a substance in blood from patients with pneumonia which reacted with and precipitated pneumococcus bacteria. This was further characterised as a protein by Abernethy & Avery (1941), who found that it bound the somatic C-polysaccharide of pneumococcus in a calcium-dependent reaction and hence named it "C-reactive protein".

C-polysaccharide (CPS) is a complex teichoic acid constituent of the cell wall of these bacteria. It has a repeating structure of sugar residues connected by ribitol units (Jennings et al, 1980), and contains phosphoryl-choline bound to galactosamine residues (figure 1.1). The studies of Volanakis & Kaplan (1971) showed that the main binding specificity of CRP was to these phosphorylcholine residues.

CRP can also bind to the type-specific polysaccharide of pneumoccocal strain 27 (Lofstrom, 1944), and to cell wall components of other bacteria and higher organisms such as fungi and parasites (Baldo et al, 1977; Capron et al,

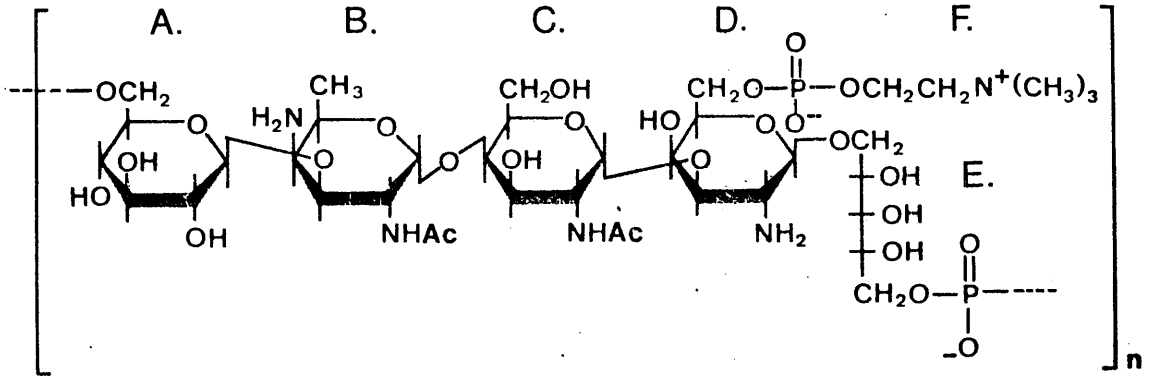


Figure 1.1. Structure of C-polysaccharide.

1964; Fletcher et al, 1980). There is a secondary binding activity to polycationic compounds, which has been demonstrated for polylysine and cationic proteins (Siegel et al, 1975; Potempa et al, 1981).

CRP is now known to be a pentameric protein with a subunit molecular weight of 21000 (Oliveira et al, 1979). It forms planar pentagonal discs in solution, and is a member of the family of proteins called pentraxins. The binding sites for phosphorylcholine are probably on one face of the disc (Roux et al, 1983). The only other known member of the pentraxin family in man is serum amyloid P (SAP) protein (Osmand et al, 1977), although these proteins have been discovered in all animal species studied. Not all are acute phase reactants, however.

Many activities have been reported for CRP, but it is uncertain what its major function is in vivo. It is a highly effective precipitin and agglutinin in man, but, since the binding sites for phosphorylcholine are all on the same face of the CRP molecule, it seems unlikely that this can be a primary function. When it is complexed to CPS, it is able to activate the complement cascade by binding to Clq (Kaplan & Volanakis, 1974). It then promotes all the functions of complement, including adherence of complement components to cells, cell lysis, and opsonisation (Osmand et al, 1975; Mortensen et al, 1976), but only in the human system (Baltz et al, 1982). In contrast, CRP inhibits the activity of the alternative complement pathway (Mold et al, 1984).

Hokama et al (1962) showed that preincubation of

bacteria with CRP stimulated their phagocytosis by neutrophils. Kindmark (1971) confirmed these results. CRP has been shown to have a protective effect against certain bacterial infections in vivo, which may be connected with this opsonisation effect (Mold et al, 1981; Yother et al, 1982; Nakayama et al, 1983). Since CRP binds strongly to chromatin and nucleosomes (Robey et al, 1984), and can be detected binding to necrotic muscle fibres after ischaemic injury (Kushner & Kaplan, 1961; Kushner et al, 1963), it is possible that the major role of CRP is to opsonise damaged tissue and target it for destruction by phagocytic cells.

Mortensen suggested that CRP bound to T lymphocytes and affected their functions (Mortensen et al, 1975; Mortensen, 1979), but it has not been possible to repeat this work. More recently, it was reported that most monocytes and a small subset of lymphocytes possessing the IgG Fc receptor also express receptors for CRP (James et al, 1981a, 1981b). It is probable that a high proportion of these latter cells were in fact natural killer cells. Baum et al (1983) detected CRP bound to the membranes of natural killer cells, which may carry out an important function in their cytotoxic action, possibly in the recognition phase, since they are closely associated with the Fc receptor. Human CRP and the murine equivalent, SAP, may also activate macrophages (Barna et al, 1984) and stimulate the release of IL-1 from monocytes (Sarlo & Mortensen, 1985).

Fiedel et al (1982) found that heat-aggregated CRP

activated platelets. There was an increase in platelet aggregation in response to several stimuli, and degranulation took place with the release of active mediators. Thromboxane synthesis was also stimulated (Simpson et al, 1982). An earlier report (Fiedel & Gewurz, 1976) stating that CRP inhibited platelet aggregation has been withdrawn, and it now appears that this impression was caused by co-purification of a low-molecular weight inhibitor of platelet aggregation, possibly a fragment of CRP itself (Fiedel et al, 1982; Fiedel & Gewurz, 1986). However, Vigo (1985) has claimed that purified unaggregated CRP prevents the activation of platelets by platelet-activating factor.

CRP concentrations in blood increase rapidly after injury. No increase is detectable for about 6 hours (Colley et al, 1983), but the level can increase to several hundred times its original value within the next 24. In rabbits, the doubling time of increase has been reported to be 7.3 hours (Yen-Watson & Kushner, 1974), while in humans after a myocardial infarction, the doubling time was 8.2 hours (Kushner et al, 1978). This increase represents de novo synthesis by the liver, since the fractional catabolic rate of CRP is unaltered by injection of endotoxin or turpentine (Chelladurai et al, 1983). The synthesis rate can increase in rabbits from 6.7 μg/kg/day in unstimulated animals to 560 after turpentine injection (Chelladurai et al, 1982).

The production of CRP is normally short lived and the level returns to normal within a few days. It is not known

whether continuing production of CRP contributes to the pathology of chronic inflammatory conditions, although its presence has been detected in rheumatoid synovial cells (Gitlin et al, 1977) and in the skin lesions of vasculitis in association with complement (Parish, 1976).

1.5.3. Serum Amyloid Protein A

Serum amyloid A protein (SAA) is a circulating protein whose amino acid sequence is closely similar to that of the major protein of reactive (secondary) amyloid (Levin et al, 1973; Rosenthal et al, 1976). The subunit molecular weight of SAA is 12000 when measured under dissociating conditions, but it normally circulates in the blood as an apolipoprotein of HDL (Benditt & Erikson, 1977). Under normal circumstances, it is a trace constituent of HDL, but following stress, SAA can comprise 50% of its apoprotein content (Benditt et al, 1982).

SAA is an even more sensitive acute phase reactant than CRP (Rosenthal & Franklin, 1975; McAdam et al, 1978; Pepys & Baltz, 1983). It has proved difficult to assign exact values to the levels of circulating SAA, since there is no agreed standard material, but it is likely that concentrations can increase by several thousand fold to over a gram per liter. Its level is increased in many chronic inflammatory conditions, particularly those in which amyloid is a recognised complication, such as rheumatoid arthritis.

SAA has been reported to inhibit the antibody response

to red blood cells, acting at an early stage of the response (Benson & Aldo-Benson, 1979, 1982), but the significance of this observation in vivo is not known. The catabolic rate of HDL containing SAA is much faster than that of native HDL (Hoffman & Benditt, 1982), and it has been suggested that this may act as a means of clearing toxic substances from the circulation. It is, for example, known that bacterial endotoxin binds to HDL (Ulevitch et al, 1981).

1.5.4. Other Serum Acute Phase Proteins

α_1-Antitrypsin and α_1-antichymotrypsin are both antiproteases. Antitrypsin is the major component of the α_1 band on electrophoresis. Both proteins increase in blood by two to three fold after surgery, but do not reach peak values for several days. They are also elevated in a variety of other inflammatory and infectious states. The rate of increase in antichymotrypsin is somewhat more rapid than that in antitrypsin, and it increases to a greater extent.

The names of both these proteins are misnomers. Although α_1-antitrypsin is the major trypsin inhibitor in blood, this is not its primary function. It inhibits the activity of a large number of serine proteases, and its preferred name is α_1-protease inhibitor. It has been shown that the greatest affinity of the protein is for neutrophil and macrophage elastase (Beatty et al, 1980). These are phagocyte proteases, which could cause severe tissue

damage if allowed to act unchecked. In the genetic defect of α_1-antitrypsin defiency, patients often present with early emphysema, which is thought to be due to the unopposed release of elastase, which degrades the elastic fibres of the alveoli (Eriksson, 1964).

Similarly, α_1-antichymotrypsin is a poor inhibitor of chymotrypsin, and its main activity is directed against lysosomal cathepsins (Ohlssen & Akesson, 1976). Its function is less obvious, since no clearly defined role is known for its major substrate, cathepsin G.

Antiproteases may have a role to play in immune regulation. They are expressed on the surface of lymphocytes (Andersen, 1983), and have been reported to inhibit generation of immunoglobulin-secreting cells (Arora et al, 1978). Antitrypsin inhibits both antibody-dependent and natural killer cell activity (Ades et al, 1982).

Orosomucoid, also known as α_1-acid glycoprotein, is a highly acidic protein, containing 45% carbohydrate. The concentration of orosomucoid increases by about two-fold after surgery, and it is very similar kinetically to α_1-antitrypsin. There are reports that the extent of sialic acid substitution as detected by crossed electrophoresis into agarose containing lectins decreases as the synthetic rate increases (Nicollet et al, 1981), though it has not been proved yet that this finding is of diagnostic use.

The function of α_1-acid glycoprotein is still not known. There have been claims that it is immunosuppressive,

binding to lymphocytes and blocking the lectin binding sites (Chiu et al, 1977; Kudo et al, 1982; Cheresh et al, 1984). It has been shown to bind progesterone and some drugs (Ryan & Westphal, 1974), it may have antiheparin activities (Andersen & Eika, 1980; Andersen et al, 1981), and it may inhibit platelet aggregation (Costello et al, 1979).

Haptoglobin is similar in its kinetic behaviour to α_1-antitrypsin, and it increases to about twice its normal concentration in inflammation. It shows phenotypic variation in man. There are two types of alpha chain, designated type 1 and type 2, and an invariant beta chain. Haptoglobin molecules containing type 2 chains have a tendency to associate to form stable polymers, and the molecular weight of haptoglobin ranges from 98000 for type 1-1 to several hundred thousand for type 2-2. This property can cause problems when it is measured by gel techniques, as there is variable penetration into the gel.

The primary function of haptoglobin is to bind to any free haemoglobin that may enter the plasma from haemolysis of red cells, and to clear it from the circulation. When haptoglobin binds haemoglobin, it is catabolised very rapidly, with a half-life of only a few minutes, so it is possible to have a normal serum concentration of haptoglobin even in the face of an ongoing inflammatory process.

1.5.5. Trace Metals

It is now thought that the decreases in serum iron and zinc seen after injury (Kampschmidt, 1980) are probably due to synthesis of specific metal binding proteins in liver and other tissues, which remove the metals from the circulation. The decrease in zinc is probably caused by an increased synthesis of metallothionein (Sobocinski et al, 1981; DiSilvestro and Cousins, 1984), and an early rise in metallothionein mRNA has been noted (Durnam et al, 1984). The decrease in iron is partly due to an increase in synthesis of tissue ferritin (Konijn et al, 1977, 1981), and partly to release of lactoferrin from activated neutrophils into the circulation (Van Snick et al, 1974).

The minor increase in serum copper after injury is due to an increase in the serum concentration of the carrier protein, caeruloplasmin, which is also synthesised by the liver.

1.5.6. Measurement of Acute Phase Proteins in Clinical Practice

The major acute phase proteins, particularly α_1-antitrypsin, α_1-acid glycoprotein, and haptoglobin, have all been used as indicators of inflammation. All are elevated during episodes of infection and after surgery. Bacterial infection, in particular, causes marked, reproducible increases, though not all increase simultaneously in many clinical situations. Measurement of

the levels of acute phase proteins can give valuable information as to the severity of an infection, and can provide a guide as to the effectiveness of antibiotic treatment. It has also been claimed that the stage and prognosis of neoplastic disease can be predicted from a knowledge of the concentrations of a selection of proteins (Cooper & Stone, 1979). It is commonly found that useful information is only obtained if several are assayed.

There are reasons for preferring certain of the acute phase proteins over others for routine use. Haptoglobin and α_1-antitrypsin both have the disadvantages of possessing variant forms, while haptoglobin is affected by haemolysis. The most reliable of the high concentration acute phase proteins are probably α_1-antichymotrypsin and α_1-acid glycoprotein. Of the two, antichymotrypsin is slightly preferable, as the concentration of α_1-acid glycoprotein is affected by administration of steroids and by renal function, and changes in the concentration of α_1-antichymotrypsin are more marked.

CRP estimation is probably the most valuable single protein estimation. It has proved to be of use in monitoring many inflammatory diseases and in the diagnosis of infection.

CRP is one of the most sensitive tests for assessing the disease activity in rheumatoid arthritis (McConkey et al, 1973, 1979; Amos et al, 1978; Mallya et al, 1982; Dixon et al, 1984), though other proteins, particularly SAA, haptoglobin, orosomucoid, and α_1-antichymotrypsin, may also be elevated. The level of CRP correlates with several

of the clinical measurements which have been developed, such as articular index. It has been claimed (Amos et al, 1977) that the level of CRP can predict the severity of bone erosions on a prospective basis. Interestingly, the concentration of CRP is not elevated to the same extent in systemic lupus erythematosis (Honig et al, 1977; Pepys et al, 1977), and it has been suggested that this test can be used to differentiate these diseases (Pereira da Silva et al, 1980).

CRP measurement has been shown to be the best objective index of inflammation in diseases as diverse as temporal arteritis (Park et al, 1981), polymyalgia rheumatica (Mallya et al, 1985), Wegener's granulomatosis (Hind et al, 1984), and vasculitis (Hind et al, 1985). Its level is increased more in severe than in moderate acute pancreatitis (Mayer et al, 1984), and it has been claimed to be a better predictor of outcome than any other clinical or biochemical score. The concentrations of orosomucoid and CRP were the only acute phase proteins found by Andre et al (1981) to correlate with a Crohn's disease activity score.

A continually raised CRP several days after surgery (Fischer et al, 1976; Schentag et al, 1984) or a myocardial infarction (Levinger et al, 1957; De Beer et al, 1982) has been claimed to be a good indicator of complications.

The use of CRP as a diagnostic test has been more limited, largely because of the problem of non-specificity. The most frequent application is in the

detection of infection. It was originally thought that CRP levels were only elevated in bacterial infection, but it is now known that a variety of viral infections can cause increases in CRP (Salonen et al, 1981; Cooper et al, 1984; Whicher et al, 1985), so data must be interpreted with care.

It does appear that CRP is capable of distinguishing bacterial from viral meningitis (Peltola, 1982), and it has also been claimed that it is a useful test for detecting infections in neonates (Sabel & Hanson, 1974; Sabel & Wadsworth, 1979; Philip & Hewitt, 1980; Alt et al, 1982). It is also of value in patients with leukaemia, who may be incapable of showing any of the usual signs of infection, such as fever or leucocytosis (Rose et al, 1981; Peltola, 1983; Harris et al, 1984; Rowe et al, 1984).

CRP measurement is now well established in routine use, and is widely employed both in monitoring and diagnosis. Its sensitivity is greater and its response time faster than those of the other commonly measured proteins, but it is not unduly affected by minor stimuli. The concentration of SAA seems to be affected by diseases as trivial as the common cold (Whicher et al, 1985), and it is much more difficult to measure, so it is unlikely to replace CRP measurement.

1.5.7. Interspecies Variation in Acute Phase Proteins

One of the most remarkable features of the acute phase

proteins is their variability between species. It cannot be assumed that if a protein is an acute phase reactant in one species, it will behave in the same way even in closely related ones. For example, CRP is a major acute phase reactant both in man and in rabbit, but it is only a trace component of mouse serum, and the major murine acute phase pentraxin is serum amyloid protein P. Similarly, CRP is a poor acute phase reactant in the rat, though its basal concentrations are higher than in man.

Rats do not possess serum amyloid A protein, but mice do, and it is a major acute phase reactant in that species. The most marked acute phase reactant of the rat is an α_2-macroglobulin, with close functional and immunological similarity to human α_2-macroglobulin. The human protein shows no tendency to act as an acute phase protein. It is not known why there should be this striking variability in response, but it does underline the fact that data obtained using one species of animal cannot necessarily be extrapolated to any other.

1.5.8. Control of Acute Phase Protein Synthesis

The details of the regulation of the synthesis of acute phase proteins by the liver are still unclear, though a number of hormones have been implicated. It has been shown in vivo that there is a marked de novo synthesis of plasma proteins following an inflammatory stress. This has been documented by the injection of radiolabelled proteins (Davies et al, 1966) and by incorporation of labelled

amino acids (Neuhaus et al, 1966; Schreiber et al, 1982) or [^{14}C]-labelled bicarbonate (Koj & McFarlane, 1968) into newly synthesised protein. Livers removed from animals at various times after injection of inflammatory agents have also been shown to synthesise and secrete an increased quantity of protein (Jamieson et al, 1975; McIntyre et al, 1983).

It seems unlikely that catabolism represents a major regulatory step. The catabolic rate of CRP has been shown to be unaltered by injection of endotoxin and turpentine (Chelladurai et al, 1983), although there does appear to be an increase in the catabolic rate of fibrinogen in man after burn injury (Davies et al, 1966).

Most current work on the control of liver protein synthesis is carried out on isolated hepatocytes (Crane & Miller, 1983). However, there are considerable difficulties in maintaining an optimal environment for secretory protein synthesis in vitro (Jeejeebhoy et al, 1975). Careful studies of the requirements for anabolism in isolated hepatocytes have shown that conventional culture media are suboptimal. Schwarze et al (1982) have developed a medium containing high concentrations of amino acids and considerable extra buffering power which is capable of supporting protein synthesis for several days in the absence of serum. High concentrations of insulin are also commonly used to maintain protein synthesis.

A different approach tried recently is to culture hepatocytes in the presence of other liver cells, on the assumption that cell-cell contact may be of importance in

maintaining a functional liver unit. Guillouzo et al (1984) claimed that culturing hepatocytes in the presence of a layer of other liver cells, probably derived from primitive biliary cells, greatly improved their viability and protein synthetic capacity. Synthesis of albumin, haptoglobin, and α_2-macroglobulin by cultures of rat cells continued for several weeks in serum free medium, and similar results have been reported for human hepatocyte cultures. It has also been recently claimed (West et al, 1985) that addition of Kupffer cells to primary cultures of hepatocytes stimulated both structural and secretory protein synthesis. Possibly the absence of accessory cells may go some way to explain why it is so difficult to maintain secretory protein synthesis in cultures of hepatocytes.

It is now thought that acute phase protein synthesis is regulated at the level of mRNA synthesis. Increases have been documented in the concentration of specific mRNA species in rat liver after an inflammatory stimulus of up to 60 fold for α_1-acute phase globulin (Baumann & Held, 1981; Ricca et al, 1981, 1982; Northemann et al, 1983; Diarra-Mehrpour et al, 1985), 66 fold for α_2-macroglobulin (Northemann et al, 1983), and 500 fold for murine SAA protein (Stearman et al, 1982). The mechanism of accumulation of SAA mRNA seems to be an increase in transcription rate (Stearman et al, 1982), but Vannice et al (1984) have claimed that the effect of glucocorticoids on α_1-acid glycoprotein mRNA is to induce a protein which stabilises the transcripts and prevents their rapid

breakdown.

Much work on the mediators which cause the increases seen in acute phase proteins has concentrated on the catabolic hormones, in particular corticosteroids, although effects of many other hormones have been demonstrated. Gordon & Limaos (1979) showed that injection of cortisone into the rat could stimulate the synthesis of several acute phase proteins, including fibrinogen, haptoglobin, and α_2-macroglobulin. However, it has been shown that the response of rat α_1-acid glycoprotein (Baumann et al, 1983a) and haptoglobin (Thompson et al, 1976) to inflammatory stress is unimpaired by adrenalectomy. In contrast, the increase in α_2-macroglobulin required the presence of corticosteroids for its full expression (Heim & Ellenson, 1965; Thompson et al, 1976; Van Gool et al, 1984).

Work of Miller using perfused rat livers (reviewed in Miller & Griffin, 1975) showed that cortisol was capable of directly stimulating the secretion of several acute phase proteins. The effects were greatest when insulin and amino acids were also perfused, as this best preserved the liver in an anabolic state, though insulin does not appear to stimulate synthesis of acute phase proteins specifically.

The studies of Baumann et al (1983a) and Koj et al (1984) on hepatocytes in tissue culture confirmed that the presence of glucocorticoids could directly stimulate the synthesis of several rat acute phase proteins, among them α_1-acid glycoprotein and α_2-macroglobulin. However,

Baumann et al (1983b, 1984) showed that dexamethasone only had a significant effect on rat hepatocytes, not on mouse or human cells.

Miller & Griffin (1975) found that perfusion of isolated rat livers with noradrenaline had no effect on protein synthesis, while there was only a small stimulation if adrenaline was used. However, Van Gool et al (1984) showed that in the intact animal the response of α_2-macroglobulin to the injection of catecholamines was greater than the response to cortisol. They also produced evidence to show that catecholamines stimulated corticosterone production by the adrenal cortex, and that the two hormones synergised in the production of α_2-macroglobulin. The overall pattern of acute phase protein secretion in response to catecholamines was closely similar to that produced by trauma.

The synthesis of many acute phase proteins is also sensitive to sex hormones (Barbosa et al, 1973; Laurell et al, 1967). In females taking oral contraceptives, some proteins are increased in concentration, particularly caeruloplasmin, while others, such as α_1-acid glycoprotein, are decreased. Diarra-Mehrpour et al (1985) have shown that oestradiol and inflammation due to turpentine injection independently increase the synthesis of rat α_1-acid glycoprotein.

Metallothionein synthesis has been recently shown to be under the independent control of glucocorticoids, metal ions, glucagon, catecholamines, and inflammatory stress, probably mediated by IL-1 (Sobocinski et al, 1981; Durnam

et al, 1984; Karin et al, 1985). Glucagon, thyroid hormones, and growth hormone also have some effects on the synthesis of α_1-acid glycoprotein in primary hepatocyte cultures (Jeejeebhoy et al, 1977). Thus, acute phase proteins seem to be under multiple hormonal control, as has previously been documented for several non-secretory liver proteins (Feigelson & Kurtz, 1978).

In spite of the ability to simulate the changes in nitrogen and glucose metabolism by triple infusions of cortisol, adrenaline, and glucagon (Bessey et al, 1984), it appears that these hormones do not elicit an acute phase protein response in man. Indeed, Watters et al (1986) demonstrated a suppression of the rise in CRP in response to aetiocholanolone inflammation if triple hormones were simultaneously infused. Neurogenic blockade sufficient to prevent increases in both cortisol and catecholamines does not abolish the synthesis of acute phase proteins (Kehlet et al, 1980), and suppression of cortisol levels by etomidate is also without effect (Fleck, personal communication). Davies et al (1970) studied patients with transections of the spine who were undergoing surgical procedures to their lower limbs. Their metabolism of radiolabelled albumin was indistinguishable from that of normal patients, and they also showed normal increases in globulin levels and in ESR.

It is necessary to postulate other factors, and much work is now being carried out to isolate mediators produced by a variety of cells, particularly macrophages, which may be directly involved in the control of hepatic

protein synthesis. So far, IL-1, tumour necrosis factor (TNF), and several different species of hepatocyte stimulating factors have been implicated.

The negative acute phase proteins, particularly albumin, may be under somewhat different control. From kinetic experiments using labelled albumin, it seems clear that a large part of the early decrease in blood albumin concentrations is due to redistribution into the extravascular space rather than to a decrease in synthesis rate (Ballantyne et al, 1973). However, there do also appear to be alterations in the synthetic rate of albumin, though there must be doubt as to their quantitative significance in vivo. In hepatocyte cultures, catabolic hormones such as corticosteroids and certain macrophage-derived cytokines tend to cause a decrease in albumin synthesis (Koj et al, 1984), and some workers have claimed that this is also the case in vivo after injury (Ballantyne et al, 1973) and after injection of turpentine (Jamieson et al, 1975). A decrease in the concentration of albumin mRNA in livers of stressed animals has been documented (Northemann et al, 1983), and Ramadori et al (1985) have shown similar effects on adding IL-1 directly into hepatocyte cultures. In contrast, Koj & McFarlane (1968) claimed that injection of endotoxin into rabbits caused a 60% increase in the rate of albumin synthesis as measured by incorporation of [^{14}C] bicarbonate in vivo.

1.6. Interleukin 1 and Tumour Necrosis Factor

One of the most significant advances in inflammation research in recent years has been the recognition that many apparently unconnected aspects of inflammation, such as the activation of the immune system, fever, and the changes in acute phase proteins and metal concentrations, could be largely explained by the activity of a small group of mediators, now known as interleukin 1α, interleukin 1β and tumour necrosis factor. All these hormone-like proteins have been known for many years, but it is only recently becoming clear how many different and important actions they have. IL-1, in particular, has been known by many different names in the past, such as endogenous pyrogen, leukocytic endogenous mediator, and lymphocyte activating factor.

Beeson (1948) showed that peritoneal exudate cells elicited by injection of saline or shellfish glycogen could produce a pyrogenic mediator, which he named granulocytic pyrogen. Atkins & Wood (1955a, b) subsequently proved that an endogenous pyrogen (EP) circulated in the blood of febrile rabbits. This was later shown to be the same as granulocytic pyrogen.

In the 1960s, Kampschmidt discovered that the same exudate cells produced other mediators which were capable of altering a wide variety of biochemical and haematological indices when injected into rabbits or rats. Injection of crude tissue culture fluid conditioned by peritoneal granulocytes caused lowering of serum iron and

zinc concentrations (Kampschmidt, 1980). There was an influx of amino acids into the liver and a marked elevation in the synthesis of certain acute phase proteins (Eddington et al, 1971, 1972; Kampschmidt & Upchurch, 1974; Merriman et al, 1975; Wannemacher et al, 1975) and in numbers of circulating neutrophilic granulocytes (Kampschmidt & Upchurch, 1980). This factor became known as leukocytic endogenous mediator (LEM). It was noted that it resembled endogenous pyrogen in its physical properties and kinetics of release.

Merriman et al (1977) copurified rabbit EP and LEM through five steps, including isoelectric focussing. Both were found to be proteins, with molecular weights of 12-15000 and distinct forms with isoelectric points (pIs) of 5 and 7. The ratio of the activities stayed constant during these steps.

It was originally assumed that the source of both activities was the granulocytes which comprised greater than 90% of the cells in most peritoneal exudates, but it was later discovered that most of the detectable activity was produced by the minor population of macrophages that was always present (Bodel, 1974; Kampschmidt & Pulliam, 1978; Cebula et al, 1979). Bodel (1978) also demonstrated that certain monocyte-like cell lines were capable of secreting pyrogen. Indeed, Hanson et al (1980) claimed that purified rabbit granulocytes derived both from blood and from exudates were incapable of secreting any pyrogen.

Gery et al (1972) and Gery & Waksman (1972) described a factor from macrophages which stimulated the proliferation

of lymphocytes, especially thymocytes, in the presence of a normally submitogenic concentration of phytohaemagglutinin (PHA) or concanavalin A (Con A). This factor was called lymphocyte activating factor (LAF). Several other cofactors for the maturation of lymphocytes were described in the following years. Some were produced by monocytes/macrophages, as was LAF, but others were lymphocyte products. There were obvious biochemical and functional similarities between many of these factors and it was decided (Aarden et al, 1979) to call the monocyte derived activities interleukin 1 (IL-1), to distinguish them from a group of those produced by lymphocytes, which were termed interleukin 2 (IL-2). IL-2, also known as T cell growth factor, is now a well characterised protein, whose major function is to cause proliferation of T lymphocytes and hence to promote the production of cytotoxic T cells and immunoglobulin-producing B cells.

It is now thought that IL-1 has at least two separate actions on T lymphocytes. It can stimulate them to produce IL-2, which in turn causes proliferation of T lymphocytes bearing IL-2 receptors (Smith et al, 1980; Gillis & Mizel, 1981; Maizel et al, 1981; Rao et al, 1983; Conlon, 1983). It has also now been shown that IL-1 can cause IL-2 receptor expression in certain cloned T cell lines (Kaye et al, 1984) and in peripheral blood cells stimulated by monoclonal antibodies (Schwab et al, 1985). IL-1 may also be active directly on B cells to stimulate proliferation and secretion of immunoglobulins (Howard et al, 1983; Lipsky et al, 1983; Falkoff et al, 1983, 1984; Giri et al,

1984).

1.6.1. Comparisons between EP, LEM, and LAF

Rosenwasser & Dinarello (1981) showed that human endogenous pyrogen and LAF copurified in a protocol which included adsorption with an antiserum against EP. The dose response curves in the two assay systems were parallel, which suggested that EP and LAF were closely related. Murphy et al (1980) similarly showed identity between rabbit EP and LAF.

An increase in SAA concentrations was documented in the blood of mice given injections of macrophage-conditioned medium containing IL-1 (Selinger et al, 1980) or of semipurified IL-1 (Sztein et al, 1981). Le et al (1982) and Le & Mortensen (1984) showed similar effects on the secretion of SAP. They found that the maximum stimulation produced by addition of macrophages to their in vitro culture system was greater than that caused by optimal concentrations of IL-1, which suggested that other macrophage-derived mediators might also be required.

Sztein et al (1981) showed that preparations of human and murine LAF and rabbit pyrogen were active in the induction of SAA. In addition, an antiserum against rabbit pyrogen inhibited both LAF activity and SAA inducing activity. Although they were not using highly purified preparations of their materials, the strong similarity between the molecules active in three independent assay systems was evidence that all these activities were caused

by identical or at least very similar molecules.

More recently, however, it has been shown that interferon α and TNF are both pyrogenic (Dinarello et al, 1984a; Dinarello et al, 1986a), and the contribution of these factors to total endogenous pyrogen activity may be significant in clinical situations. TNF has no LAF activity, though it has been shown to increase production of some acute phase proteins in vitro (Dinarello, 1986).

1.6.2. Other Effects on the Immune System

IL-1 has strong chemotactic activity for neutrophils, monocytes (Luger et al, 1983a,b; Sauder et al, 1984), and lymphocytes (Miossec et al, 1983; Sauder et al, 1985). It synergises with IL-2 and interferon to cause differentiation of natural killer cells (Dempsey et al, 1982), and also seems to be essential for their activity, apparently by acting on the target cells (Herman et al, 1985). It may also be able to activate macrophages to become tumouricidal (Onozaki et al, 1985a) and be cytocidal itself (Onozaki et al, 1985b), though the cytotoxic activity of activated macrophages seems to be mediated by TNF (Philip & Epstein, 1986). The killing of tumour cells was, of course, the first action of TNF to be described. IL-1 and TNF can cause neutrophil degranulation (Klempner et al, 1978; Smith et al, 1985) and can stimulate a respiratory burst (Klempner et al, 1979; Shalaby et al, 1985; Klebanoff et al, 1986). Both IL-1 and TNF cause release of GM-colony stimulating factor from a

variety of cell types (Munker et al, 1986; Zucali et al, 1986).

1.6.3. Connective Tissue

IL-1 has multiple effects on connective tissue. Dayer et al (1977, 1979, 1981) reported that a "mononuclear cell factor" (MCF) stimulated the release of prostaglandins and proteases from rheumatoid synovial tissue. This activity was later proved to be identical to IL-1 (Mizel et al, 1981). Normal synovial cells and chondrocytes also responded in the same way (Evequoz et al, 1984; McGuire-Goldring et al, 1984). Murphy et al (1985a) found that there was concurrent stimulation of the secretion of the collagenase inhibitor known as the tissue inhibitor of metalloproteases.

An activity derived from pig synoviocytes and leukocytes which stimulated the net breakdown of cartilage, and was originally named catabolin, has been shown to be the porcine equivalent of IL-1 (Saklatvala et al, 1983, 1984). Catabolin acts to decrease proteoglycan synthesis and also increases the activity of proteoglycanase (Tyler, 1985a,b).

IL-1 is a powerful resorbing agent for bone (Gowen et al, 1983). Its action on osteoclasts may be indirect, since purified osteoclasts will not respond to it unless osteoblasts are present on the same bone slice (Thomson et al, 1985). It has been claimed that osteoclast activating factor is identical in sequence to the pI7 form of IL-1

(Dewhirst et al, 1985). TNF has also been shown to have strong MCF-like activity (Dayer et al, 1985).

IL-1 has been shown to be mitogenic for fibroblasts (Postlethwaite et al, 1984; Schmidt et al, 1984), osteoblasts (Gowen et al, 1985), mesangial cells (Lovett et al, 1983), astrocytes (Giulian & Lachman, 1985), and smooth muscle cells (Libby et al, 1985a,b). It is likely, therefore, that IL-1 is involved in tissue remodelling, both in physiological and pathological states.

1.6.4. Vascular Cells

IL-1 and TNF have been shown to stimulate vascular endothelial cells to secrete procoagulant activity (Bevilacqua et al, 1984; Nawroth & Stern, 1986) and to increase their adhesiveness for leukocytes (Bevilacqua et al, 1985; Cavender et al, 1986; Pober et al, 1986; Pohlman et al, 1986). The prothrombotic effects of IL-1 also include inhibition of the protein C/protein S fibrinolytic system (Nawroth et al, 1986) and induction of the synthesis of an inhibitor of plasminogen activator (Emeis & Kooistra, 1986). IL-1 can cause release of prostacyclin from vascular endothelium (Albrightson et al, 1985; Rossi et al, 1985) and also of PGE_2 from vascular smooth muscle cells (Albrightson et al, 1985).

1.6.5. Protein Metabolism

The role of IL-1 in the control of protein catabolism in

vivo is uncertain. Clowes et al (1983) detected a muscle degrading activity in the serum of patients following burn injury and other traumatic insults. They named this factor, which caused increases in protein catabolism of both rat and human muscle samples in vitro, proteolysis inducing factor (PIF). Its concentration in blood correlated with the release rate of amino acids from peripheral tissue (Clowes et al, 1985). This activity may be caused by a fragment of IL-1, since it could be neutralised by antisera against IL-1 and antisera raised against PIF inhibited the activity of IL-1, though it has an apparent molecular weight of only 4000. PIF has also been reported to be active in inducing the synthesis of liver acute phase proteins (Loda et al, 1985), and it augmented the activity of natural killer cells in a similar way to IL-1 (Morrison et al, 1985). Only partially purified preparations are available, however, and their activity in the LAF assay is poor, so final proof of identity will have to await purification and sequence studies.

Baracos et al (1983) claimed that purified IL-1 could cause proteolysis of rat skeletal muscle in vitro. The major effect was on protein degradation. They showed that the increase in degradation was dependent on prostaglandin synthesis and could be blocked by indomethacin or by inhibitors of thiol proteases. These workers have also shown that the major alteration in rat muscle metabolism following endotoxin injection was an increase in protein degradation, in keeping with a role of IL-1 (Goldberg et

al, 1984). Jepson et al (1985) confirmed these results, though they did note some decrease in protein synthesis as well. However, IL-1 has low activity in the PIF assay, and it has proved difficult to demonstrate effects of IL-1 on skeletal muscle metabolism in vivo.

1.6.6. Miscellaneous Activities

IL-1 has been reported to stimulate ACTH secretion from pituitary cells (Woloski et al, 1985) and to induce slow wave sleep in rabbits (Krueger et al, 1984), both activities consistent with a role during infections in vivo.

It is as yet unclear whether IL-1 has any direct effects on intermediary metabolism. Some work (George et al, 1977) has suggested that LEM-containing supernatants caused insulin and glucagon release from pancreas, but these authors used unpurified material, which contained many other mediators. It now appears, in fact, that IL-1 is cytotoxic for insulin-secreting pancreatic islet cells (Dinarello, 1986). Roh et al (1986) have demonstrated a stimulatory effect of IL-1 in vivo on hepatic gluconeogenesis, but this effect seemed to be indirect, as it was not demonstrable on hepatocytes incubated in vitro. In contrast, Hill et al (1986) have claimed that IL-1 prevents the fasting-induced increase in PEPCK, the rate-limiting enzyme for gluconeogenesis.

1.6.7.Molecular_Characterisation_of_IL-1_and_TNF

The genes for two forms of human IL-1 have now been sequenced (Auron et al, 1984; March et al, 1985), as have a murine (Lomedico et al, 1984) and a rabbit IL-1 (Furutani et al, 1985). These proteins can be divided into two groups on the basis of their amino acid sequences. One group, with a pI of 5, comprises the murine and rabbit proteins and one of the human gene sequences, which has been designated IL-1α by March et al (1985). The other group, so far only described from human material, has a pI of 7, and has been named IL-1β. This is identical to a protein previously purified by Dinarello et al (1977), and the sequence has been confirmed by direct analysis of purified protein (Cameron et al, 1985; March et al, 1985). This seems to be the major form in many species, since rabbits also possess a substantial amount of a pI7 form of IL-1, and it has also been described in mice and rats. A previously detected pI6 IL-1 appears to be produced by post-translational modification of the pI5 protein.

There is remarkably little similarity between the primary structures of IL-1α and β. The overall homology is only 30%, though the homology between the various members of the pI 5 family is considerably greater. The primary transcripts of all these proteins are larger precursor molecules of 31000 molecular weight, which have now been detected in macrophage culture medium (Giri et al, 1985, Sahasrabuddhe et al, 1985) and inside macrophages (Kock et al, 1986; Matsushima et al, 1986). They are then cleaved

to forms with molecular weights of about 17500, which are the major circulating forms. It has also been discovered that probable fragments of IL-1 with molecular weights of 4000 and 1500 circulate in blood (Dinarello et al, 1984b; Cannon & Dinarello, 1985), which may be related to the PIF activity demonstrated by Clowes. Fragments with LAF activity have also been reported to occur in urine from normal volunteers (Kimball et al, 1984), but their relationship to IL-1 is far from clear. A membrane bound form of IL-1 has also been described (Kurt-Jones et al, 1985), which may have great importance in the cell-cell interactions of the immune system.

Gimenez-Gallego et al (1985) have shown that there is some homology between the amino acid sequence of IL-1 and those of the acidic and basic forms of brain-derived fibroblast growth factor. Although the homology is quite weak (25%), it is significant, and suggests that these growth factors are derived from a common ancestor.

The sequence of TNF has also been derived from murine and human gene clones. It is also a protein with a molecular weight of 17000, though it aggregates in solution. There is only one gene, and cleavage products have not been described. It has been found that TNF is identical to cachectin, a protein which inhibits lipid uptake and synthesis by adipocytes in culture (Beutler & Cerami, 1986). TNF may therefore have many as yet undescribed effects on intermediary metabolism.

1.6.8. Sources of IL-1

The major sources of both IL-1 and TNF are tissue macrophages and blood monocytes. However, a wide variety of cells is now known to secrete IL-1-like activity (table 1.2), though TNF production does not appear to be so widespread. It has been found that macrophages from all tissues studied are capable of producing IL-1, including alveolar macrophages (Ulrich, 1977; Simon & Willoughby, 1981), Kupffer cells of the liver (Haeseler et al, 1977), placental macrophages (Flynn et al, 1982) and microglial cells of the brain (Giulian et al, 1985). Activated B lymphocytes (Matsushima et al, 1985a,b; Scala, 1984a) and natural killer cells (Scala, 1984b) are also potent sources.

A very similar factor, known as epithelial cell derived thymocyte stimulating factor (ETAF), has been shown to be produced by skin keratinocytes, keratinocyte cell lines (Luger & Oppenheim, 1983), and corneal epithelial cells (Grabner et al, 1982). It is present in high concentrations in stratum corneum (Gahring et al, 1985). It also appears that some IL-1 can be produced by synovial cells (Duff et al, 1985; Elford et al, 1985; Wood et al, 1985), by brain glial cells (Fontana et al, 1982), and by vascular endothelial cells (Stern et al, 1985; Miossec et al, 1986). Fibroblasts (Okai et al, 1982; Heath et al, 1985) and neutrophils (Harris, 1982a; Tiku et al, 1986) may also produce IL-1-like activity.

It has not yet, however, been proved that the IL-1

Table 1.2

Sources of IL-1

Macrophages:

Peripheral blood monocytes

Peritoneal macrophages

Alveolar macrophages

Synovial cells

Kupffer cells

Microglia

Large granular lymphocytes

Hodgkin's cells

B lymphocytes

Neutrophils

Keratinocytes

Corneal epithelial cells

Langerhans cells

Renal mesangial cells

Endothelial cells

Astrocytes

Fibroblasts

derived from these various cell types is identical to either of the macrophage species so far characterised. Indeed, Matsushima et al (1985b) have claimed that "IL-1" derived from a B lymphocyte cell line does not react with an antiserum raised against human leukocytic pyrogen. A form of IL-1 derived from Epstein-Barr virus activated B lymphocytes has been shown to have no homology in its N-terminal sequence to the previously described forms of IL-1 (Rimsky et al, 1986). In contrast, Kock et al (1986) have claimed that antisera raised against IL-1 do inhibit the activity of ETAF. It is certainly possible that what is commonly known as IL-1 is in fact a closely related family of proteins expressed in different tissues, in a similar way to the interferon family. Definitive answers to this question must await sequencing of other forms of IL-1 and studies with monospecific antisera.

1.6.9. Stimuli for IL-1 Production

Many different stimuli have been reported to cause IL-1 release (Dinarello, 1984, table 1.3). The most potent are bacterial in origin. The endotoxin component of the cell walls of Gram-negative bacteria, which consists of a lipopolysaccharide (LPS) material attached to protein, is one of the most powerful stimuli known (Duff & Atkins, 1982a). Unfortunately, it is a ubiquitous contaminant of reagents and apparatus, and is extremely difficult to remove. The antibiotic polymyxin B binds to purified LPS and inactivates it (Duff & Atkins, 1982b), but is

Table 1.3

Stimuli for IL-1 Release

Bacterial lipopolysaccharide

Bacterial lipoprotein

Peptidoglycans

Staphylococci and Staphylococcal cell walls

Toxic shock syndrome toxin 1

Muramyl peptide

Antigens/lectins (acting via lymphokines)

Colony stimulating factor

Interferons (potentiate other stimuli)

Collagen

Urate crystals

Silica dust

Mast cell products

Thrombin

C5a

Aetiocholanolone

Leukotrienes B and C

Phorbol esters

Calcium ionophores

Latex beads

Vitamins A and D (differentiating agents)

Tumour necrosis factor

ineffective against many naturally occurring endotoxins, especially if substantial quantities of bacterial lipoprotein are present (Kluger et al, 1985). Many experiments claiming to show that some substance stimulates IL-1 release have not been controlled for endotoxin contamination, and it seems likely that some of the more non-specific agents used, such as latex beads, were in fact contaminated. The minimal fragment of endotoxin which has activity is the lipid A component (Arend et al, 1985a). Synthetic fragments of E. coli bacterial lipoprotein are active, though less so than LPS (Bessler et al, 1984).

The toxic shock toxin produced by certain strains of Staphylococcus aureus is an exceptionally powerful stimulus (Ikejima et al, 1984; Parsonnet et al, 1985). Cell wall components of Staphylococcal species are good stimuli (Oken et al, 1981), and Oppenheim et al (1980) have shown that muramyl peptide derivatives from mycobacterial cell walls are also effective, and may represent the active component of Freund's complete adjuvant.

Immune complexes are capable of eliciting IL-1 release, but only in the presence of serum (Arend et al, 1985b). Presumably one of the complement components is the active factor, and since C5a has been reported to cause IL-1 secretion in vitro (Goodman et al, 1982), this may well be the factor involved.

It has been reported that collagen subtypes II and III (Dayer et al, 1982) are able to cause IL-1 release. This

may be of importance when they are exposed by tissue damage. Stimulation of IL-1 release by urate crystals (Malawista et al, 1985) and silica dust (Schmidt et al, 1984) may be implicated in the pathogenesis of gout and silicosis respectively. Products of mast cells and basophilic granulocytes have been reported to cause IL-1 release (Yoffe et al, 1985), and IL-1 may cause mast cell degranulation, so it may be involved in type 1 hypersensitivity reactions.

Lymphokines have been described in supernatants from lectin or antigen stimulated lymphocytes (Atkins & Francis, 1978; Atkins et al, 1980; Bernheim et al, 1980) and mixed lymphocyte cultures (Dinarello, 1981) which cause IL-1 production. Colony stimulating factor may be one such mediator (Moore et al, 1980). Interferons have been reported to enhance IL-1 production in the presence of low levels of LPS and CSF (Arenzana-Seisdedos & Virelizier, 1983; Boraschi et al, 1984; Candler et al, 1985; Newton, 1985), but it has been shown that the mediator from mixed lymphocyte cultures is not interferon (Dinarello & Kent, 1985). There seem to be complex interactions between IL-1, TNF, and interferons (Philip & Epstein, 1986).

It is now clear that most IL-1 synthesised is retained within the cell (Bayne et al, 1986). It seems likely that the release of IL-1 from macrophages is controlled by Ia antigens, since release, but not synthesis, is blocked by antibodies against them (Durum et al, 1984, 1985; Gilman et al, 1983).

Both vitamin A (Trechsel et al, 1985) and vitamin D (Amento et al, 1984) have been shown to stimulate IL-1 production. The effect of vitamin D is indirect, since it acts to cause maturation of macrophages.

Little is known about the feedback mechanisms which control the level of IL-1. Both sympathetic stimulation in vivo (Hirsch et al, 1985) and administration of cortisol (Snyder & Unanue, 1982; Staruch & Wood, 1985) have been reported to inhibit its secretion, as have PGE_2 (Kunkel & Chensue, 1985) and several other agents which increase cAMP in cells (Knudsen & Strom, 1985). There is also some evidence for suppressor cells in the spleens of specifically tolerised animals (Atkins & Francis, 1985).

1.7. Other Macrophage-derived Mediators

It has now been shown that pure and recombinant IL-1 (Bauer et al, 1985; Gauldie et al, 1985; Ramadori et al, 1985) are capable of stimulating acute phase protein synthesis in vivo and in primary cultures of rat and mouse hepatocytes. In contrast, Koj et al (1984) and Baumann et al (1984) have suggested that the macrophage-derived factor responsible for stimulation of liver protein synthesis was not IL-1. They found discrepancies between the peaks of LAF and LEM activity on gel filtration, with LEM activity running at an apparent molecular weight of 25-30000. Ritchie & Fuller (1983) and Woloski & Fuller (1985) also detected a hepatocyte stimulating factor (HSF) which caused an increase in the secretion of fibrinogen by

rat hepatocytes, had a similar molecular weight to that described by Baumann et al (1984), and did not copurify with LAF activity. Purified IL-1 had no effect in their system. More recently, Baumann et al (1986) have purified three HSF species from squamous carcinoma cells and one from monocytes. These factors appear to be biochemically and functionally distinct from IL-1.

One possible explanation for these discrepancies was suggested by Le & Mortensen (1986). They developed a single cell assay for SAP production by hepatocytes, which allowed them to distinguish between factors which caused recruitment of cells for protein synthesis and those which increased the protein synthesis rates of committed cells. They claimed that IL-1 increased the level of mRNA in recruited cells, while other factors present in macrophage supernatants increased the number of hepatocytes capable of synthesising SAP. These latter factors had molecular weights of 30000 and 70000, so they may be related to HSF. In addition, TNF has been reported to stimulate acute phase protein production both directly and by stimulating IL-1 release (Dinarello, 1986).

Other mediators, as yet undescribed, may well also be involved in the metabolic and protein responses to trauma. For example, it has been shown by Whicher et al (1980) that infusion of prostaglandin E_1 causes a brisk acute phase protein response. Leukotriene B_4 has also been reported to stimulate IL-1 release in vitro (Polla et al, 1985). The control of inflammation is very complex, and interactions of IL-1 and TNF with other growth factors,

interferons, and arachidonic acid metabolites are likely to prove a fruitful, but very difficult, field for study.

1.8. Modification of the Response to Trauma

It is still controversial whether the acute phase response is on balance beneficial or harmful to the patient. In rheumatoid arthritis and other autoimmune diseases, the inflammatory response clearly causes the symptoms of the disease, so it is desirable to treat the acute phase response as such, commonly by suppressing the response with glucocorticoids.

However, it is much less certain that it is necessary or even advisable to do so after uncomplicated surgical procedures or during simple infections. Many aspects of the response, including fever and acute phase protein synthesis, are probably of benefit to the patient. The survival of lizards infected with bacteria was enhanced if they were housed at higher temperatures (Kluger et al, 1975). In vitro, the response of lymphocytes to IL-1 was enhanced by incubation at temperatures of 38°C to 40°C, corresponding to fever temperatures (Duff & Durum, 1983; Murphy et al, 1985b). Also, many acute phase proteins may be protective, either as antiproteases, as scavengers of potentially toxic materials, or as immunoregulators. The degree of protein catabolism found after moderate injury may be beneficial in providing a supply of amino acids and gluconeogenic precursors. Preventing these phenomena may well be harmful in some cases.

There is some evidence for this from the work of Keenan et al (1982), who measured the ability of severely ill patients to synthesise monocyte IL-1. There was a subgroup of patients who produced subnormal amounts of IL-1 in response to a standard stimulus, and the prognosis of these patients was considerably worse than that for patients who were capable of mounting a response. Moldawer et al (1984) have made out a case based on these findings for the therapeutic administration of IL-1 in such patients. Luger et al (1984) also claimed that septic patients who had high circulating levels of IL-1 had a better prognosis than those who did not show this response, and Dominioni et al (1985) demonstrated that septic patients who died during intensive care synthesised lower quantities of acute phase proteins than those who survived. However, an excessive catabolic response is without doubt harmful to the patient, and attempts are normally made to minimise the extent of the systemic disturbance after surgery, on the assumption that this will allow a more rapid convalescence, with fewer complications.

Attempts to modify the acute phase response can be classified into two categories. The environment in which patients are nursed may be altered or various exogenous agents may be administered.

Caldwell and his colleagues (1959, 1962) maintained burned experimental animals at elevated environmental temperatures, and noted that their metabolic rate and nitrogen output were both reduced. Barr et al (1968)

confirmed these findings in burned patients treated at elevated temperatures. The clinical improvement was accompanied by changes in the catabolic rate of albumin and γglobulin (Davies et al, 1969) and in the excretion of catecholamines (Birke et al, 1972). In similar experiments on traumatically injured patients, Cuthbertson et al (1972) and Ballantyne & Fleck (1973) demonstrated reductions in nitrogen and zinc excretion and in the concentrations of serum α_1-acid glycoprotein and CRP.

Many studies have shown that it is possible to reduce nitrogen losses by adequate feeding postoperatively. For a given input of amino acids, the effect in cases of starvation depends on the number of calories infused (O'Connell et al, 1974). It is, however, difficult to show much clinical benefit in uncomplicated situations.

The infusion of insulin seems to be a highly effective way of reducing nitrogen excretion and promoting anabolism (Woolfson et al, 1979), though again, it is difficult to show clinical benefit. Growth hormone has also been shown to be effective (Wilmore et al, 1974b).

Several drugs, including naftidrofuryl (Burns et al, 1981) and anabolic steroids (Blamey et al, 1984) have been claimed to reduce nitrogen excretion. They have not yet, however, found their way into clinical practice.

In the special case of rheumatoid arthritis, there are certain drugs which appear to alter the course of the disease and to affect the acute phase response directly. These are commonly known as "second-line" drugs, or "disease-modifying" drugs, to distinguish them from the

non-steroidal anti-inflammatory drugs, which only affect the symptoms of the disease. Examples of "second-line" drugs are gold salts, penicillamine, and antimalarials.

In the future, there will be much more emphasis on attempting to control the response to trauma directly, by manipulating the secretion and action of mediators such as IL-1 and TNF. This work is still in its infancy, but there are suggestions that arachidonic acid metabolites may be involved in both IL-1 production and action (Dinarello et al, 1984c), and modification of the arachidonic acid pathways may prove a fruitful area to explore. Now that the sequences of IL-1 and TNF are known, it will also be possible to investigate the properties of analogues and antagonists.

1.9. Studies Described in this Thesis

The studies which will be described here concentrate on three of the possible ways described above of modifying the acute phase response. In the first section, the effect of environmental temperature on the response of patients undergoing elective surgery will be described. The purpose of this study was to demonstrate whether the effects previously demonstrated in severely injured or burned patients could be duplicated in the context of routine surgery. This was part of a larger study on the effects of temperature on the metabolic rate and hormone concentrations.

The second experiment involved a study of the effect of

gold salts on the acute phase protein response of patients with rheumatoid arthritis. Treatment with injectable gold and placebo was compared with a newly available oral gold preparation, known as auranofin. Their effects on acute phase protein concentrations were measured and compared with a selection of clinical indices.

In the third section, a series of experiments will be described which explored the possibility of inhibiting IL-1 production from monocytes with drugs active on various parts of the metabolic pathways of arachidonic acid.

In the final section, the preliminary steps towards developing an immunoassay for IL-1 will be described. This is an essential preliminary for any future work in this field, as the role of IL-1 production in vivo is still unproven, largely because of the lack of sensitive, precise, and specific assays.

CHAPTER 2

METHODS

2.1. Immunonephelometric Assays

The concentrations of CRP, α_1-antitrypsin, α_1-acid glycoprotein, α_1-antichymotrypsin, haptoglobin, α_2-macroglobulin, prealbumin, and transferrin were measured by immunonephelometry. The principles of this technique are described by Kusnetz & Mansberg (1978) and by Whicher et al (1983). All except transferrin were measured using the Hyland LAS-R nephelometer PDQ (Whicher et al, 1978, figure 2.1). This is a manual nephelometer, with a helium-neon laser source emitting light at a wavelength of 632.8 nm and optics which allow readings to be taken at a forward angle of 31°. This ability is claimed to increase the sensitivity of the technique by increasing the ratio of light scattered by the large immune complexes of the sample to that scattered by smaller interfering molecules. Disposable cylindrical glass tubes are used both as reaction tubes and as cuvettes. The instrument is capable of automatically subtracting both reagent and sample blanks.

2.1.1. Optimisation

The procedure used for optimising the assay was essentially the same for all the proteins studied and was similar to that recommended by Whicher & Blow (1980).

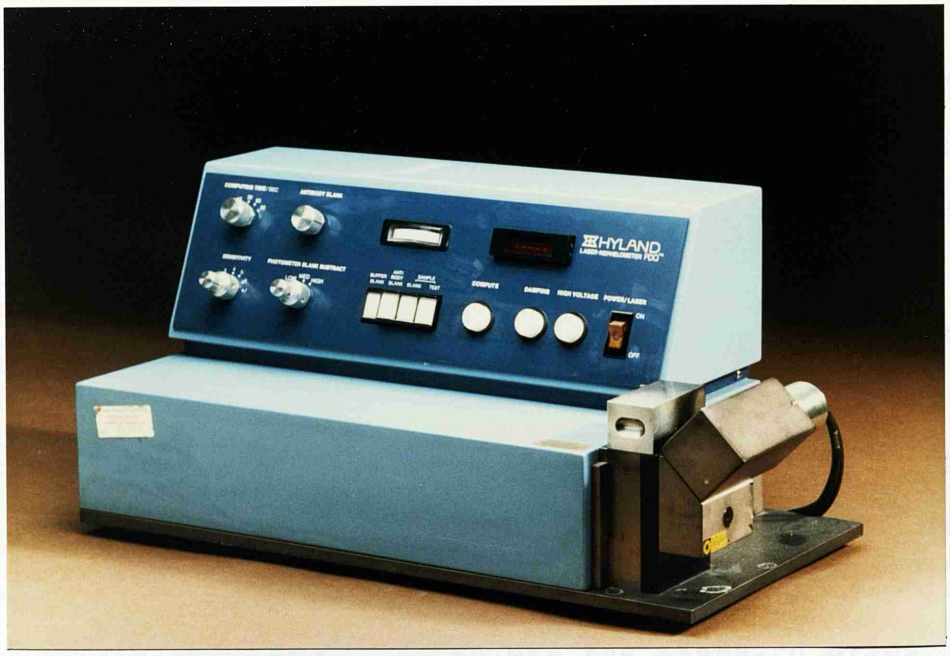


Figure 2.1. Hyland LAS-R nephelometer.

The buffer used was 10 mmol/l phosphate, pH 7.0, containing 150 mmol/l NaCl, 40 g/l polyethylene glycol (PEG) 6000, and 1.5 ml/l Tween 20.

Antibody reagent consisted of antibody diluted in buffer, while blank reagent consisted of buffer alone. Both reagents were filtered through disposable 0.22 μm filters (Millipore or Gelman) before use. Samples were diluted in sterile 150 mmol/l NaCl (0.9% saline, Travenol).

Antiserum (swine) against CRP was obtained from Orion Diagnostika, and a standard preparation from Behringwerke. The antibody against α_1-antichymotrypsin was also obtained from Orion Diagnostika, as was the standard preparation (normal standard serum). Antiserum and standards for prealbumin were purchased from Seward Laboratories. All other antisera and standards were obtained from Atlantic Antibodies.

For each protein, a standard curve was constructed in doubling dilutions to span a wide range of concentrations. 100 μl of each dilution was added to 1 ml of antibody or blank reagent. A tube containing antiserum alone was used as an antibody blank, and an individual sample blank tube was assayed for each test sample. After an incubation period of 2 hours at room temperature, the sample tubes were placed in the instrument and the light scattering (%RLS) was read. The instrument was zeroed on saline, and the full scale deflection was set using the dilution of standard chosen to represent the top of the standard curve. The "compute time" chosen for integration of the scattering signal was 5 seconds.

At least two concentrations of antiserum were tested for each protein. Several of the higher concentrations of standard were used in turn to set the full scale deflection of the nephelometer, a procedure which allowed the whole dynamic range of the instrument to be evaluated.

The dilution curves for α_1-antitrypsin are given as a typical example of the results obtained. It can be seen (figure 2.2) that the scattering obtained followed the expected shape of the precipitin curve, rising to a maximum value at the equivalence point, the position of which depended on the concentration of antibody used. In conditions of antigen excess, the light scattering decreased once more as the complexes became soluble. Care was taken to avoid using the portion of the curve near to the equivalence point.

Satisfactory standard curves could be obtained at several different sensitivity settings of the instrument, so the final choice of conditions depended on criteria such as the economy of using high dilutions of antiserum, the impracticability of using very high dilution factors for samples, and the inaccuracy of the instrument at the higher sensitivity settings. It was found that the response time of the instrument was slow if sensitivity settings of greater than 4 were used, and noise due to dust particles also became troublesome, even if all reagents used were filtered. A sensitivity setting of 5 only proved necessary for CRP measurement.

The final conditions used for the proteins measured are shown in table 2.1. The antibody dilutions used were 1:50 or 1:100, and the final sample dilutions ranged from 1:500

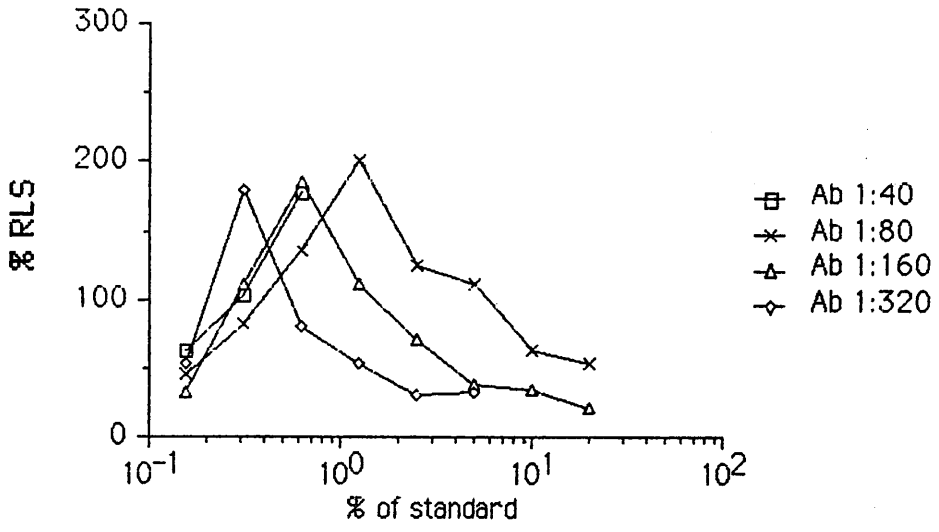


Figure 2.2. Antibody dilution curves for antitrypsin.

Serial dilutions of antiserum and standard were analysed using the Hyland Laser nephelometer

Table 2.1

Optimised Conditions for Immunonephelometry

Protein	Antibody dilution	Sample dilution	Volume added to cuvette (μl)
CRP	1:100	1:100	100
ACT	1:100	1:100	50
AT	1:100	1:100	50
AG	1:50	1:100	50
HP	1:100	1:100	50
AM	1:100	1:100	100
Prealbumin	1:50	1:50	100
Albumin	1:100	1:200	10

CRP: C-reactive protein; ACT: α_1-antichymotrypsin; AT: α_1-antitrypsin; AG: α_1-acid glycoprotein; HP: haptoglobin; AM: α_2-macroglobulin

to 1:20000. All proteins were found to reach the plateau of light scattering by 120 minutes (figure 2.3).

2.1.2. Measurement of CRP

Some initial difficulty was found in the measurement of CRP by nephelometry. Because the concentrations being measured were very low, many samples had high blank values, which prevented their measurement even at high dilutions of sample. It was decided to test the effect of diluting the antiserum in PEG 4000 instead of PEG 6000, in an attempt to reduce non-specific precipitation.

The results in table 2.2 show that the use of 40 g/l PEG 4000 was effective in reducing blank values, so it was used routinely.

2.1.3. Method Precision

Within-batch precision was less than 10% for all the methods. For example, the standard deviation of a commercial QC serum (Precipath) analysed 10 times for α_1-antitrypsin in a single batch was 0.06 g/l at a level of 1.15 g/l (5.2% CV), and that of a patient sample was 0.18 g/l at a mean level of 2.47 g/l (7.3% CV). The between-batch precision for the acute phase reactants is shown in table 2.3, and was comparable with that for other manual methods for measuring specific proteins. For α_1-antichymotrypsin, the methodology was more precise at higher levels; at the level of 0.43 g/l, the between batch precision was 16%, as compared to 4.5% at 1.33 g/l. A

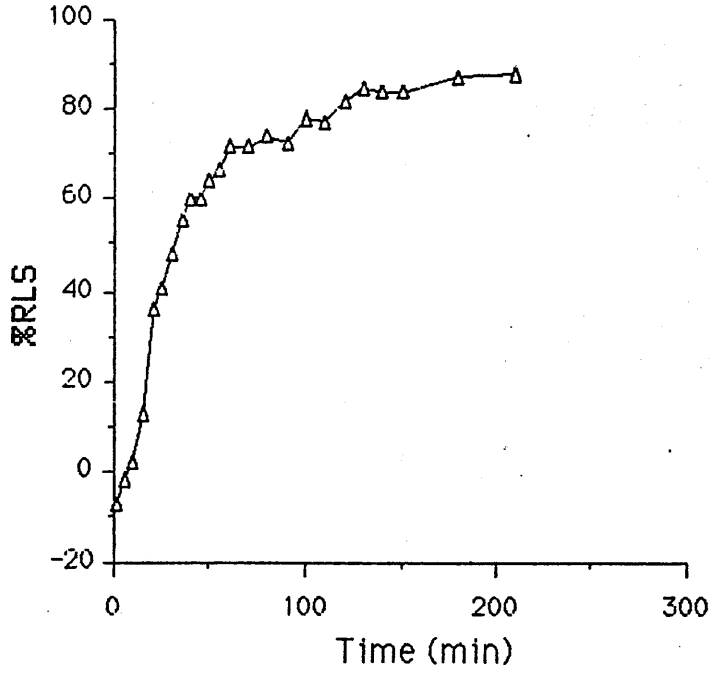


Figure 2.3. Time course of development of light scattering.

The development of light scattering was followed at intervals using an anti-serum against antichymotrypsin and the Orion reference standard.

Table 2.2

CRP Measurement by Nephelometry

Comparison of PEG 4000 and 6000

Light Scattering of Sample Blank

	PEG 4000	PEG 6000
	1.8	8.1
	1.5	6.1
	1.1	6.7
	0.2	3.2
	-0.4	2.5
	0.2	4.5
	-0.3	2.7
	5.7	12.7
	14.3	19.2
	5.9	13.9
	8.6	26.8
Mean	3.51	9.67
SD	4.66	7.78

($p<0.05$)

Table 2.3

Between Batch Precision (Nephelometry)

Protein	Mean (g/l)	Standard deviation	Coefficient of variation	n
CRP	0.0701	0.0076	10.8	12
ACT	1.33	0.06	4.5	12
	0.43	0.07	16.2	9
AT	1.08	0.16	14.8	25
AG	0.48	0.07	14.5	14
HP	0.69	0.10	14.4	15

CRP: C-reactive protein; ACT: α_1-antichymotrypsin; AT: α_1-antitrypsin; AG: α_1-acid glycoprotein; HP: haptoglobin

limited number of estimations of this QC material for CRP showed that the precision of the method at levels less than about 20 mg/l was very poor (>40%).

2.1.4. Reference Ranges

Reference ranges for all the acute phase proteins except α_1-antichymotrypsin were derived using specimens from 55 normal volunteers (30 male, 25 female). Their ages ranged from 14 to 76 years. A separate study was carried out using blood obtained from 79 normal laboratory staff (41 male, 38 female) to estimate a reference range for α_1-antichymotrypsin. Their ages ranged from 20 to 55 years. The ranges derived were those between the 2.5th percentile and the 97.5th percentile of the frequency distributions, since the form of the distributions was not Gaussian. The ranges found are shown in table 2.4.

2.2. Transferrin Measurement

Transferrin concentrations were measured using the Technicon Automated Immunoprecipitin System, which is based on their AA-2 continuous flow system. A fluorimeter with 90° optics and a wavelength of 340 nm is used for detection. Samples were diluted 1:40 with 150 mmol/l NaCl before aspiration into a predilution manifold. The antiserum was diluted 1:80 into 150 mmol/l NaCl with 40 g/l of polyethylene glycol 6000.

Table 2.4

Reference Ranges (g/l)

α_1-antichymotrypsin	0.3 - 0.8
α_1-antitrypsin	1.0 - 3.5
Haptoglobin	0.2 - 2.0
α_1-acid glycoprotein	0.3 - 1.0

2.3. Retinol Binding Protein Measurement

Retinol binding protein was measured by radial immunodiffusion, using commercial plates and standard materials obtained from Behringwerke. The diameter of the rings was measured at 48 hours and a calibration graph was constructed of the square of the diameter plotted against the concentration of standard.

2.4. C-Reactive Protein Measurement

2.4.1. Radial Immunodiffusion

Radial immunodiffusion plates for CRP were purchased from Behring and used according to the manufacturer's instructions as above. The sensitivity of the assay was stated to be 6 mg/l. The between-batch precision was 14.1% at a mean level of 33.4 mg/l (n=14).

2.4.2. Enzyme-linked Immunoassay

A homogeneous enzyme-linked immunoassay (EMIT) kit marketed by Syva (Palo Alto) was used (figure 2.4). This kit is based on the inhibition of activity of an enzyme-CRP complex by antiserum. The enzyme β-galactosidase is coupled to CRP and its substrate, o-nitrophenyl-β-galactoside, is coupled to a high molecular-weight dextran polymer. A specific antibody against CRP binds to the CRP attached to the enzyme and prevents the hydrolysis of the macromolecular substrate by

steric hindrance. If free CRP is present in samples, it binds to the antibody and prevents this inhibition (Gushaw et al, 1982).

The antibody and substrate were added to samples and incubated for 45 seconds, after which the enzyme reagent was added. The course of the reaction was followed for 45 seconds at 405 nm using a Gilford Stasar III spectrophotometer. All samples were assayed in duplicate. The stated sensitivity of the assay was 10 mg/l. The within-batch precision of duplicate measurements was 17.7% at a mean serum concentration of CRP of 10.4 mg/l, and 6.1% at a concentration of 43.9 mg/l (n=20). The between-batch precision was 4.8% at a level of 33.2 mg/l (n=5).

2.4.3. Immunoradiometric Assay

There were found to be problems in practice with the nephelometric assay for CRP. The precision of the method was unacceptable at concentrations of less than 20-25 mg/l, and even this performance was only obtainable with the particular antiserum chosen. When this antiserum was withdrawn from the market, it proved impossible to find a suitable alternative. I therefore decided to develop an assay using isotopically labelled reagents.

The two major classes of such assays either use labelled antibody or labelled analyte. The latter form of radioimmunoassay (RIA) is also known as a competitive binding assay, because sample and label compete for a limited amount of antibody. The sensitivity of the assay

is strongly dependent on the affinity constant of the antiserum used (Ekins, 1976), and a constant supply of highly purified antigen is required for labelling.

The alternative design of assay, using a labelled antibody, is known as an immunoradiometric assay (IRMA). The principles of IRMAs are described in detail by Hales & Woodhead (1980). In the most commonly used variant of labelled antibody techniques (figure 2.5), the antibody is bound to a solid phase, such as a plastic tube or a well of a microtitre plate, or to a finely divided solid material, and used to extract the antigen from a sample. The complex is then washed, and a labelled antibody is used to detect bound antigen. Alternatively, the label and the sample may be allowed to come to equilibrium first, and a solid phase antibody used to precipitate the complex. The latter is the version which was used in this study. These assays are commonly known as "two-site" assays, because the antigen must be capable of binding to two antibodies simultaneously.

The advantage of IRMA assays is that they can be driven to completion by the use of excess reagents, and can offer great speed of assay and high sensitivity. They are less critically dependent on the supply of highly purified antigen than are RIAs, as this is not required for labelling purposes, though some is still required to extract high affinity antibodies from the antiserum to be used as label. Because the supply of pure CRP was limited at the start of this study, I decided to develop an IRMA. The sample capacity of an IRMA is high, and it can easily be semi-automated.

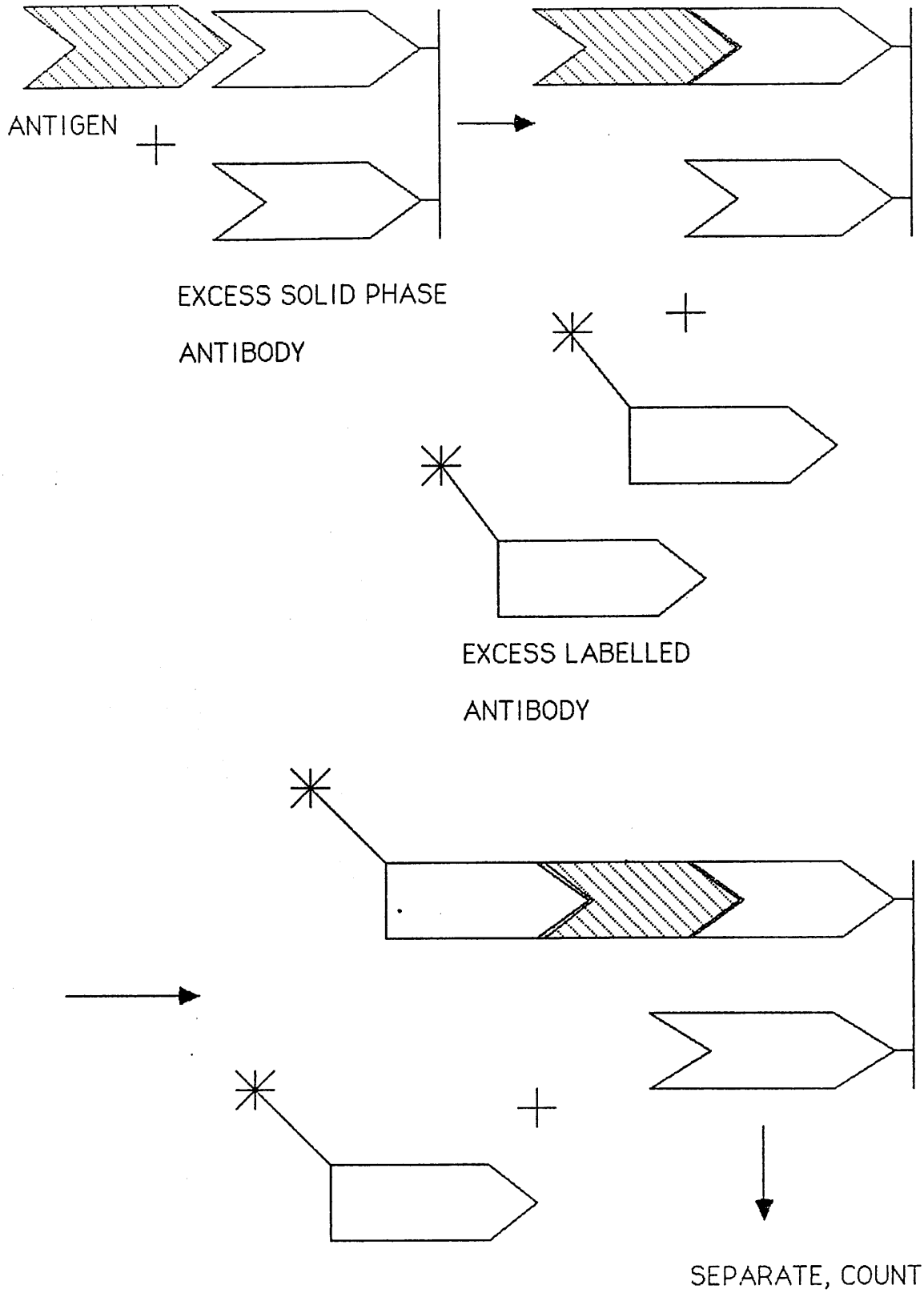


Figure 2.5. Schematic representation of an immunoradiometric assay (IRMA) Solid phase antibody may be used to extract samples before label is added, or the label may be allowed to complex with the sample before the complex is precipitated using solid phase.

a) Purification of CRP

The method of Pontet et al (1978) was used, in which phosphoryl ethanolamine bound to Sepharose acts as an affinity ligand for CRP. Although the binding of CRP to this ligand in the presence of calcium is weaker than it is to C-polysaccharide or to phosphorylcholine, which have also been used for this purpose (Osmand et al, 1975; Volanakis et al, 1978), the chemical is readily available, and it is simple to attach it to the gel by means of the amino group.

Purity of the CRP preparation was monitored by discontinuous polyacrylamide gel electrophoresis. Because it was necessary to separate CRP from the closely related protein, SAP, which has a very similar subunit molecular weight, it was decided not to use denaturing conditions. The buffers and gels used were those described by Frings et al (1971). The separating gel was 4% acrylamide, 0.1% bis, pH 8.9, and the concentrating gel was 5% acrylamide, 1.25% bis, pH 6.7. The reservoir buffer was 50 mmol/l Tris/glycine buffer, pH 8.3. The gels were photo-polymerised in tubes, the separating gel with TEMED and ammonium persulphate, and the concentrating gel with TEMED and riboflavin. They were run for 35 minutes at 5 mA/tube, and stained with Coomassie Blue.

The CRP content of column eluates was monitored by means of the Syva EMIT kit.

Activated CH-Sepharose-4B (Pharmacia) consists of Sepharose CL-4B with a spacer arm of 6-aminohexanoic acid attached to N-hydroxysuccinimide (figure 2.6). Amino

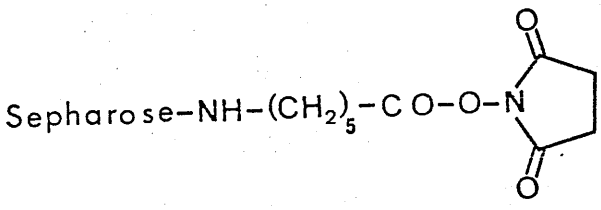


Figure 2.6. Structure of activated CH-Sepharose.

groups will couple to this ligand under mild conditions. 3.3 g of activated CH-Sepharose were swollen in 1 mmol/l HCl and washed in the same solution. 200 mg of O-phosphorylethanolamine were dissolved in 15 ml of 100 mmol/l $NaHCO_3$ and added to the gel. The coupling reaction was allowed to proceed for 1 hour at room temperature. Excess ligand was washed off with coupling buffer, and remaining active groups were blocked by an incubation for 1 hour with 100 mmol/l Tris-HCl buffer, pH 8.0, containing 500 mmol/l NaCl. The gel was washed in a Buchner funnel under reduced pressure with four 100 ml aliquots of 100 mmol/l Tris buffer, pH 8.0, containing 500 mmol/l NaCl, and four 100 ml aliquots of 100 mmol/l acetate buffer, pH 4.0, containing 500 mmol/l NaCl. The gel was then equilibrated overnight with 20 mmol/l Tris buffer, pH 8.0, containing 100 mmol/l NaCl and 10 mmol/l $CaCl_2$ and packed into a 1 x 15 cm column.

A sample of 10 ml of serum from a patient 2 days post surgery was allowed to percolate on to the column. The column was then washed with the equilibrating buffer until the absorbance of the eluate measured at 280 nm was <0.02, and then washed overnight at a flow rate of 10 ml/hour with 20 mmol/l Tris buffer containing 150 mmol/l NaCl, without calcium. The CRP was then eluted by means of a gradient of sodium citrate in Tris buffer to a final concentration of 10 mmol/l. Fractions of 3 ml were collected. CRP was eluted at the end of the gradient.

The preparation was further purified by gel filtration. The pooled eluate from the phosphoryl ethanolamine column was concentrated using a Minicon B15 cell (Amicon) and

passed down a 0.9 x 60 cm column of Sephacryl S-300. The buffer used was 10 mmol/l Tris HCl, pH 7.4, 150 mmol/l NaCl, and the flow rate was 10 ml/hour. Fractions of 2 ml were collected. The elution profile is shown in figure 2.7, and the presence of only one major band on polyacrylamide gel electrophoresis (figure 2.8) confirmed the purity of the preparation. The overall amount of CRP recovered was 695 μg, which represented 39% of the original amount present in the serum.

b)Coupling_of_Antibody_and_Antigen_to_Cellulose

The coupling method chosen was that of Chapman and Ratcliffe (1982). This employs 1,1' carbonyldiimidazole (CDI) to attach proteins to a carrier by their free amino groups (figure 2.9).

CDI was stored desiccated at -20°C. 5g of microparticulate cellulose (Sigmacell 20) were added to 50 ml of acetone containing 1.22 g of CDI in a dry Erlenmeyer flask with a ground glass stopper. The slurry was mixed with a magnetic stirrer for 1 hour and filtered through glass fibre paper (Whatman GF/A) under reduced pressure. The cellulose was washed with 3 x 100 ml aliquots of acetone and allowed to dry for 15 minutes under suction. The CDI-cellulose was then spread out on a filter paper and air-dried for a further hour. It was stored in a tightly stoppered container at -20°C.

The antiserum used for coupling was obtained from Seward Laboratories. It was the immunoglobulin fraction of a sheep antiserum, with a protein concentration of 24 g/l as

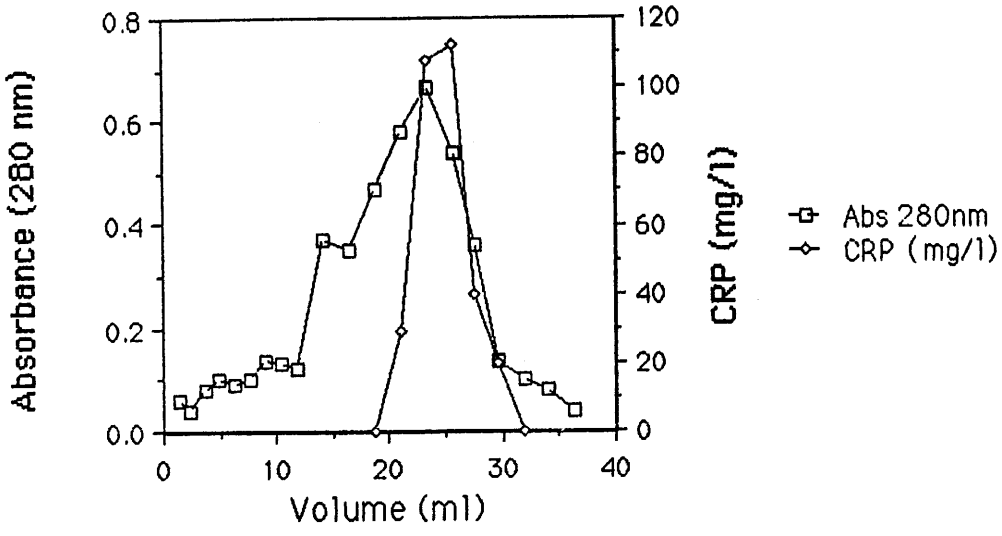


Figure 2.7. Elution profile of CRP on Sephacryl S-300.

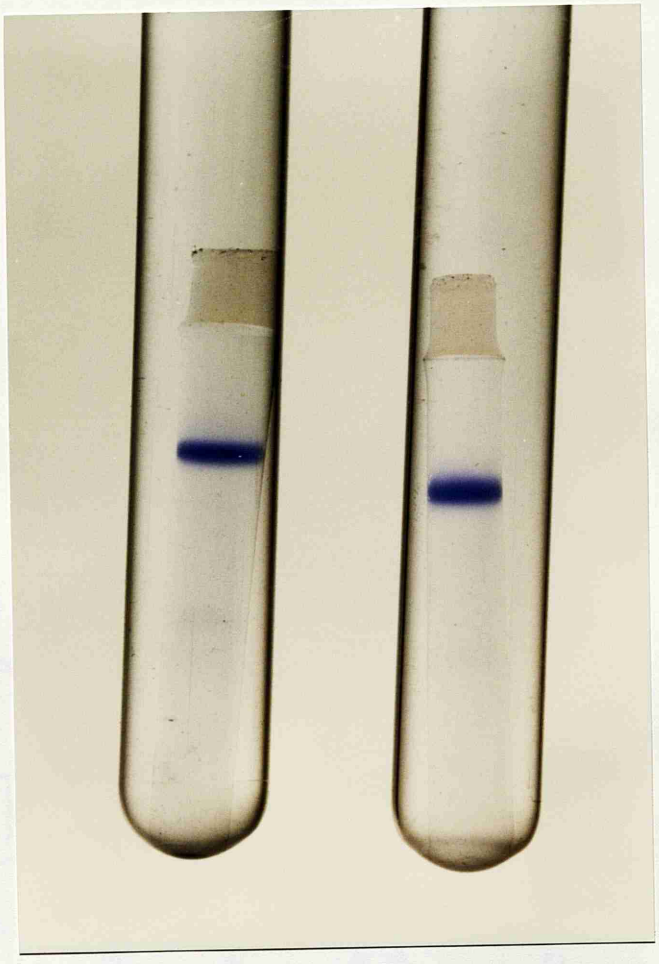
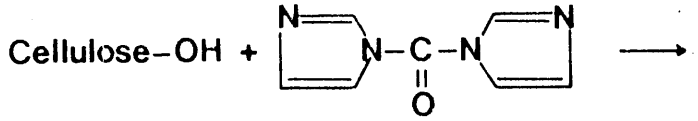
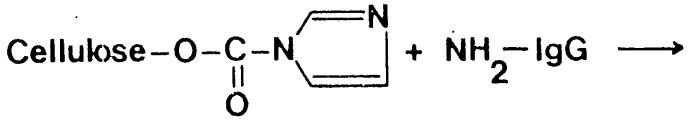


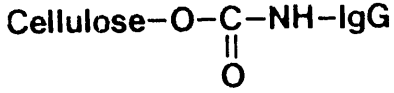
Figure 2.8. Polyacrylamide gel electrophoresis of purified CRP.
a) After affinity column.
b) After gel filtration.



1-1' Carbonyldiimidazole



Imidazolyl Carbamate



Alkyl Carbamate
(Solid Phase IgG)

Figure 2.9. Reaction of 1,1'-carbonyldiimidazole with protein.

measured by the Biuret reaction. 400 μl of antibody were added to 200 mg of CDI-cellulose in 600 μl of 50 mmol/l barbitone buffer, pH 8.0. The slurry was mixed end-to-end overnight at room temperature.

Similarly, 50 μg of CRP in 300 μl were coupled to 100 mg of CDI-cellulose in 700 μl of coupling buffer for use as an immunoextraction reagent for the preparation of specific labelled antibody.

After overnight reaction, the cellulose was washed according to the method of Wide (1969). After each wash, the cellulose was pelleted by centrifugation at 2000 rpm for 5 minutes. The sequence of washes was as follows:

1. $NaHCO_3$, 500 mmol/l for 20 minutes (x3)
2. Acetate buffer, 100 mmol/l, pH 4.0 for 60 minutes (x1)
3. Acetate buffer for 16 hours (x1)

The cellulose was recovered by centrifugation, suspended in 500 μl of 0.1 mol/l borate buffer, pH 8.0, and stored at 4°C. The method of Schachterle & Pollack (1973) was used to measure the quantity of antibody protein bound to the cellulose. This was found to range from 1.8 to 2.8% (w/w).

CRP bound to cellulose was used to extract high affinity antibodies from the antiserum to be used as label. Fifty mg of CRP-cellulose were added to 200 μl of 10 mmol/l phosphate buffer pH 7.4, containing 150 mmol/l NaCl and 50 μl of anti-CRP. After overnight mixing, the cellulose was washed three times with 50 mmol/l phosphate buffer, pH 7.4 containing 150 mmol/l NaCl, once with 150 mmol/l NaCl and 2 ml/l Tween 20, and three times with 50 mmol/l phosphate buffer, pH 7.4, omitting the NaCl.

c) Iodination of Antibody

The extracted antibodies were iodinated by the chloramine T method while still bound to CRP-cellulose. This procedure protects the antibody binding site from iodination. 30 μl of 0.5 mol/l phosphate buffer, pH 7.4, were added to 50 mg of the immobilised antibody, followed by 1 mCi of $Na^{125}I$ in 10 μl. Chloramine T, 4 g/l in 50 mmol/l phosphate buffer, pH 7.4, was added to the reaction mixture in a volume of 10 μl. The tube was agitated vigorously for 30 seconds, and the reaction was quenched with 500 μl of assay buffer.

The iodination mixture was washed three times with 2 ml of 1 mmol/l HCl, and the iodinated antibody was eluted with 1 ml each of 10 mmol/l HCl and 500 mmol/l $NaHCO_3$. In initial experiments, free iodine was removed with a 1 x 10 cm column of Sephadex G-25M.

d) Optimisation Experiments

Sample was added in a volume of 10 μl to 100 μl of labelled antibody (20000 cpm) diluted in assay buffer in 55 x 12 mm polystyrene tubes (Sarstedt, no 55.484). After an incubation of 90 minutes at room temperature, 0.5 mg of antibody-cellulose was added in 100 μl of assay buffer and the tubes incubated for a further 90 minutes with agitation on a rotary mixer. At the end of this period, the cellulose was washed three times by repeated cycles of centrifugation at 1200xg and resuspension in 2 ml of 150 mmol/l NaCl, containing 0.2% Tween 20. After the final

wash, the tubes were counted in a NE 1600 multi-well counter (New England Nuclear).

In the following experiments, the sample contained 200 μg/l of Behring standard CRP. A tube containing no CRP was also included. The antibody used as label was a rabbit antiserum obtained from Dako. Binding was expressed as a percentage of the total label added to the tube. The percentage binding of the test sample was divided by that of the zero and was used as an index of the sample to noise ratio of the assay.

The assay buffers investigated are listed in table 2.5. All contained 0.2% Tween 20 and 0.5% normal sheep serum. It can be seen that the main effect of changing the composition of the buffer was to alter the zero binding rather than that of the standard. The borate buffer, pH 8.0, and the barbitone buffer, pH 8.6, both gave acceptable blank values, of less than 3% bound, and the borate buffer was used subsequently.

It was found that the presence of serum and detergent decreased the non-specific binding of label to the assay tubes. The effects of normal sera from rabbit, sheep, goat, and donkey, and of human and bovine serum albumin were tested (table 2.6). Rabbit serum had a severely deleterious effect on the blank, while sheep serum was ineffective. Albumin, donkey serum, and goat serum all improved the blank. The most marked effects were seen with 0.1 and 0.5% donkey serum. A concentration of 0.2% was chosen for further use.

Table 2.5

CRP IRMA - Assay Buffers

Buffer		B_T	B_o	B_T/B_o
Phosphate	pH 7.4	23.9	5.0	4.7
	pH 7.8	23.2	3.3	7.0
Borate	pH 8.0	21.3	2.6	8.0
	pH 8.6	24.6	9.3	2.6
Barbitone	pH 8.0	32.8	5.4	6.1
	pH 8.6	21.7	2.7	7.9

All buffers were at a concentration of 100 mmol/l. B_T: % binding of standard CRP at 82 μg/l. B_o: % binding of zero standard. B_T/B_o: signal/noise ratio.

Table 2.6

CRP IRMA - Effect of Protein in Buffer

Protein	Concentration (%)	B_T	B_o	B_T/B_o
None	-	21.2	2.8	8.3
Serum				
Sheep	0.1	21.3	3.6	5.9
	0.5	19.3	3.2	6.1
	1.0	23.4	2.3	10.2
Donkey	0.1	20.7	1.4	14.9
	0.5	19.0	1.2	15.8
	1.0	17.9	4.4	4.0
Goat	0.1	21.6	2.3	9.4
	0.5	18.5	1.9	9.6
	1.0	17.8	1.5	11.8
Rabbit	0.1	22.1	5.4	4.1
	0.5	24.6	4.7	5.2
	1.0	22.2	14.1	1.6
Albumin				
Human	0.1	23.1	3.7	6.3
	0.5	23.3	2.5	9.2
	1.0	22.5	2.2	10.1
Bovine	0.1	22.3	2.5	8.8
	0.5	21.7	1.9	11.4
	1.0	21.1	2.6	8.0

Abbreviations as in table 2.5.

The detergents tested were the non-ionic detergents Tween 20, Brij 35, Nonidet P40, and Triton X-100, the anionic detergents sodium decyl sulphate and Triton GR-5M, and the cationic detergent hexadecyltrimethylammonium bromide (cetrimide). The results are shown in tables 2.7 and 2.8.

Triton GR-5M, cetrimide, and sodium decyl sulphate all had severely detrimental effects on binding of the standard and on the blank values. The non-ionic detergents all improved the blank values if included at their optimal concentrations. The greatest effect was shown by Tween 20 at 0.1%, which was used subsequently.

Seven commercial antisera (table 2.9) were tested for potential use as the labelled antibody. They were all iodinated using chloramine T under the previously described conditions.

Satisfactory binding and an acceptable slope of standard curve were obtained using all the antisera except those produced by Behring and Seward (figure 2.10). Since the Seward antiserum was the one which had been attached to the solid phase, this finding implies that all the binding sites of CRP for this antiserum were saturated during the incubation period with label, and that none were then available to react with the same antiserum when it was attached to cellulose. The Behring antiserum also did not bind in a two-site assay, so its binding profile was presumably similar to that of the Seward antiserum. The Atlantic Antibodies antiserum was used in all further experiments.

Table 2.7

CRP IRMA - Effect of Non-ionic Detergents

Detergent	Concentration (%)	B_T	Bo	B_T/Bo
None	-	22.9	5.2	4.4
Tween 20	0.1	23.6	1.6	14.6
	0.2	23.8	2.0	11.9
	0.5	24.3	2.1	11.6
	1.0	24.6	9.9	2.5
Brij 35	0.1	23.9	1.7	14.2
	0.2	24.3	2.9	8.5
	0.5	23.7	9.4	2.5
	1.0	23.5	2.1	11.2
Nonidet P40	0.1	23.7	11.3	2.1
	0.2	24.1	1.9	12.7
	0.5	24.0	2.1	11.6
	1.0	25.3	4.2	6.0
Triton X-100	0.1	24.6	2.2	11.1
	0.2	24.4	2.4	10.0
	0.5	24.7	5.1	4.8
	1.0	24.9	1.8	13.8

Abbreviations as in table 2.5.

Table 2.8

CRP IRMA - Effect of Ionic Detergents

Detergent	Concentration (%)	B_T	B_O	B_T/B_O
SDS	0.01	32.2	15.5	2.1
	0.05	29.3	10.5	2.8
	0.10	26.6	8.2	3.2
Triton GR	0.01	6.6	8.4	0.8
	0.05	21.9	7.6	2.9
	0.10	15.5	5.5	2.8
Cetrimide	0.01	6.9	4.1	1.7
	0.05	16.4	12.4	1.3
	0.10	13.7	8.6	1.6

Abbreviations as in table 2.5.

Table 2.9

Antisera Tested for Use in CRP IRMA

Supplier	Species
Atlantic Antibodies	Goat
Immuno	Goat
Kallestad	Goat
Seward	Sheep
Behring	Rabbit
Orion	Pig
Dako	Rabbit

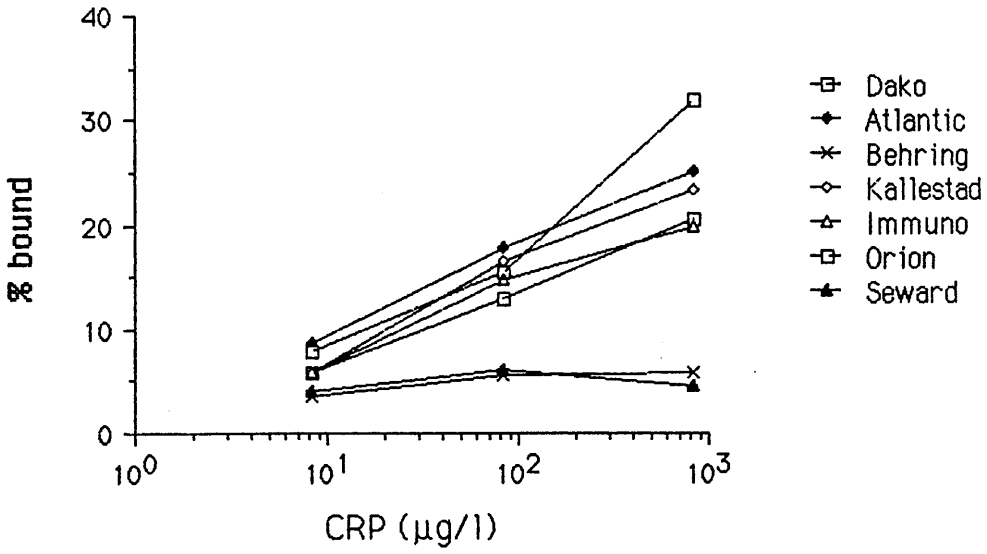


Figure 2.10. Selection of antisera for CRP IRMA.

The length of time required for binding of sample to the labelled antibody was investigated by adding label to a high (820 μg/l) and to a low (8.2 μg/l) standard for various lengths of time. At the end of the incubation period, solid-phase antiserum was added and incubation continued for 90 minutes with agitation before the final washing and counting steps.

The low standard required a longer period of incubation than the high standard to reach equilibrium, but both had reached equilibrium after 2 hours. In a similar experiment in which sample and label were incubated for 2 hours before addition of solid phase (figure 2.11), it was found that the incubation with solid-phase antibody also required 2 hours.

It was found that the assay was quite insensitive to the concentration of tracer added, so 40000 cpm per tube were routinely added to give sensitivity of counting coupled with economy of reagent use. One mg of solid phase antibody was used per tube.

The simple desalting step originally used to remove free iodine from the label was not capable of removing any aggregated or damaged material which might have been present. To investigate whether there was any such material in the labelling mixture, a sample of label was passed down a column of Sepharose CL-6B (figure 2.12). There was a shoulder (A) before the main peak (B), possibly due to dimerisation of the antibody or to the presence of CRP-antibody complexes which may have been

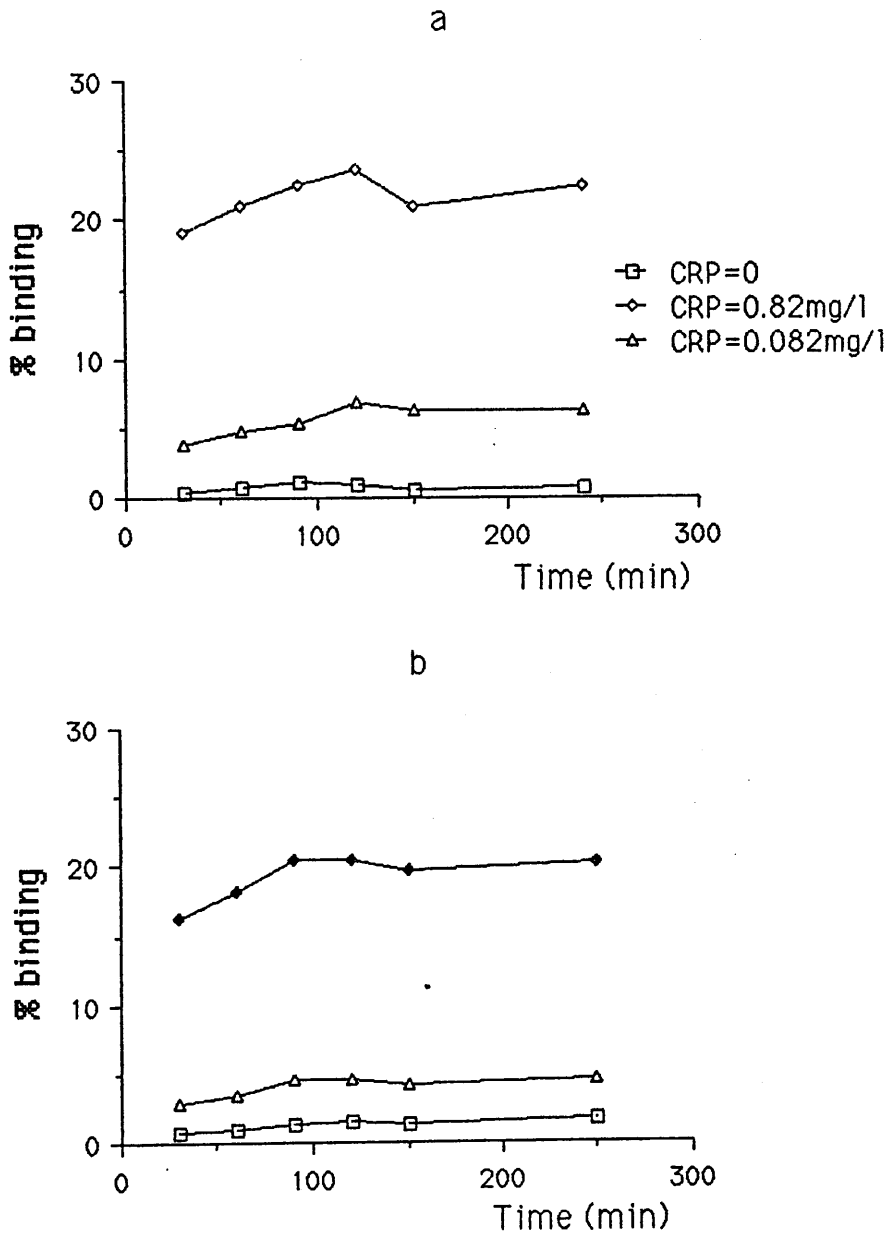


Figure 2.11. Time course of IRMA reactions.

a. Differing times of incubation of label.
b. Differing times of incubation of solid phase.

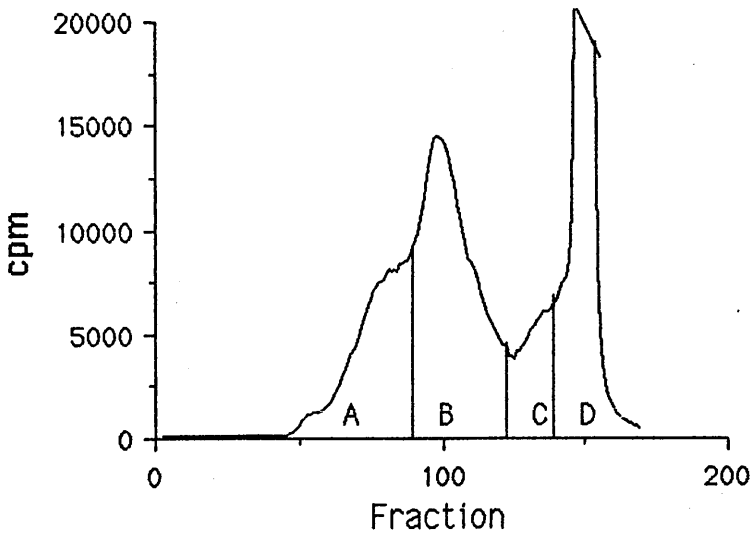


Figure 2.12. Elution profile of anti-CRP antibody on Sepharose CL-6B after iodination.

stripped from the cellulose. Some low-molecular weight material (C) was also sometimes eluted before the iodide peak (D).

The binding capacity of the shoulder was inferior to that of the main peak (maximum binding 20% compared to 23%), and the non-specific binding was 5% as compared to 1%, so the major peak was used routinely.

e)Precision

A typical standard curve is shown in figure 2.13. Precision profiles were calculated by the method of Ekins & Edwards (1983). A coefficient of variation of less than 15% was obtainable between CRP concentrations of 5 μg/l and 100 μg/l. All serum samples were diluted with saline to lie within this range, and were assayed in duplicate.

Two patient samples were assayed ten times in duplicate in an assay batch. The precision values obtained were 9.0% at a serum concentration of 6.3 mg/l and 8.1% at 35.1 mg/l.

Two commercially produced quality control materials were assayed in each batch. These were the Syva control material which was provided with the EMIT kit and the Orion Diagnostika low CRP control. The between-batch precisions of these materials were 11.9% at a mean value of 35.4 mg/l (n=9) and 13.6% at a mean value of 20.0 mg/l (n=9) respectively.

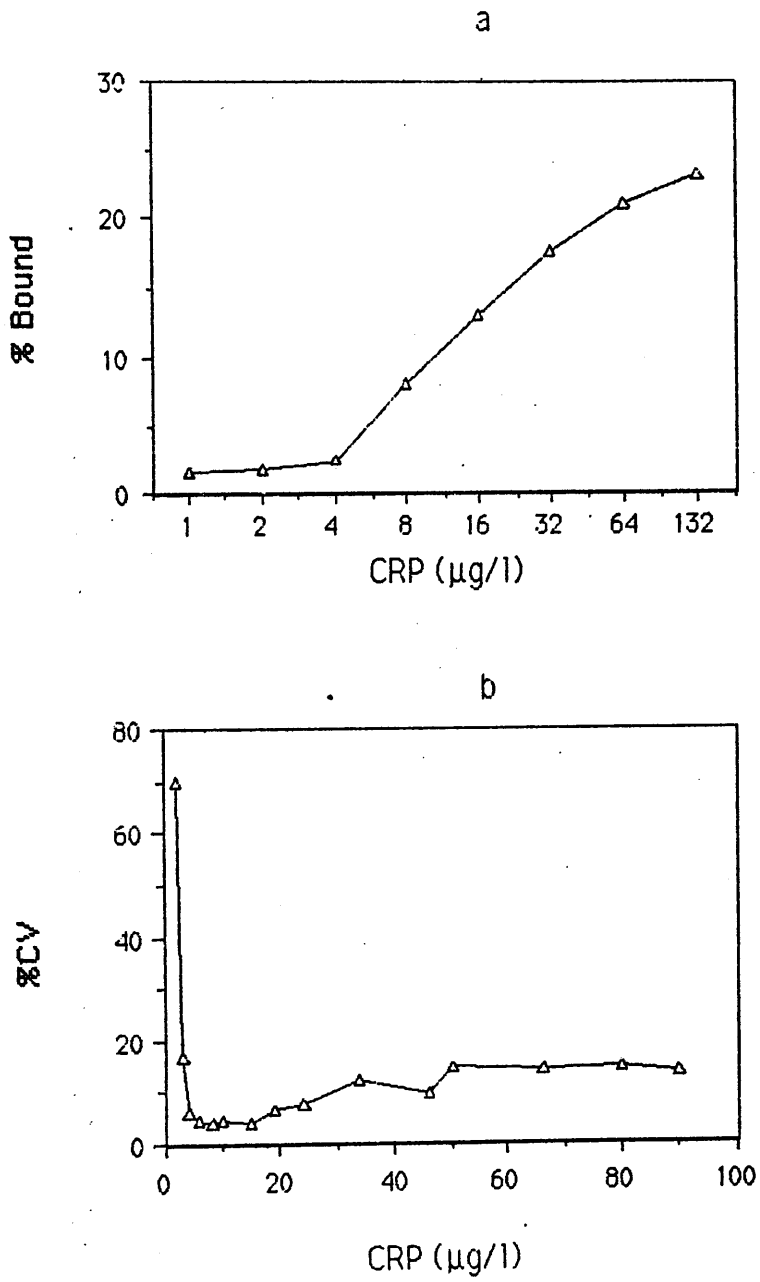


Figure 2.13. a) Standard curve and

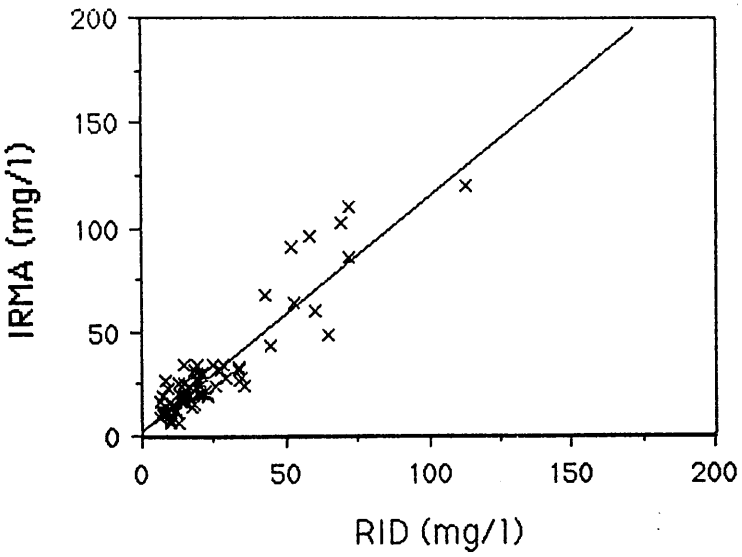
b) Precision profile of CRP IRMA

f)Method_Comparisons

The IRMA was compared with radial immunodiffusion, EMIT, and immunonephelometry (figures 2.14-2.16). Correlation was good with both the RID and EMIT methods (r = 0.916 and 0.935 respectively). Correlation with the laser method was less close (r = 0.878), although the slope of the regression line was very close to 1.0. This may reflect the poor precision of nephelometry at low concentrations.

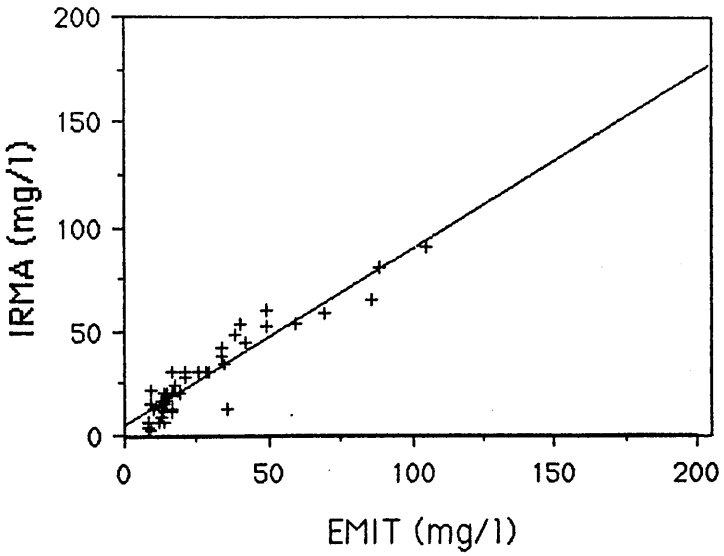
g)Reference_Range

Sera from 86 normal volunteers (40 male, 46 female) were analysed by the IRMA method. The frequency distribution of CRP concentrations was markedly skewed towards high values (figure 2.17), the median CRP concentration being 0.40 mg/l and the mean 1.31 mg/l. The percentiles which enclosed 95% of the values corresponded to 0.1 mg/l and 4.0 mg/l. There was considerable uncertainty of the exact position of the upper percentile, since there were few samples at this level. For comparison, the data points were transformed logarithmically and a normal range derived using Gaussian statistics. The range from 2 standard deviations below the mean to 2 standard deviations above the mean was 0.068 - 5.1 mg/l, with a mean of 0.593 mg/l. This range was similar to that obtained from the untransformed values.



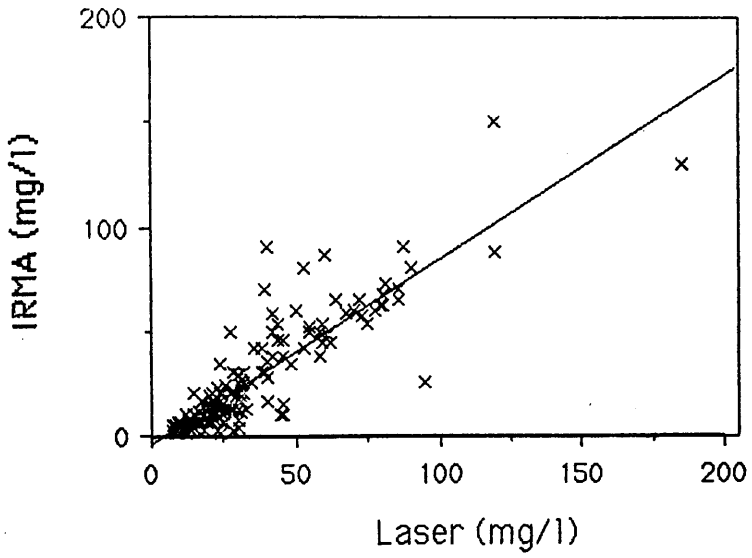
$y = 2.46 + 1.14x, \quad r=0.92$
(SE 1.99) (SE 0.06)

Figure 2.14. Comparison between IRMA and RID methods for C-reactive protein.



$y = 4.76 + 0.84x, \quad r=0.93$
(SE 0.76) (SE 0.05)

Figure 2.15. Comparison between IRMA and EMIT methods for C-reactive protein.



$y = -4.05 + 0.89x, \quad r=0.88$
(SE 1.66) (SE 0.04)

Figure 2.16. Comparison between IRMA and laser nephelometric methods for C-reactive protein.

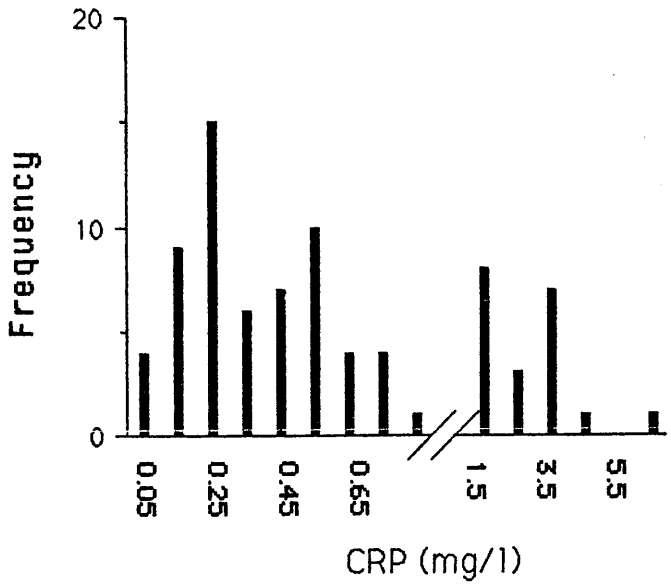


Figure 2.17. Histogram of CRP values in normal volunteers.

h)Summary of Characteristics of Methods Used

During the course of these studies, four methods were used to measure CRP. These were radial immunodiffusion, immunonephelometry, EMIT, and IRMA. The advantages and disadvantages of each method will now be compared with those of the Abbott TDX system, which we currently use routinely. This is a fluorescence polarisation assay, similar in principle to the drug assays which have been developed for the same instrument. The assays are compared in terms of ease of use, rapidity, sensitivity, and cost.

The simplest methods to use are TDX and RID. The TDX system is automated, and gives a direct readout of concentration. The RID methodology is almost as simple, since only a ruler and a magnifying glass are required to measure ring diameters, and a simple graph is constructed for calculation of concentrations.

Sample preparation for nephelometry is also not difficult, and the Hyland nephelometer itself is not complicated. However, since it is a manual system, operation becomes tedious if large numbers of samples are assayed. Antibody variability is also a problem, since few antisera are suitable, and the assay therefore suffers from long-term lack of robustness.

The EMIT methodology for CRP is more complicated than that for most of the drugs assayed by the same principle. There are two reagent additions, and their timing is critical. Because of this, considerable attention to detail is required if reproducible results are to be

obtained, and the method is not suitable for the analysis of large batches of samples, particularly since all standards and samples must be assayed in duplicate. A fully automated system is available, but is very expensive.

The IRMA assay was not difficult to run, and it was easy to assay large batches of samples. The pipetting and wash steps could be automated, providing large savings in time and effort. However, there was a substantial amount of reagent preparation, particularly since an iodine label was employed, which was only usable for 4-6 weeks. Immunoselection and iodination took 2 days' work, which is comparable to the effort required for other radio-immunoassays.

The most precise assays were the EMIT, TDX, and IRMA assays, though both the EMIT and IRMA required duplicate determination of samples. The nephelometric method was less precise, especially at low levels, and RID was the least precise method. Only the IRMA method was capable of measuring levels within the normal range.

The cheapest methods to run are the nephelometric and IRMA methods. Both use small amounts of expensive antisera and few consumables. The IRMA does need technician time to be spent on reagent preparation, but the nephelometric method requires an instrument dedicated to protein analyses.

The RID, EMIT, and TDX methods all use commercial kits, so are much more expensive. The EMIT system is possibly the most expensive, since duplicate determinations are made and standards must be run in each assay, while the

TDX standard curve is stable for weeks. Both EMIT and TDX use dedicated instruments, but these can also be used for a variety of drug assays.

The final choice of method depends on the use to which the assay will be put and the equipment available in the laboratory. The IRMA may be the most suitable for large-scale research work, since it can handle large numbers of samples and can measure levels within the normal range. It is also a rapid method, so might also be suitable for rapid turnround of a high routine workload.

RID requires little expertise, but is expensive, and too slow for routine use in a clinical setting. The EMIT, TDX, and nephelometric assays all offer a same day availability of results. Both the EMIT and TDX are costly, but the TDX, in particular, is very easy to use and reliable. Nephelometric methods have in the past been insufficiently robust for routine use, but developments in methods, such as the Beckman ICS rate nephelometric analyser and the Baker latex enhanced CRP kit, may solve these problems, and offer cheap, rapid, and readily available CRP measurements in clinical practice.

2.5. Urine Analyses

Urine nitrogen was measured by an automated micro-Kjeldahl procedure (Fleck, 1967). Urine was digested to ammonium sulphate with concentrated H_2SO_4 containing potassium sulphate, mercuric oxide, and hydrogen peroxide. Digestion was carried out at 380°C for 25 minutes, and the ammonium generated was estimated by an automated Berthelot

reaction.

Urine 3-methylhistidine was measured on a Chromospek amino acid analyser (Rank Hilger). Urine was deproteinised with sulphosalicylic acid (50 mg/ml). The resin used was Dowex AG50-X8, 200-400 mesh, and the internal standard was S-β-(4-pyridylethyl)-L-cysteine. Detection and quantitation were carried out using the ninhydrin reaction.

Urinary free cortisol was measured using a modification of the fluorimetric method of Mattingly et al (1964). 17-hydroxycorticosteroids were measured by the colourimetric method of Norymberski et al (1953) as modified by Few (1961) and James & Caie (1964). This method depends on the cleavage of the side chains of cortisol metabolites with metaperiodate to form 17-ketosteroids, which are then estimated with dinitrobenzene. Urine catecholamines were measured by a semi-automated fluorimetric method based on the method of Wood & Mainwaring-Burton (1975). Adrenaline was estimated by difference from the values of total catecholamines and noradrenaline. Full details of the methods for 3-methylhistidine and catecholamines are given in Gedeon (1981).

2.6. Measurement of Interleukin 1

In the absence of sensitive immunoassays, bioassays based on the multiple activities of IL-1 are used to measure its concentration. The most practical assays are those which use isolated cells in vitro rather than entire

animals, both from the points of view of ease and expense.

The lymphocyte activation (LAF) assay was chosen to measure IL-1, as it is capable of handling large numbers of samples and is comparatively inexpensive. On the other hand, it is known to suffer from interferences from many biological fluids. The responder cells used were murine thymocytes, and the label was tritiated thymidine. The culture medium used in all experiments was RPMI 1640, containing 2 mmol/l glutamine, 50 U/l of penicillin, 50 μg/l of streptomycin, and 10% foetal bovine serum. Solutions of phosphate-buffered saline and balanced salts solution were made up from 10x concentrates (Gibco, Irvine) and autoclaved before use. All plastic ware used was sterile, and all glassware was baked in an oven at 160°C for 2 hours before use. All manipulations of cells were carried out using sterile technique in a laminar flow cabinet.

2.6.1. IL-1 Preparation

Human peripheral blood mononuclear cells were prepared by centrifugation over Ficoll-Hypaque (Pharmacia) by the method of Boyum (1968). They were suspended at 10^7 cells/ml in RPMI 1640 containing glutamine, 20 mmol/l Hepes buffer, and 2% foetal calf serum and stimulated to produce IL-1 with 50 ng/ml of E. coli endotoxin, phenol extracted, serotype 055:B5 (Sigma) for 24 hours at 37°C. The cells were removed by centrifugation at 1000xg for 15 minutes, and low-molecular weight materials removed by desalting over a Pharmacia PD10 column into assay medium.

2.6.2. IL-1 Assay

It became evident in preliminary experiments that the strain of mouse considerably affected the response to IL-1. Mice of 5 different inbred strains were obtained from Banton and Kingman. The strains tested were Balb/c, C57Bl/6, CBA/Ca, C3H/He, and AKR. All mice were 6-8 weeks old when tested.

Concanavalin A and Phytohaemagglutinin (PHA-P) were obtained from Sigma. They were dissolved in balanced salts solution and diluted to the required concentration in assay medium. A range of final concentrations of 0.5 - 10 μg/ml was used.

Mice were killed by cervical dislocation. Their thymi were removed aseptically and a single cell suspension made by passing them through a plastic sieve or by grinding them between the frosted ends of glass microscope slides. Debris was allowed to settle for 10 minutes. The cells were washed twice and resuspended in assay medium at a concentration of 10^7 cells/ml. They were pipetted into 96-well microtitre plates to give 10^6 cells per well, and mitogen added to the required final concentration. The outer rows and columns were not used, to avoid the possibility of edge effects. Samples of IL-1 were added to give a final dilution of 1:48, which had been shown to be optimal in preliminary experiments.

After incubation for 72 hours in an atmosphere of 5% CO_2 in air at 37°C, the cells were pulsed with 0.5 μCi of [^{3}H] thymidine (5 Ci/mmol, Amersham) for 4-6 hours, and harvested using a semiautomatic harvester (Titertek) on to

glass fibre discs. These were washed with 5% trichloroacetic acid and methanol, air dried, and counted in a liquid scintillation counter, using Beckman Filter-Count scintillation fluid.

Most strains of mouse tested did not show a marked comitogenic effect of IL-1 in the presence of Con A (table 2.10). For the Balb/c and C57Bl/6 mice, the direct mitogenic effect of IL-1 was greater than the comitogenic effect at all concentrations tested. CBA mice did, however, show a definite peak of activity at 1 μg/ml of Con A. There was little response from AKR mice at any mitogen concentration.

The effects of PHA were more marked (table 2.11), although the absolute level of counts was lower than that for Con A. CBA, C3H/He, Balb/c, and C57Bl/6 mice all showed a peak of comitogenic activity at 2 μg/ml. However, the comitogenic effect was substantially greater than the direct mitogenic effect only for CBA mice (stimulation index 10.2 for comitogenesis versus 2.8 for direct mitogenesis). Once more, AKR mice showed the poorest responses at all doses tested.

CBA mice were chosen for further use, as they were the only ones tested which showed a clear response to the combination of IL-1 and PHA. Although the optimum concentration of PHA was 2 μg/ml in this experiment, this varied somewhat with the source of mice. In later experiments, mice from the breeding colony of Strathclyde University were used, and it was found that the optimum concentration of PHA was 4 μg/ml.

In the final version of the assay, samples were serially

Table 2.10

Response of Mouse Strains to Concanavalin A

Mouse Strain	Concentration of Mitogen (μg/ml)	Difference ± IL-1 (cpm)	SI
C57Bl/6	0.0	158	4.4
	0.5	139	3.3
	1.0	260	3.1
	2.0	164	3.6
	5.0	2760	2.1
	10.0	1050	2.1
Balb/c	0.0	452	9.2
	0.5	450	7.1
	1.0	2295	6.8
	2.0	3750	4.3
	5.0	208	1.5
	10.0	-377	0.6
C3H/He	0.0	675	6.3
	1.0	1662	5.7
	2.0	2513	2.5
	5.0	1109	1.5
	10.0	973	1.4
CBA	0.0	216	2.8
	0.5	318	4.3
	1.0	1881	7.4
	2.0	3968	3.6
	5.0	1621	2.2
	10.0	2101	1.8
AKR	0.0	25	1.6
	0.5	87	2.0
	1.0	61	1.6
	2.0	54	1.5
	5.0	40	1.4
	10.0	68	1.6

SI: stimulation index, ratio of counts ± IL-1

Table 2.11

Response of Mouse Strains to Phytohaemagglutinin

Mouse Strain	Concentration of Mitogen (μg/ml)	Difference ± IL-1 (cpm)	SI
C57Bl/6	0.0	131	3.8
	0.5	159	4.6
	2.0	254	5.7
	5.0	204	4.1
	10.0	102	2.4
Balb/c	0.0	452	9.2
	0.5	366	6.7
	1.0	306	6.0
	2.0	343	6.3
	5.0	304	5.2
	10.0	323	4.5
C3H/He	0.0	675	6.3
	1.0	476	4.7
	2.0	597	5.9
	5.0	340	3.2
CBA	0.0	216	2.8
	0.5	299	4.2
	1.0	507	6.3
	2.0	956	10.2
	5.0	610	5.6
	10.0	625	4.8
AKR	0.0	25	1.6
	0.5	49	1.5
	1.0	263	3.1
	2.0	2777	2.6
	5.0	2007	1.5
	10.0	192	1.0

SI: stimulation index: ratio of counts ± IL-1

diluted at a ratio of 1:3 in assay medium, and added in 50 μl aliquots to wells of microtitre plates. Phytohaemagglutinin was added in 50 μl to a final concentration of 4 μg/ml, and 100 μl of cells were added to all wells. All samples were assayed in triplicate, and blank wells were included, either without sample or without PHA or sample. Incubation and labelling were as above, except that 1 μCi of [^{3}H]-thymidine was used.

CHAPTER 3

EFFECT OF ELEVATED ENVIRONMENTAL TEMPERATURE ON THE ACUTE PHASE RESPONSE

3.1. Environmental Temperature and the Response to Trauma

It is well established that the magnitude of certain aspects of the response to trauma can be reduced by maintaining the injured animal at an elevated environmental temperature. Caldwell et al (1959) showed that the metabolic rate of burned rats depended on the temperature at which they were housed. They claimed that the increase in metabolic rate normally seen following injury could be abolished by maintaining the animals at 32°C. In 1962, Caldwell also reported that weight loss and nitrogen excretion were considerably reduced if rats were housed at a temperature of 30°C after injury.

Barr et al (1968) showed that burned patients who were maintained in warm, dry conditions (32°C, 30% humidity) had a substantially lower metabolic rate and mortality than those nursed at 22°C. This was accompanied by reductions in the rates of albumin and gamma globulin catabolism and in the amount of albumin found in the extravascular space, and an increase in the synthesis rate of albumin (Davies et al, 1969). Catecholamine excretion also fell at the higher temperature (Birke et al, 1972).

These marked effects on the metabolic rate and nitrogen excretion of burned patients have not been confirmed by

all groups of workers. Wilmore et al (1974a) failed to alter the metabolic rate of patients with a burn of less than 40% surface area by varying the temperature between 25°C and 33°C. There was, however, an increase in metabolic rate if the temperature was lowered as far as 21°C, and they could demonstrate a small difference between 25°C and 33°C in more severely burned patients. They were never able to bring the metabolic rate to normal by increasing the temperature.

Cuthbertson et al (1968, 1972) also demonstrated a reduction in metabolic rate and in nitrogen excretion in patients with long bone fractures maintained at 30°C as compared to 20°C. Studies by Ballantyne & Fleck (1973) showed that the effects of injury on the concentrations of albumin, α_1-acid glycoprotein, and CRP were also much less marked in patients maintained at 30°C, although no change in the catabolic rate of albumin could be demonstrated (Ballantyne et al, 1973).

It has been difficult to repeat the findings of Cuthbertson et al (1972). Spivey and Johnston (1972), studying patients undergoing elective surgery, failed to detect any effect of raising the temperature from 24°C to 29°C.

Carli et al (1982), on the other hand, studying an older group of surgical patients undergoing major surgery, were able to reduce heat loss during the operation and nitrogen excretion over the subsequent 48 hours by using a heated mattress and warming all fluids given intravenously to 37°C. Ryan (1983) and Jones et al (1984) reported similar

results, using a fluidised warmed bed at 32°C. They showed that there was a decrease in total body protein breakdown and in liver secretory protein synthesis.

To investigate further whether there was an effect on surgical patients of raising the environmental temperature, we tested the effect of maintaining a group of patients undergoing abdominal surgery at either 20°C or 28°C. Energy and nitrogen intake were controlled, and measurements were made of the metabolic rate and of a wide variety of biochemical parameters. These included several hormones and acute phase proteins.

3.2. Study Design

3.2.1. Patients

Twenty male patients, aged between 20 and 71 years, undergoing elective cholecystectomy or vagotomy and drainage, were studied at an environmental temperature of either 20° or 28°C. Informed consent was obtained from all patients. Throughout the study the patients were nursed in purpose built metabolic cubicles with an air conditioning system which maintained ambient temperature within ± 1°C of the desired level and relative humidity of 35 - 40%. The patients wore light cotton bedgowns and were covered by a sheet and "duvet" which they were at liberty to put on or remove for comfort. There were no major post-operative complications in either group of patients and the group did not differ in the incidence of minor

complications. Three patients in the 20°C group and four patients in the 28°C group developed post-operative chest infections and one patient developed a mild urinary tract infection. No patient required transfusion with either blood or plasma during the post-operative period.

The patients were studied on one control day preoperatively and on the first four postoperative days. From the evening before the control study the patients received a constant intravenous (IV) infusion of 5% dextrose at a rate of 83 ml/hr (21/24h). The IV infusion was discontinued at the end of the control study and was re-established postoperatively. The remainder of the patients' fluid requirements were met by calorie free fluids and water only was allowed by mouth when clinically indicated.

3.2.2. Samples

A 24 h urine collection was taken commencing at 9 a.m. on the control day. Postoperatively four consecutive 24 h collections were made commencing at 9 a.m. on the first postoperative day. Urine was collected in clean bottles with no added preservative. On completion, urine volume was determined from weight and specific gravity and aliquots were taken for determination of 17-hydroxycorticosteroids (17 OHC) and free cortisol. The urine was then acidified with concentrated sulphuric acid and aliquots made for catecholamine, total nitrogen, and 3-methylhistidine estimation. The samples were frozen and

stored at -20°C for subsequent analysis.

Blood samples for protein estimation were taken via a Venflon catheter, inserted into an antecubital vein, at 9 a.m. on each study day. Serum was separated by centrifugation and stored at -20°C until analysis.

3.2.3. Biochemical Analyses

The proteins measured were C-reactive protein, α_1-antichymotrypsin, α_1-antitrypsin, α_1-acid glycoprotein, haptoglobin, albumin, transferrin, prealbumin, α_2-macroglobulin, and retinol binding protein. All except transferrin and retinol binding protein were measured by immunonephelometry using the Hyland LAS-R nephelometer PDQ. Transferrin was measured using the Technicon AIP system, and retinol binding protein was measured by radial immunodiffusion. Details of the methods used to measure proteins and urine constituents are described in chapter 2.

3.2.4. Temperature Measurement

An estimate of the patients' core temperature was made using a thermistor (YSI 44011, Yellow Springs Instruments Ltd) which was fitted into a silicone rubber earpiece and inserted into the external auditory canal. Mean skin temperature was measured using thermistors (YSI 409, Yellow Springs Instruments Ltd) with flat metal surfaces, which were taped to the patients' chest, thigh, and foot.

3.2.5. Statistical Analysis

Except where otherwise stated, results are quoted as mean ± 1 standard deviation. The results were analysed by two-way analysis of variance with repeated measures on one factor - days (Winer. 1971a; Cohen & Holliday, 1982a). Tukey's test for honestly significant differences (Winer, 1971b; Cohen & Holliday, 1982b) was used to test for differences between study days. When significant differences were found between temperatures, individual days were compared by means of unpaired t-tests.

3.3. Results

There were no significant differences between the groups of patients in age, weight, or operative procedure (table 3.1). The only significant difference in initial biochemical measurements was that the concentration of transferrin was lower in the 20°C group than the 28°C group ($p<0.05$).

The effect of the surgical procedure on all the biochemical variables measured was highly significant ($p<0.001$).

3.3.1. Temperatures

The mean external auditory temperature in the 28°C group was 35.4°C ± 0.4. This was significantly higher than the temperature for the 20°C group, which was 34.8°C ± 0.4

Table 3.1

Clinical Details of Patients

	Environmental Temperature	
	20°C	28°C
number of patients	10	10
age (years): mean ± SD	47±18	48±15
range	20-71	25-69
weight (kg): mean ± SD	68±9	71±10
range	61-85	56-86
operative procedures:		
cholecystectomy	4	4
vagotomy and drainage	6	6

($p<0.01$). The mean skin temperature (33.9°C ± 0.64) was also higher than the value at 20°C (32.2°C ± 0.67).

3.3.2. Urine Volume

Urine volume increased after the operation, but not significantly until the third day. The volume passed on days 1 and 2 was greater in the 20°C group than in the 28°C group (2046 ml ± 676 vs 1197 ml ± 748 on day 1, $p<0.05$; 2423 ml ± 735 vs 1410 ml ± 1013 on day 2, $p<0.05$).

3.3.3. Urine Nitrogenous Constituents

Since there was no nitrogen intake over the course of the study, figures quoted for nitrogen excretion in urine were taken to be equivalent to nitrogen balance. No account was taken of possible losses in faeces. Nitrogen excretion (table 3.2), was significantly greater than the control values on postoperative days 1 - 3. The maximum daily excretion was observed on day 1, after which the levels started to fall. The mean maximum excretions, calculated from the maximum value recorded for each patient, were 221% of the control value and 183% of control for the 20°C and 28°C groups respectively. There was no detectable effect of environmental temperature.

The excretion of 3-methylhistidine and the ratio of 3-methylhistidine to creatinine increased postoperatively and showed no sign of decreasing again during the course of the study. The mean maximum values of 3-methyl-

Table 3.2

Excretion of Nitrogen and 3-Methylhistidine after Operation (mean ± SD; n=10)

	Control		Day 1		Day 2		Day 3		Day 4	
	20°C	28°C	20°C	28°C	20°C	28°C	20°C	28°C	20°C	28°C
Volume (ml)	1277 ±387.9	1662 ±726.6	2046 ±676.2	1197 ±748.5	2423 ±735.5	1410 ±1013.0	2566 ±896.5	2065 ±913.5	2817 ±1035.1	1790 ±1272.4
Nitrogen (g/24h)	6.2 ±2.2	8.2 ±2.3	12.7 ±3.3	12.3 ±4.1	10.6 ±3.2	10.7 ±2.9	9.5 ±2.8	12.2 ±2.6	9.0 ±2.5	9.4 ±3.6
3-MH (μmol/24h)	223.7 ±81.1	246.2 ±113.4	471.5 ±154.2	362.3 ±113.0	485.3 ±184.3	399.2 ±166.2	472.5 ±105.4	435.5 ±123.4	395.6 ±108.4	394.2 ±163.4
3-MH/creatinine (μmol/mmol)	21.8 ±6.3	23.0 ±12.0	25.9 ±7.3	20.7 ±9.2	38.1 ±13.4	24.1 ±9.7	39.5 ±15.0	32.4 ±11.0	37.3 ±18.8	34.0 ±13.2

3-MH: 3-methylhistidine.

histidine excretion were 262% of control at 20°C and 224% at 28°C. The maximum ratios of 3-methylhistidine to creatinine were 199% of control at 20°C and 160% at 28°C. There were no significant differences between the two temperature groups.

3.3.4. Hormones

The excretion of noradrenaline was maximal on the first post-operative day (table 3.3), and decreased thereafter, ceasing to be significantly different from control on day 4. The mean maximum values were 460% of control in the 20°C group and 285% of control in the 28°C group. The mean daily excretion of noradrenaline over the four days was 24% higher at 20°C than at 28°C (82.6 μg/24 hr $\pm$ 40.4 vs 66.4 μg/24 h $\pm$ 27.4), but this did not reach statistical significance because of the considerable variation between patients. Similarly, the peak excretion of adrenaline occurred on day 1 and the mean maximum excretions were 584% of control and 628% of control for the two groups. Though the mean daily excretion of adrenaline was 31% higher at 20°C than at 28°C (35.3 μg/24 h $\pm$ 34.8 vs 27.0 μg/24 h $\pm$ 16.5), this again did not reach statistical significance. The percentage changes suggest that much of this difference was due to differences between the groups on the control day. The overall analysis of variance confirmed that there were no significant differences between 20°C and 28°C at any time.

The excretions of cortisol and 17-hydroxycorticosteroids

Table 3.3

Excretion of Hormones after Operation (mean ± SD; n=10)

Control		Day 1		Day 2		Day 3		Day 4			
20°C	28°C	20°C	28°C	20°C	28°C	20°C	28°C	20°C	28°C		
Noradrenaline (μg/24h)		28.3 ±10.2	35.8 ±18.8	128.5 ±75.2	96.3 ±44.8	94.5 ±53.7	66.5 ±27.7	64.3 ±27.7	62.5 ±29.2	52.4 ±23.9	41.6 ±26.0
Adrenaline (μg/24h)		10.8 ±6.1	8.6 ±8.2	62.8 ±44.6	54 ±32.5	39.2 ±42.2	19.2 ±12.4	22.9 ±13.5	20.3 ±15.7	16.2 ±5.4	14.6 ±11.9
Cortisol		0.29	0.30	3.45	1.61	1.13	0.58	1.19	0.94	1.02	0.61
				(μmol/24h) Medians; n=9							
17-OHC (μmol/24h)		52.3 ±26.5	57.3 ±17.1	194.3 ±93.7	106.4 ±31.4	128.4 ±59.9	58.9 ±13.6	83.7 ±22.8	85.9 ±38.5	79.4 ±28.9	52.4 ±23.2

17-OHC: 17-hydroxycorticosteroids

were also maximal on the first day. The mean maximum excretion of cortisol was 1592% (20°C) and 664% (28°C), and that of 17-OHC was 381% and 198% of control respectively. The frequency distributions of the cortisol and 17-OHC results were highly skewed, so the results were transformed to their logarithms before analysis (Winer, 1971c; McNeil, 1977). Analysis of variance then showed significant differences between temperatures for both hormone measurements ($p<0.05$ and $p<0.01$ respectively), but the interaction between day of study and temperature was also significant. This suggests that the operation had different effects on the pattern of cortisol excretion at the two temperatures. Unpaired t-tests on the transformed values showed that there were significant differences between the temperature groups on days 1, 2, and 4.

The cortisol and 17-OHC data was also analysed non-parametrically. The median cortisol values on day 1 were 3451 and 1610 nmol/24 h and the excretions of 17-OHC were 201 μmol/24 h and 115 μmol/24 h at 20°C and 28°C respectively. The excretion of both compounds was significantly greater at 20°C on days 1 and 2 when tested by the Mann-Whitney test for unpaired samples.

3.3.5. Serum Proteins

The serum concentrations of CRP, haptoglobin, α_1-antichymotrypsin, α_1-acid glycoprotein, and α_1-antitrypsin all rose significantly following operation (table 3.4). The peak daily mean concentrations of CRP and

Table 3.4

Serum Concentrations of Proteins Which Increased after Operation (mean ± SD; n=10)

	Control		Day 1		Day 2		Day 3		Day 4	
	20°C	28°C	20°C	28°C	20°C	28°C	20°C	28°C	20°C	28°C
CRP (mg/l)	10 (median)	<10 (median)	149 ±37	152 ±47	209 ±64	209 ±58	157 ±62	163 ±52	100 ±49	113 ±51
ACT (g/l)	0.78 ±0.27	0.78 ±0.31	1.71 ±0.40	1.65 ±0.43	2.26 ±0.37	2.27 ±0.52	2.20 ±0.40	2.07 ±0.60	1.96 ±0.30	2.03 ±0.49
Haptoglobin (g/l)	1.52 ±0.68	1.45 ±0.65	1.59 ±0.58	1.58 ±0.53	2.36 ±0.69	2.39 ±1.04	2.71 ±0.86	2.83 ±1.17	2.83 ±0.71	2.78 ±0.84
Antitrypsin (g/l)	1.48 ±0.48	1.56 ±0.42	2.22 ±0.44	2.26 ±0.40	2.91 ±0.52	3.00 ±0.69	3.12 ±0.55	3.38 ±0.86	2.95 ±0.62	3.16 ±0.69
AG (g/l)	0.72 ±0.30	0.74 ±0.27	1.01 ±0.28	0.99 ±0.21	1.27 ±0.27	1.26 ±0.31	1.30 ±0.37	1.32 ±0.18	1.29 ±0.17	1.28 ±0.34

ACT: α_1-antichymotrypsin AG: α_1-acid glycoprotein

α_1-antichymotrypsin occurred on the second post-operative day, after which levels fell, but they did not return to normal by the end of the study period. CRP rose by greater than 20 fold to a mean maximum of 211 mg/l $\pm$ 62 at 20°C and 214 mg/l $\pm$ 58 at 28°C. The increase in α_1-antichymotrypsin was to 295% of control at 20°C and 303% of control at 28°C.

The concentration of haptoglobin showed a delayed rise on the second day, and continued to rise slowly thereafter. The mean maximum concentration was 193% of control at 20°C and 205% at 28°C. The concentrations of α_1-acid glycoprotein and α_1-antitrypsin increased until day 2 and then showed no further significant increase. The maximum values for α_1-acid glycoprotein were 196% at 20°C and 186% at 28°C, and those for α_1-antitrypsin were 217% at 20°C and 222% at 28°C. The relative magnitude of the increase of these proteins was lower than that of CRP and α_1-antichymotrypsin.

The temperature at which the patients was nursed did not affect the rate or amount of increase of these proteins.

The concentrations of albumin, transferrin, prealbumin, retinol binding protein, and α_2-macroglobulin all decreased significantly until day 2, after which there was no further significant fall (table 3.5). The most marked decreases occurred in the levels of prealbumin and RBP. The minimum levels of prealbumin were 53% and 50% of control at 20°C and 28°C respectively, while the mean minimum levels of RBP were 50% and 44%.

There was a more moderate degree of decrease of albumin

Table 3.5

Serum Concentrations of Proteins Which Decreased After Operation (Mean ± SD; n=10)

	Control		Day 1		Day 2		Day 3		Day 4	
	200C	280C	200C	280C	200C	280C	200C	280C	200C	280C
Albumin (g/l)	36.8 ±5.7	37.0 ±4.4	35.3 ±6.7	33.9 ±2.7	29.3 ±3.9	31.0 ±3.5	25.7 ±3.3	29.8 ±3.4	26.2 ±4.8	28.2 ±3.4
Transferrin (g/l)	2.66 ±0.38	3.02 ±0.60	2.34 ±0.38	2.63 ±0.33	1.92 ±0.25	2.22 ±0.37	1.75 ±0.19	2.05 ±0.32	1.75 ±0.16	2.08 ±0.36
Prealbumin (mg/l)	305 ±55	353 ±54	267 ±52	290 ±57	199 ±37	214 ±42	173 ±31	184 ±39	168 ±36	183 ±32
RBP (mg/l)	51.9 ±10.8	59.4 ±15.6	35.9 ±7.6	37.9 ±7.9	27.9 ±6.1	29.2 ±5.1	28.0 ±6.4	27.8 ±4.6	31.4 ±6.5	27.4 ±5.8
AM (g/l)	2.30 ±1.43	1.86 ±0.44	2.17 ±1.08	1.73 ±0.38	2.01 ±0.99	1.64 ±0.39	1.80 ±0.78	1.58 ±0.30	1.90 ±1.02	1.50 ±0.36

RBP: retinol binding protein AM: α_2-macroglobulin.

and transferrin, to 68% (20°C) and 76% (28°C) for albumin and to 64% and 67% for transferrin respectively. The mean maximum fall in α_2-macroglobulin was only to 76% of control at 20°C and to 80% of control at 28°C.

The environmental temperature again did not affect the concentrations of these proteins. There was an apparently significant difference between the concentrations of transferrin at 20°C and 28°C ($p<0.05$), but this corresponded to the difference between the groups before operation, and the percentage change caused by surgery was not altered by temperature.

3.4. Discussion

The pattern of response of the acute phase proteins to surgery was as expected from previous studies (Aronsen et al, 1972; Werner & Cohnen, 1969). The most rapid and marked increase was exhibited by CRP, followed by α_1-antichymotrypsin. CRP increased by at least 20 fold from undetectable levels in most of the patients, and reached a peak concentration by the second post-operative day. The concentration of α_1-antichymotrypsin also reached a peak value on the second day, though it only tripled in value. Both proteins had started to decrease again before the end of the four day study period, which suggests that either might be useful in detecting complications of surgery arising in the first week. As none of the patients in this study had any severe complications, this hypothesis could not be tested. The other acute phase

proteins showed increases of a lesser magnitude, which did not show any sign of returning towards normal. It is unlikely, therefore, that they would be of any value in clinical management of patients.

The decreases in the negative acute phase proteins were also of the magnitude expected. The greatest decreases were seen in prealbumin and RBP, with lesser decreases in albumin, transferrin, and α_2-macroglobulin. It is difficult to distinguish the effects of short term starvation from trauma when interpreting the results of prealbumin and RBP. It has been claimed that they can respond to dietary deficiencies very rapidly (Shetty et al, 1979), but decreases of the order of magnitude seen in this study have also been reported in patients receiving full nutritional support (Young et al, 1979). The fall in α_2-macroglobulin has not been reported before, but may merely represent changes in hydration of the patients. Colley et al (1983) have suggested that the concentrations of all plasma proteins should be corrected for the haematocrit, but this was not done in this study.

The endocrine changes seen in this study were also in accord with previous studies. There was a marked, transient increase in the excretion of both cortisol and catecholamines, and both had almost returned to normal by the end of the study period.

Nursing patients at 28°C had few effects on any biochemical measurements. None of the proteins were at all affected by the environmental temperature. The excretion of nitrogen and 3-methylhistidine in urine and the

metabolic rate were similarly unchanged (Harris et al, 1985).

There was a significant decrease in urine output at the higher temperature. The main biochemical differences between the groups were decreases in the excretion of cortisol and 17-hydroxycorticosteroids at 28°C. A similar trend was noted in the excretion of catecholamines, but this failed to reach significance.

This overall lack of response to alterations in environmental temperature is in agreement with the results of Spivey & Johnston (1972), but contrasts with the observed effects on both metabolic rate and biochemical measurements reported by Barr et al (1968), Davies et al (1969) and Cuthbertson et al (1972). There are several possible reasons for these discrepancies.

The difference in temperature experienced by the two groups of patients may not have been sufficiently great to cause metabolic alterations. Although the patients were in a controlled environment, they were allowed a duvet, and were able to regulate their own microclimate. However, a significant difference was noted between the two groups in both core and skin temperatures, so this factor may not have been important.

The degree of injury experienced by the patients in the various studies may be an important variable. Elective surgery may not cause a sufficient degree of metabolic disturbance for any effect of environmental temperature to be noticeable. The groups who have reported effects of environmental temperature have all studied either thermal

burns or accidental long bone injury, and Wilmore et al (1974a) were only able to alter the metabolic rate of the most extensively burned of their patients. However, the more thorough heating produced by a warmed mattress or by warming fluids during the operation does seem to produce metabolic effects even in patients undergoing surgery (Carli et al, 1982; Jones et al, 1984).

The effects noted on cortisol may be directly due to adaptation of the patients to the environmental temperature. It is known that one of the effects of subjecting healthy volunteers to cold is to cause increases in the excretion of cortisol and catecholamines (Budd et al, 1970; Lamke et al, 1972), so the increase seen here may merely represent the effect of a mild cold stress at 20°C, and not be related at all to any fundamental alteration in the response to surgery.

The changes seen in this study on the excretion of cortisol were not reflected in nitrogen metabolism, metabolic rate, glucose concentrations, or acute phase proteins (Harris et al, 1985). This must call into question the likelihood of a direct controlling role of adrenal hormones in the acute phase response in man. Alberti et al (1980) only demonstrated a correlation of cortisol with intermediary metabolites after burns, not elective surgery, and they did not prove a cause and effect relationship.

The direct evidence for an important role of steroid hormones in the stimulation of the liver protein response has come entirely from animal experiments, mostly carried

out in rats. The limited work which has been done on cultures of murine and human cells would suggest that these hormones are not an important mediator of the protein response to trauma in other species (Baumann et al, 1984), and this is supported by the lack of response of CRP to the infusion of catabolic hormones carried out by Bessey et al (1984). Indeed, these workers even showed a suppression of CRP synthesis in response to inflammation when a combination of cortisol, adrenaline, and glucagon was infused (Watters et al, 1986).

CHAPTER 4

EFFECT OF TREATMENT WITH GOLD SALTS ON THE ACUTE PHASE RESPONSE IN RHEUMATOID ARTHRITIS

4.1. Use of Gold Salts In Rheumatoid Arthritis

Gold salts were originally used in the nineteenth century as antituberculous drugs. It was noticed that certain patients who also suffered from rheumatoid diseases had relief of their symptoms while they were being treated. Forestier (1935) published the first clinical report on the use of gold for the treatment of rheumatoid arthritis. The efficacy of gold compounds was later confirmed in double-blind trials (Fraser, 1945; Research Sub-Committee of the Empire Rheumatism Council, 1961).

The gold salts which were first developed were administered by intramuscular injection. The ones which are still in clinical use, gold sodium thiomalate and aurothioglucose, are rapidly absorbed into the circulation and are effective in relieving the signs and symptoms of rheumatoid arthritis. Unfortunately, they are significantly toxic, and in 1974, Girdwood reported that the mortality associated with their use was higher than that for any other drug available at that time. For this reason, it is essential to monitor patients very closely. The complications which have been reported include skin rashes, renal and hepatic damage, and thrombocytopenia

(Gottlieb and Gray, 1978).

The problems of toxicity and the inconvenience of intramuscular injections prompted the development of auranofin (figure 4.1), an orally absorbed gold compound (Finkelstein et al, 1976). This compound contains gold (I) in combination with an acetylated thioglucose ligand and triethyl phosphine. From 15-25% of an oral dose is absorbed from the gut (Gottlieb, 1982), and blood concentrations are proportional to the dose given. Unlike the water soluble injectable gold preparations, the main route of excretion is in the faeces. It is thought that the sulphur and phosphine ligands are removed in the gastrointestinal mucosa and that gold in an unknown form is bound in blood to proteins. The gold derived from auranofin enters cells readily, and is almost equally partitioned between plasma and erythrocytes (Herrlinger et al, 1982; Lewis et al, 1983). It is hoped that this could improve its efficacy compared to the more hydrophilic compounds, which do not enter cells readily.

Several clinical trials have now reported on the use of auranofin, though only a few groups have compared the efficacy of this drug with a placebo, because of the difficulty of maintaining patients with active disease on placebo (Katz et al, 1982; Ward et al, 1983; Wenger et al, 1983). However, there is general agreement that auranofin is effective in controlling the activity of rheumatoid arthritis, and its efficacy is similar to or somewhat less than that of gold thiomalate. It has been claimed that treatment with auranofin reduces the rate of progression

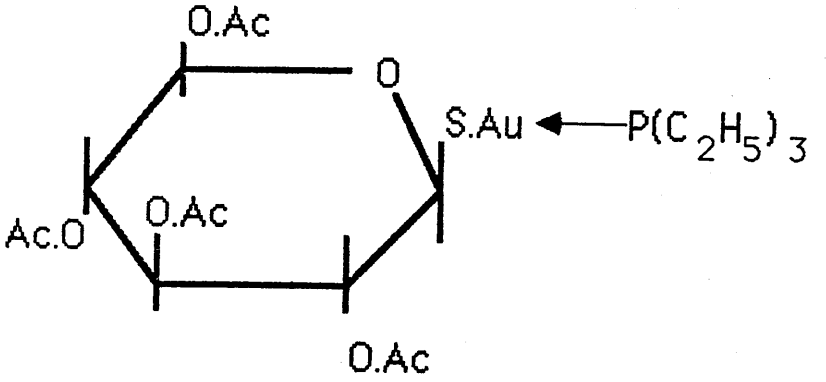


Figure 4.1. Structure of Auranofin. (2,3,4,6,-tetra-O-acetyl-1-thio- -D-glucopyranosato-S-triethylphosphine) gold

of radiologically detectable bone lesions, suggesting that it may be classified as a disease modifying drug. The number of toxic complications is considerably lower than for injectable gold, consisting mainly of diarrhoea and skin rashes. In general, auranofin seems to be promising as a possible replacement for injectable gold.

It has been shown that the concentrations of acute phase proteins in blood reflect the clinical activity of rheumatoid arthritis. This suggests that the effect of a disease modifying drug could be monitored by measurement of these proteins. The studies of Dixon et al (1980, 1981, 1984) would tend to confirm that measurement of a number of biochemical variables can allow treatment to be followed and can give an objective basis for classifying a drug as being "disease modifying". They claimed that CRP was the most sensitive indicator of disease improvement, as measured by its correlation with clinical indices of improvement.

It is not known how these drugs affect liver protein synthesis, but it is thought that they have effects on both lymphocytes and macrophages (Gordon et al, 1985). It is possible that they interfere with IL-1 production either directly or by preventing stimulatory signals coming from lymphocytes (Griswold et al, 1985).

The biochemical investigation of the effects of auranofin has been incomplete. Most published studies have concentrated on clinical indices of efficacy, such as a score for the number of swollen joints, or the amount of pain felt. At best, the levels of haemoglobin, ESR, and

immunoglobulins have been measured to give a more objective index of improvement, and very few measurements of acute phase proteins have been made. It is not certain which of the acute phase proteins is best to monitor this particular condition. Dixon et al (1984) recommend the measurement of CRP, but haptoglobin has often been used to monitor the intensity of inflammation. We decided to measure the levels of several commonly employed acute phase proteins during the course of a double blind trial, with a placebo group, of auranofin versus gold thiomalate.

4.2. Study Design

4.2.1. Patients

Ninety patients (28 male, 62 female) with classical or definite rheumatoid arthritis according to the criteria of the American Rheumatism Association (Ropes et al, 1959) were studied. All patients were receiving optimal doses of non-steroidal anti-inflammatory drugs and none was taking steroids or had previously received treatment with gold in any form. In addition, none had received penicillamine, levamisole, or immunosuppressive drugs in the six months preceding the trial.

Initially patients were randomly allocated into three treatment groups. The clinical details are shown in table 4.1. Thirty patients were given an initial 10 mg intramuscular injection of sodium aurothiomalate followed by 50 mg weekly for the first 20 weeks or until clinical

Table 4.1

Initial Clinical Characteristics of Patients.

Median and range

Characteristic	Placebo	Gold Thiomalate	Auranofin
Age (years)	54.5 (31-72)	47.0 (25-69)	55.0 (22-71)
Duration of Disease (years)	4.5 (1-24)	5.0 (1-40)	5.5 (1-30)
Male:female	11:19	9:21	8:22

response. Thereafter, the frequency of injection was reduced to fortnightly, three weekly, and ultimately four weekly. Thirty patients received auranofin tablets (3 mg twice daily) throughout the study. Thirty patients were given placebo tablets identical in appearance with auranofin twice daily. No attempt was made to give placebo injections. Patients discontinuing placebo therapy were allocated randomly to either of the gold compounds.

4.2.2. Assessment of Disease Activity

Patients were initially monitored weekly for possible side effects and assessments of disease activity were performed at three-monthly intervals.

At each assessment, the duration of morning stiffness was recorded in hours, and pain score was measured in millimeters on a 100 mm visual analogue scale. Grip strength was measured with an anaeroid manometer attached to a small cuff inflated to 20 mm of mercury, and the Ritchie articular index was calculated (Ritchie et al, 1968). These parameters were also combined with haemoglobin and ESR (Westergren) according to the method of Mallya & Mace (1981) to derive a disease activity index.

Venous blood samples were obtained three monthly for acute phase protein estimation and the serum stored at -20°C until analysis. The serum concentration of CRP was measured by the two-site immunoradiometric assay.

The other acute phase proteins which were measured were

α_1-antichymotrypsin, haptoglobin, α_1-antitrypsin, and α_1-acid glycoprotein, which were assayed by immuno-nephelometry.

4.2.3. Statistical Methods

Non-parametric statistics were used, because the data for several variables was skewed. Measurements at different time points within treatment groups were compared using the Wilcoxon signed rank test, and measurements across treatment groups at particular time points were compared by the Kruskal-Wallis test. If an overall significant variation was found between the drug groups, the source of the variation was further investigated using the Mann-Whitney U test. All data was analysed using the SPSS and SPSSx statistical packages.

Discriminant function analysis was carried out using the Discriminant routine of SPSSx (Norusis, 1985; Tabachnik & Fidell, 1983). Stepwise analysis was employed, with the criterion for inclusion of variables being the minimisation of Wilks's lambda.

4.3. Results

At the start of the trial the three treatment groups were similar in terms of age, sex ratio, duration of disease and clinical variables (tables 4.1, 4.2). Although there were no predefined entry criteria, the method of

Table 4.2

Initial Values of Clinical Measurements.

Median and interquartile range

Measurement	Placebo	Gold Thiomalate	Auranofin
Duration of Morning Stiffness (hours)	1.0 (1.0-3.0)	2.0 (1.0-4.0)	1.5 (1.0-3.2)
Pain score on 100mm analogue scale	54 (33-83)	65 (51-87)	66 (48-81)
Ritchie Articular Index	21 (21-31)	16 (10-32)	15 (9-21)
Grip Strength (mm Hg)	82 (58-114)	91 (76-117)	87 (73-113)
Haemoglobin (g/dl)	12.6 (11.5-13.6)	11.6 (11.0-14.2)	12.2 (11.0-13.7)
Rheumatoid factor Number positive	27	26	26

assessing disease activity as derived by Mallya & Mace (1981) showed that the majority of patients had moderately active disease (70/90), while 14/90 had slightly active and 6/90 had very active disease. There were also no significant differences in initial protein concentrations between the treatment groups (table 4.3).

The number of patients whose concentrations of acute phase proteins and whose ESR were within the reference range at the start of the trial is shown in table 4.4. Only 2 patients had normal values for all 6 measurements. From the data in the table, the proteins can be divided into 2 groups. Fewer than 20% of patients had values of ESR, CRP, or α_1-antichymotrypsin within the reference range at week 0. On the other hand, haptoglobin, α_1-acid glycoprotein and α_1-antitrypsin were considerably less sensitive in detecting active disease. In particular, only 30% of patients entered the study with abnormal levels of α_1-antitrypsin.

The relative sensitivity of each of the proteins for the detection of active disease was compared with that of CRP, which was expected to be the most sensitive. There were 7 patients in whom CRP was normal in the face of an abnormal ESR and 9 in whom CRP was normal but α_1-antichymotrypsin was raised. The numbers of patients in whom CRP was raised but ESR or α_1-antichymotrypsin was normal were only 2 and 3 patients respectively. CRP was much more sensitive than any of the remaining proteins measured. There were 23 patients in whom haptoglobin concentrations were normal, 16 patients in whom α_1-acid glycoprotein was normal, and

Table 4.3

Initial Values of Biochemical Measurements

Median and interquartile range

Measurement	Placebo	Gold Thiomalate	Auranofin
ESR	56	58	51
	(40-69)	(28-79)	(28-78)
Haptoglobin (g/l)	2.3	2.2	2.1
	(2.0-3.3)	(1.5-3.2)	(1.3-2.8)
Antitrypsin (g/l)	2.3	2.3	2.1
	(2.1-2.6)	(2.0-2.7)	(1.8-2.8)
AG (g/l)	1.2	1.2	1.1
	(1.1-1.6)	(1.0-1.7)	(1.0-1.5)
CRP (mg/l)	30	20	33
	(17-50)	(13-58)	(9-45)
ACT (g/l)	1.2	1.1	1.1
	(1.0-1.4)	(1.0-1.4)	(1.0-1.4)

AG: α_1-acid glycoprotein; ACT: α_1-antichymotrypsin.

Table 4.4

Patients with Initial Measured Values within Reference Range

Acute Phase Protein	Number of Patients	% of total Patients
ESR	11	12
C-Reactive Protein	17	19
α_1-Antichymotrypsin	10	11
Haptoglobin	36	40
α_1-Acid Glycoprotein	26	29
α_1-Antitrypsin	64	71

48 in whom α_1-antitrypsin concentrations were normal in spite of a raised CRP.

Withdrawal from the trial due to adverse reactions to therapy or to lack of beneficial effect occurred in 17/30 (57%) of patients on placebo by 24 weeks. After this period the numbers in the placebo group declined rapidly and became insufficient for statistical analysis. No patients in this group completed 48 weeks. Twenty-two (73%) on thiomalate and 17 (57%) on auranofin completed 48 weeks.

The reasons for dropout are shown in table 4.5. In the placebo group, 27 were removed from the study because of lack of efficacy of the drug, compared with none in the group treated with gold thiomalate and 6 in the group treated with auranofin. Toxic effects of treatment requiring withdrawal were seen in 2 patients treated with placebo, 6 with gold thiomalate, and 3 with auranofin.

Paired comparisons were made of those patients who continued treatment until 12, 24, and 48 weeks with their initial values (figures 4.2-4.8). None of the patients treated with placebo showed any significant changes at any time. In the auranofin group, none of the proteins showed any significant change until 24 weeks, at which time ESR, haptoglobin, α_1-antitrypsin, α_1-acid glycoprotein, α_1-antichymotrypsin, and CRP were all decreased compared to the initial sample. Pain score was decreased at 12 weeks, but none of the other clinical measurements showed any change until 48 weeks, when grip strength was also improved. The overall activity index was decreased at 24

Table 4.5

Reasons for Withdrawal from Therapy

	Placebo		Gold Thiomalate		Auranofin	
	n	%	n	%	n	%
Adverse effect	2	7	6	20	3	10
Inefficacy	27	90	0	0	6	20
Intercurrent illness	1	3	1	3	3	10
Non compliance	0	0	1	3	1	3

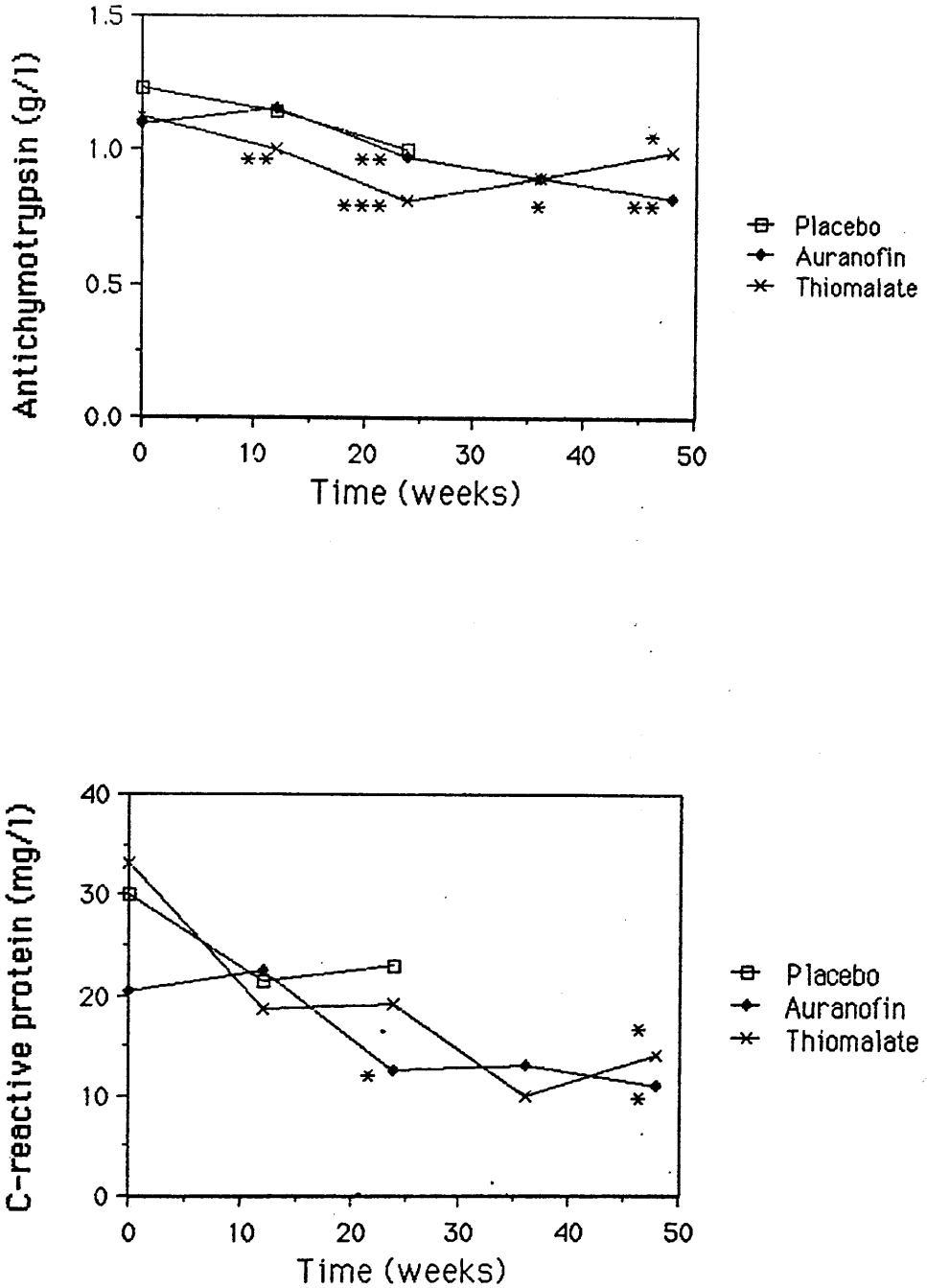
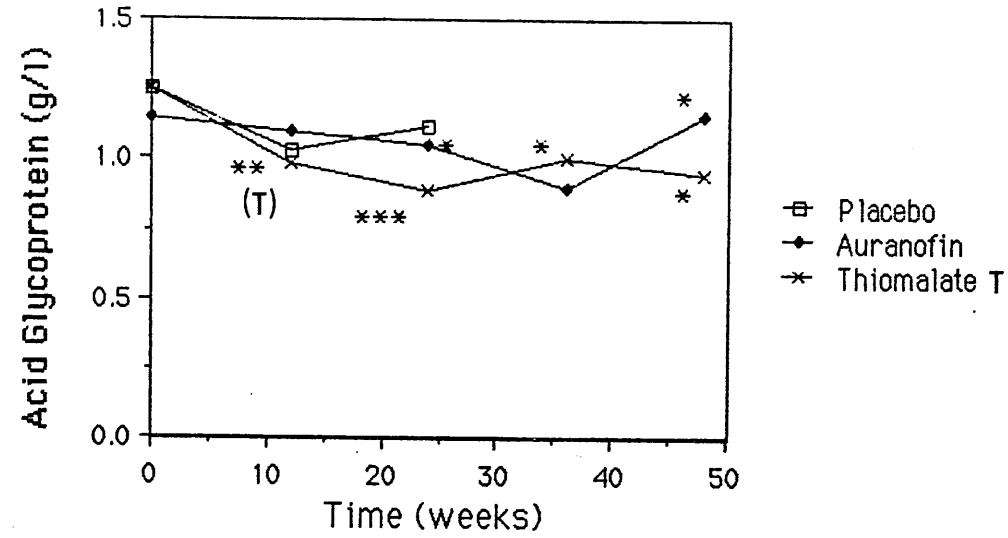


Figure 4.2. Concentrations of antichymotrypsin and C-reactive protein after treatment with gold compounds.

Asterisks show significances of time points as compared to zero time.

*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$



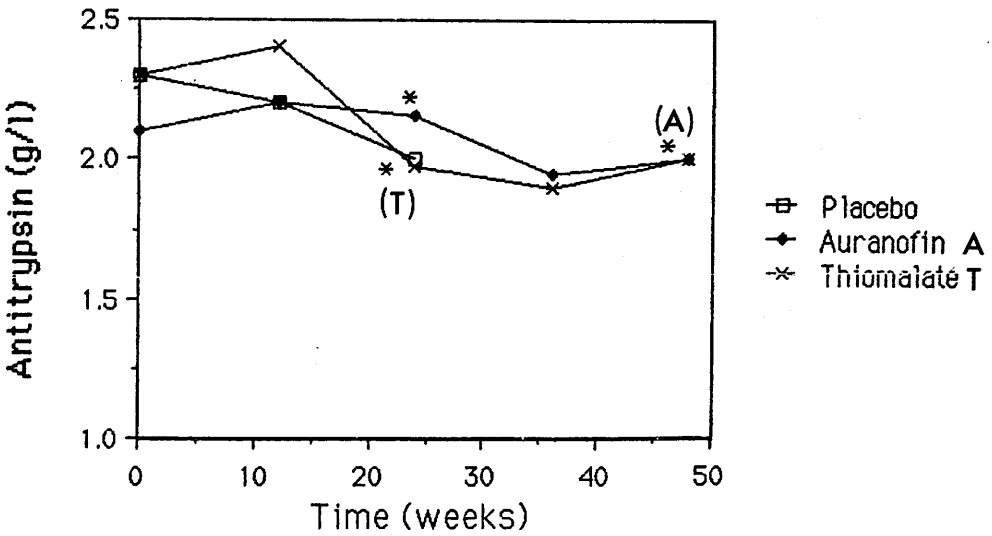
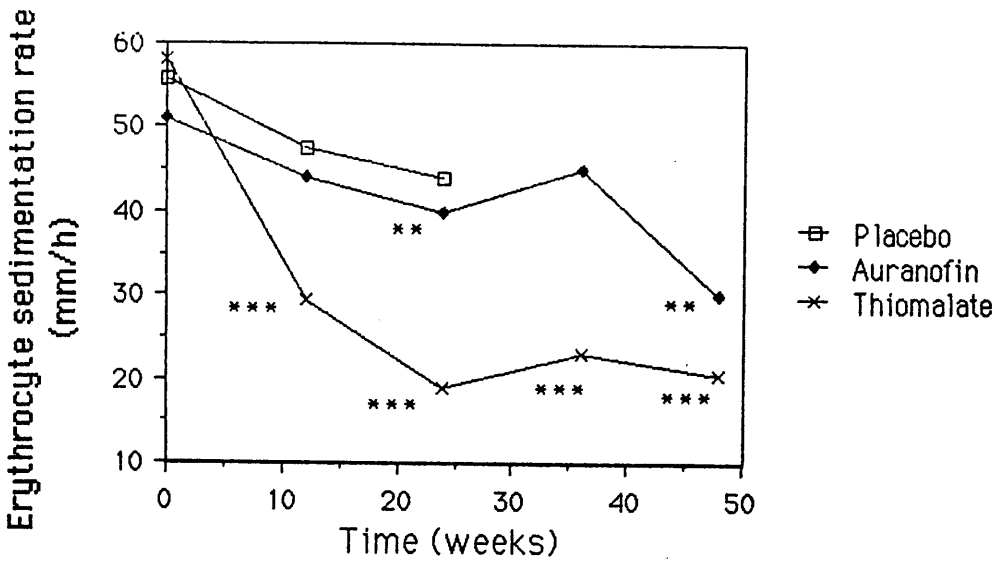


Figure 4.3. Concentrations of acid glycoprotein and antitrypsin after treatment with gold compounds.



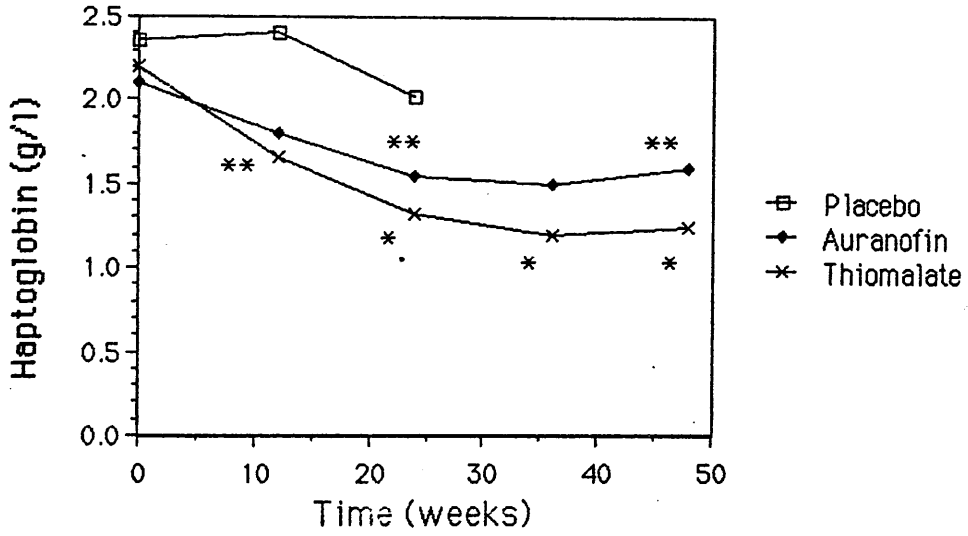
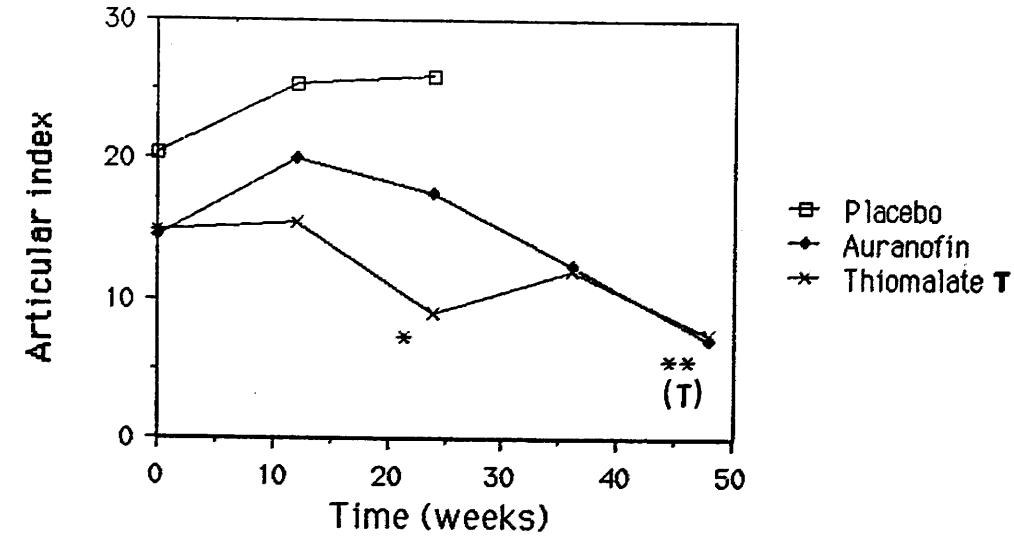


Figure 4.4. Erythrocyte sedimentation rate and haptoglobin concentration after treatment with gold compounds.



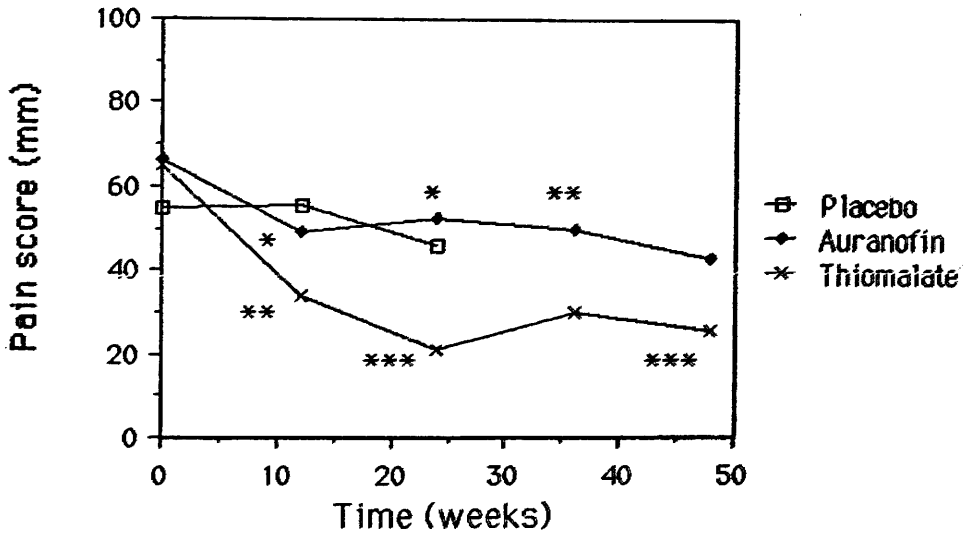


Figure 4.5. Articular index and pain score after treatment with gold compounds.

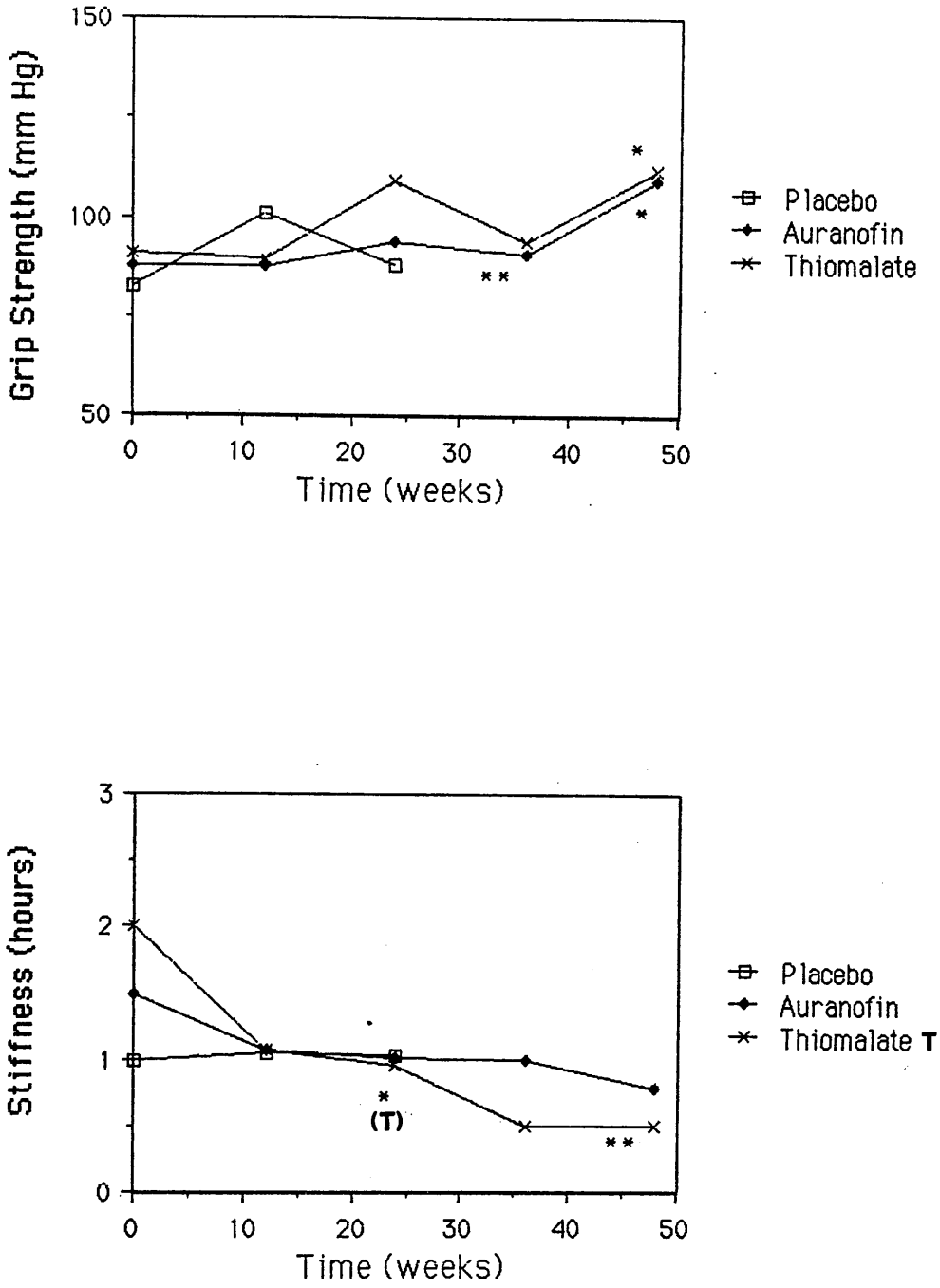


Figure 4.6. Grip strength and stiffness after treatment with gold compounds.

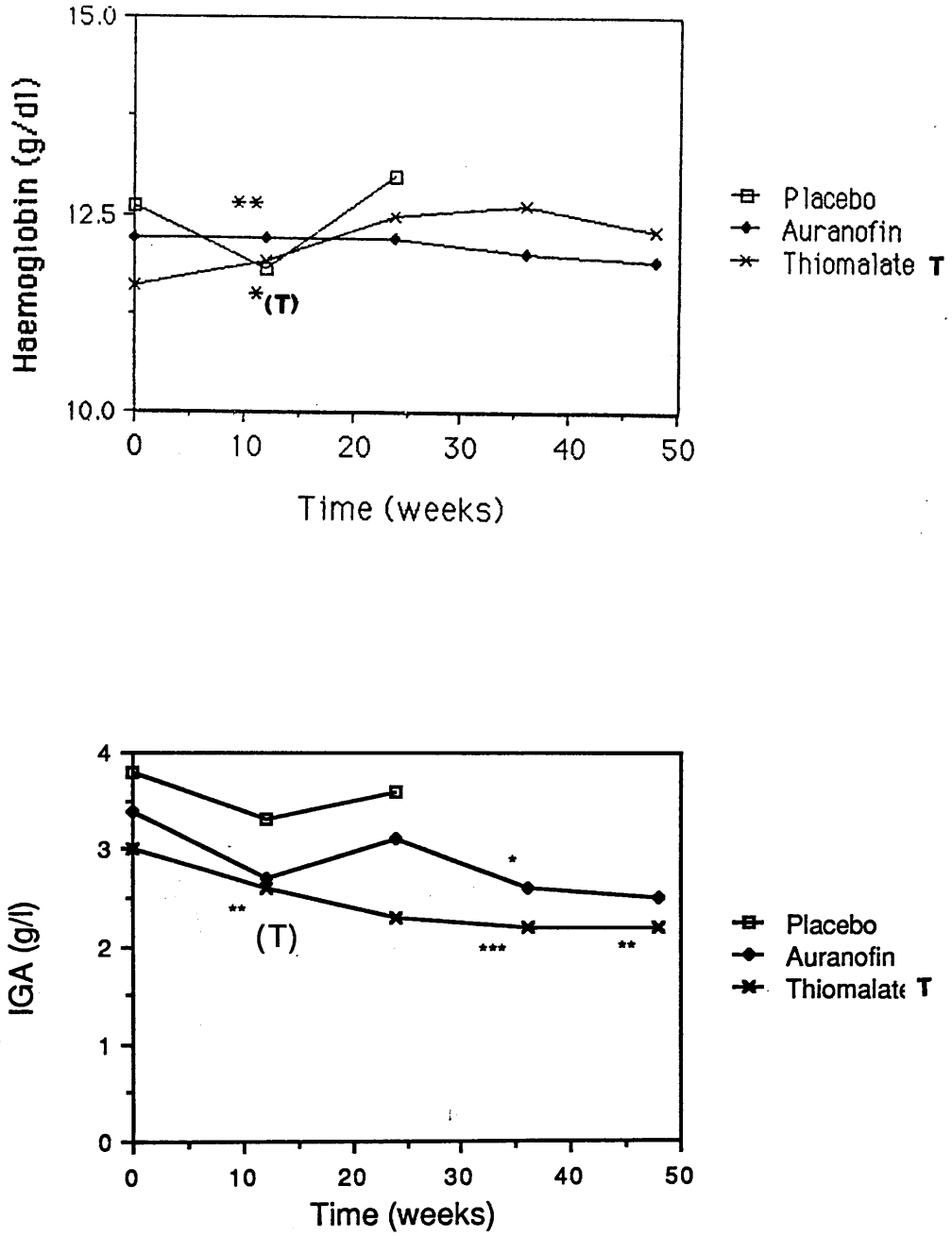


Figure 4.7. Concentrations of haemoglobin and IgA after treatment with gold compounds.

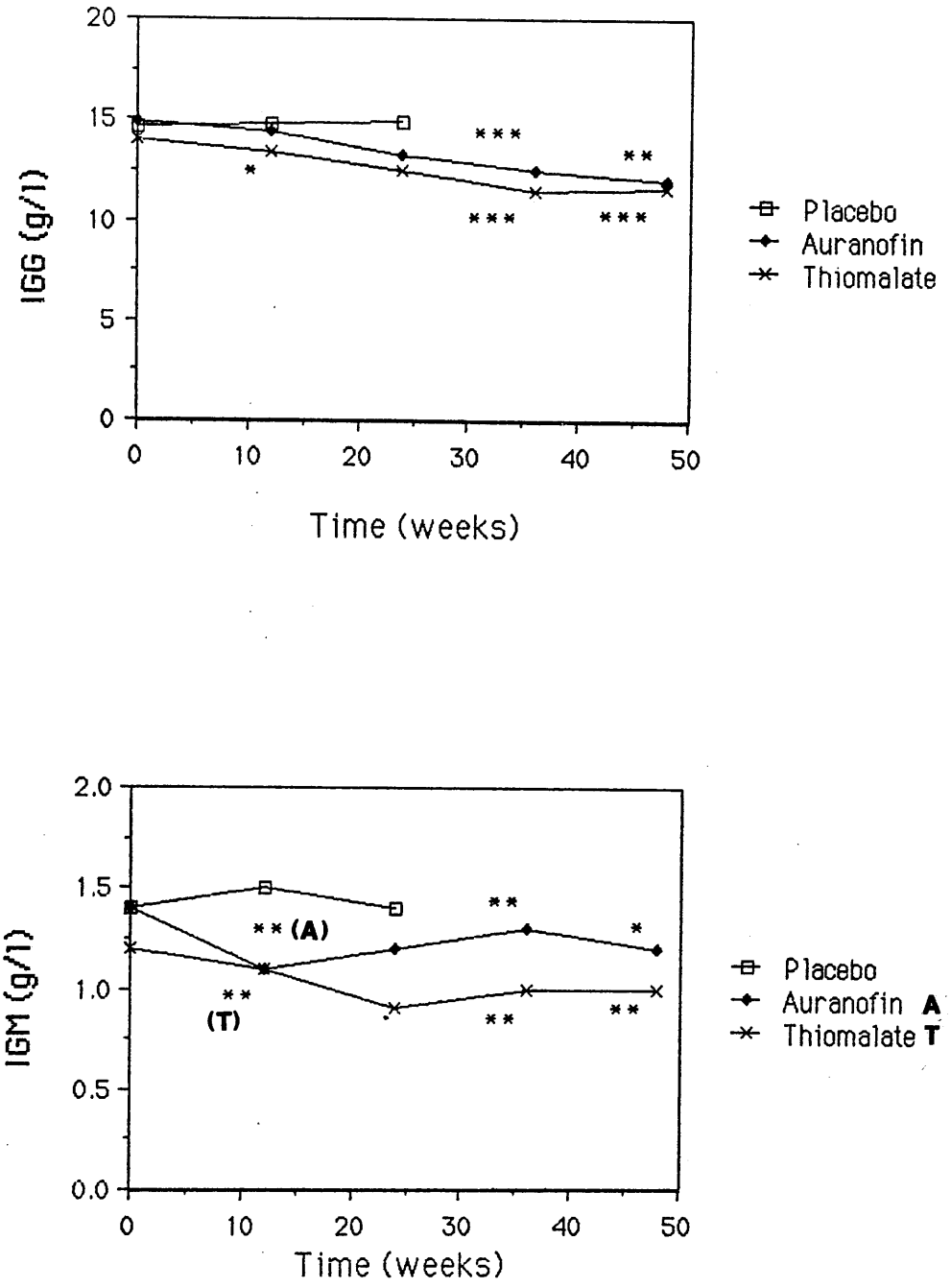


Figure 4.8. Concentrations of IgG and IgM after treatment with gold compounds.

weeks.

In the group treated with gold thiomalate, there were significant changes in ESR, haptoglobin, α_1-acid glycoprotein, α_1-antichymotrypsin, activity score and pain score at 12 weeks. By 24 weeks, α_1-antitrypsin, morning stiffness and Ritchie articular index had also decreased, but CRP and grip strength did not change until 48 weeks.

There was considerable variability of proteins across patients at particular time points, so it was more difficult to detect significant differences. The only significant difference across treatment groups at 12 weeks was for haptoglobin. At 24 weeks, ESR, haptoglobin, pain score, and activity index were all significantly different between the groups. All the significant differences were shown by Mann-Whitney tests to be between the actively treated groups and the placebo group. There were no significant differences between the gold-treated groups at any time. At 48 weeks, by which time there were no patients remaining in the placebo group, no significant differences were found.

In an attempt to determine whether a combination of biochemical and clinical measurements would give better separation of the groups than any individual index, a discriminant function analysis was carried out. The variables initially used were the acute phase proteins, ESR, pain score, stiffness, grip strength, articular index, and haemoglobin, all measured at week 24.

A significant discriminant function was obtained by stepwise analysis. The canonical correlation of group

membership with this function was 0.565 ($p<0.001$), and the function accounted for 81.9% of between-group variability. From the matrix of correlations of individual measurements with the function, it could be seen that the measurements which contributed to the function significantly ($r>0.3$) were pain score, articular index, and stiffness. A second function, which incorporated the acute phase proteins, was not significant. When the patients were classified using the significant function, only 61% were correctly classified. The greatest separation was between the group treated with aurothiomalate and the other two.

A similar analysis was carried out using the change in the variables from week zero to week 24. There was no improvement in efficiency of classification. The correlation coefficient was 0.473 ($p<0.02$), and 60% were correctly classified. The variables contributing to separation of the groups were pain score, articular index, α_1-antichymotrypsin, and haptoglobin.

Because of the poor separation using either clinical or biochemical variables, it was decided to include various other measurements which were made on the patients, but are not reported in detail in this study. These included immunoglobulins, plasma and red cell lysate thiol concentrations, superoxide dismutase, caeruloplasmin, and white cell and platelet counts. There was an improvement of classification efficiency. The correlation coefficient increased to 0.732 ($p<0.0001$), and 74% of patients were correctly classified. However, the only measurements out of all those carried out which contributed to this

separation were pain score and IgA, and the separation was primarily between the group treated with parenteral gold and the others. The graphs of the immunoglobulins with time confirmed that they showed early decreases with treatment (figures 4.7, 4.8), particularly in the group treated with gold thiomalate.

Because it was known that the distribution of several of the variables was skewed, they were transformed to their logarithms and the analysis was rerun. The percentage correctly classified then decreased to 62%, with a correlation coefficient of 0.664.

4.4. Discussion

The results of this study do give evidence for the efficacy of both of the gold compounds studied in the treatment of rheumatoid arthritis and for their ability to modify the concentrations of acute phase reactant proteins. This allows the classification of auranofin as another of the class of "second line" drugs. The response to aurothiomalate was more rapid than that to auranofin. Several of the acute phase proteins decreased in concentration by three months in this group, while a significant response did not occur in the auranofin group until six months. Similarly, fewer of the clinical indices showed improvement in the group treated with auranofin. Both drugs were, however, clearly superior to placebo.

It proved extremely difficult to persuade patients to continue on treatment with placebo. The dropout rate was,

as expected, much higher in this group than in either of the other two (100% by 40 weeks). Most were removed from the study because of lack of efficacy of treatment, but there were 2 patients who showed toxic effects while taking placebo. One patient developed haematuria and one complained of diarrhoea. These effects may have been coincidental, but the patients were withdrawn from the study.

In the group treated with aurothiomalate, 6 patients had to be withdrawn because of a variety of side-effects, most commonly skin rashes. No patients complained of lack of efficacy. In contrast, 6 patients were unable to continue taking auranofin because it did not relieve their symptoms, though only 3 patients showed side-effects, some of which may again have been coincidental, as they were in the placebo group. This confirms that auranofin is slightly less effective than aurothiomalate, though its toxicity is less.

It is conceivable that the differences in dropout rate between the groups could bias the statistical analysis, particularly the paired Wilcoxon tests between time points. However, if the most severely ill patients were the ones to drop out of the study, as might be expected, there might be a corresponding change in the clinical and biochemical indices in the direction of improvement in the placebo group. This was not noted. It was also not possible to predict whether a patient would respond to treatment from their initial results. This would suggest that bias was small, and that the changes observed in the

measured variables in the actively treated groups did reflect an effect of the drugs.

Patients who were reallocated to active treatment after being removed from the placebo group showed decreases thereafter in activity index and ESR. At the 3-year follow-up (Capell et al, 1986), it was found that there were no significant differences between the patients who were originally actively treated and those who were started on placebo and then switched to gold, which makes it unlikely that those who dropped out of the placebo group were an unusually severe group of patients. The large number of gaps in the data did, however, make it impractical to analyse the results by analysis of variance.

It was found that the scatter of both biochemical and clinical results about the mean value at any time point was very wide. Because of this, it proved difficult to detect significant differences between the groups. The only acute phase protein which did show significant differences was haptoglobin. It is difficult to decide why this protein should be so much more sensitive than the others measured, especially since its sensitivity for the detection of active disease was not good. It is possible that the levels of haptoglobin may have been responding to haematological stimuli rather than reflecting the status of the acute phase reaction.

The relative magnitude of the change in CRP was the greatest of all the acute phase proteins, so it may be the most suitable to use for routine monitoring of disease

activity. Haptoglobin also decreased markedly, so its measurement may also be of value.

However, it was more difficult to use the concentrations of acute phase proteins to distinguish groups of patients being treated with different drugs. In the discriminant function analyses, no acute phase protein or combination of them proved to be effective for separation of the groups. Although haptoglobin did appear in the significant functions, its contribution to separation was minor once the clinical variables had been taken into account. Even the best combination of clinical indices and acute phase proteins was not particularly effective in distinguishing the treated groups from placebo.

The most sensitive biochemical indices for detecting active disease in untreated patients seemed to be CRP and α_1-antichymotrypsin. A combination of the two would detect almost all patients. The measurement of α_1-antitrypsin was notably ineffective. ESR was also a sensitive indicator of activity. This variable also exhibited marked decreases on treatment, possibly because its level depends on the concentrations of both immunoglobulins and acute phase proteins.

The marked effects of gold treatment on immunoglobulins were somewhat surprising. IgA proved to be one of the most effective measurements for distinguishing between the groups at 24 weeks. This may represent a direct action of gold, since it has been reported that both auranofin (Salmeron & Lipsky, 1982) and gold thiomalate (Lipsky & Rosenberg, 1979) inhibit the generation of immuno-

globulin-secreting cells in vitro.

CHAPTER 5

MODIFICATION OF INTERLEUKIN 1 RELEASE BY AGENTS ACTING ON ARACHIDONIC ACID METABOLISM

5.1. Control of Synthesis of IL-1

The molecular mechanisms of the induction of IL-1 release are still obscure. Although a large number of diverse substances is known to stimulate its synthesis, a common pathway of action is yet to be identified. There is, however, great clinical interest in the control of IL-1 synthesis, and it is to be expected that definitive answers will be available before long. Already, however, there are suggestions that IL-1 synthesis may be controlled by the recently described phosphatidylinositol pathway.

The evidence for the existence of this pathway is reviewed in Berridge & Irvine (1984) and in Nishizuka (1984), and its outline is shown in figure 5.1. It is thought that binding of many hormones to their receptors induces hydrolysis of polyphosphatidyl inositol compounds in cell membranes with release of diacylglycerol and inositol trisphosphate. The inositol trisphosphate acts to induce release of calcium from stores in the endoplasmic reticulum, while the diacylglycerol activates a novel calcium- and phospholipid-dependent protein kinase C. Protein kinase C phosphorylates a wide variety of proteins, and has many effects on cell metabolism and

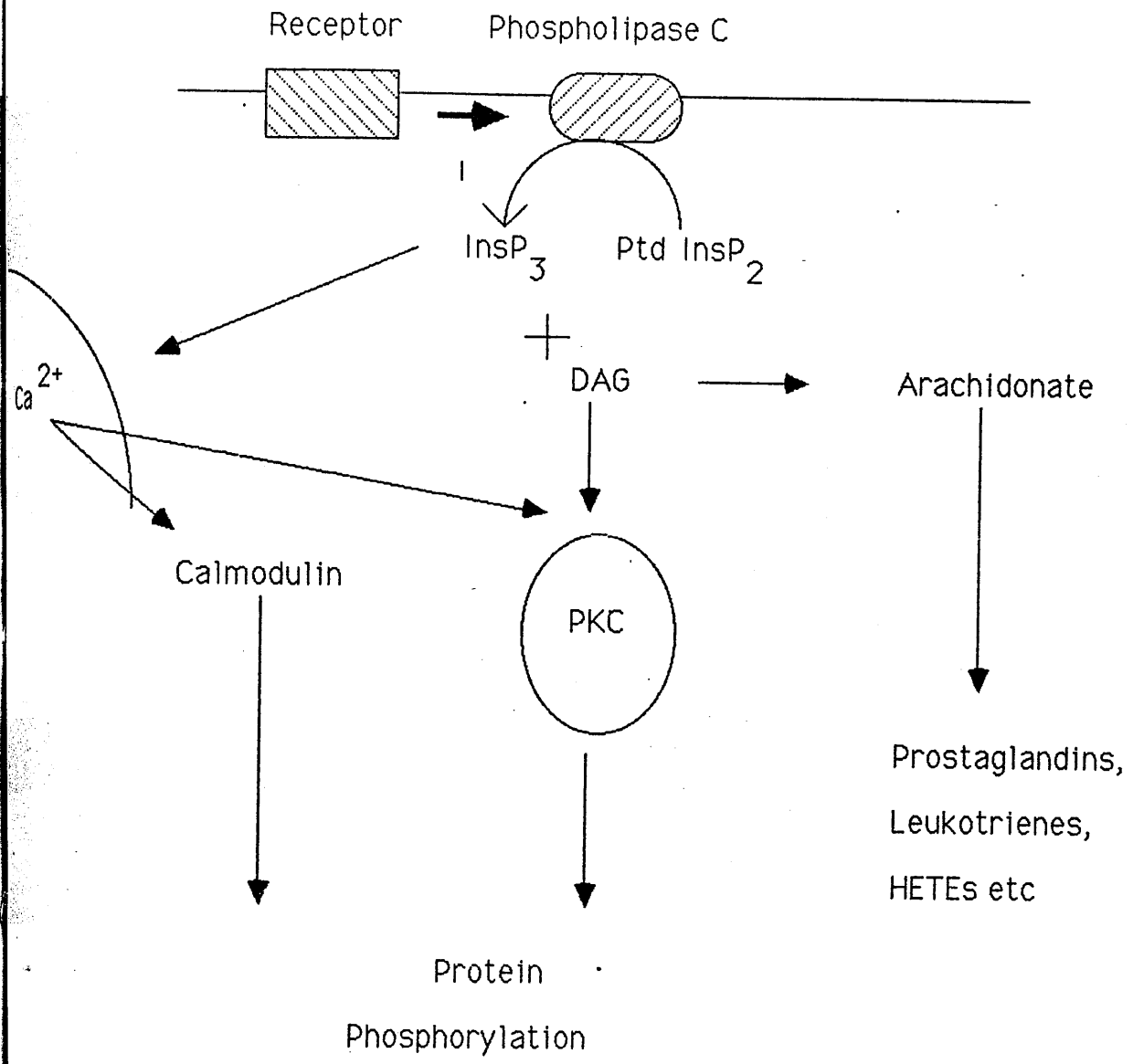


Figure 5.1. Inositol phosphate/protein kinase C pathway. Hormones activate phospholipase C, which releases inositol trisphosphate and diacylglycerol (DAG). The inositol trisphosphate causes release of calcium from stores, and this calcium and the DAG activate protein kinase C. Protein phosphorylation can be carried out both by calmodulin-dependent protein kinases and PKC. Arachidonate may be produced from metabolism of DAG.

growth. It has recently been reported that there are several genes coding for different variants of this enzyme (Coussens et al, 1986), so activating the phosphatidyl-inositol pathway may result in different effects depending on the particular kinase which is activated. LPS is known to activate phosphoinositol turnover (Rosoff et al, 1985), so this transducing system may be involved in its action. Direct evidence, however, is not yet available.

Phorbol esters, such as phorbol myristate acetate (PMA) bind directly to protein kinase C and activate it. It has been reported that phorbol esters can activate several cell lines to secrete IL-1 (Mizel et al, 1978; Palacios et al, 1982; Wakasugi et al, 1984). However, interpretation of these results is greatly complicated by the severe interference of PMA in IL-1 assays (Krakauer et al, 1982; Orosz et al, 1983). Williams et al (1985) showed that the supposed IL-1 activity elicited from K562 cells by PMA represented protein-bound PMA, so the results produced by many groups of workers must be suspect.

However, PMA does genuinely stimulate the release of IL-1 from macrophage-like cells. Mizel and Mizel (1981) have successfully purified the IL-1 stimulated by PMA from the murine macrophage cell line P388D$_1$, and Dukovich & Mizel (1985) have shown that these cells rapidly convert PMA to an inactive form. Porcine catabolin with the same molecular weight and isoelectric point as that elicited by other stimuli was produced by incubation of synovial cells with PMA (Pilsworth and Saklatvala, 1983). These studies offer some evidence for a role of protein kinase C in the

transduction of signals for IL-1 production.

In agreement with the proposed combined role of calcium and the inositol pathway, calcium ionophores have been reported to stimulate IL-1 release from peripheral blood monocytes (Matsushima and Oppenheim, 1985) and from $P388D_1$ cells (Simon, 1984), and to act synergistically with lipopolysaccharide.

Arachidonic acid can be released from membrane phospholipids during hormonal stimulation by at least two mechanisms. The diacylglycerol may be hydrolysed, liberating arachidonic acid from the 2' position. Alternatively, the phospholipase A_2 inhibitor lipocortin may be inactivated by the action of protein kinase C, allowing release of arachidonic acid from cell membranes (Touqui et al, 1986). It is not clear what the relative importance of these pathways is in vivo. The arachidonic acid can then be metabolised to a range of prostaglandins, leukotrienes, hydroperoxyacids, or lipoxins, depending on the target tissue.

There is evidence, mostly from experiments involving the use of enzyme inhibitors, for the involvement of arachidonic acid metabolites in the stimulation of IL-1 release. It is known that glucocorticoids inhibit IL-1 release, though not totally. One of the major actions of this class of compounds is to inhibit phospholipase A by stimulating the synthesis of lipocortin, though there is also evidence for a direct action on transcription of the TNF gene (Beutler et al, 1986). The combined cyclo- and lipoxygenase inhibitor BW755C has been reported to inhibit

IL-1 production (Dinarello et al, 1984c). The cyclooxygenase inhibitors indomethacin and ibuprofen had no effect, so the enzyme involved may be the lipoxygenase. There is also evidence that leukotrienes B_4 and C_4 stimulate IL-1 synthesis by peritoneal (Chensue and Kunkel, 1985) and alveolar (Polla et al, 1985) macrophages and blood monocytes (Rola-Pleszczynski & Lemaire, 1985).

In the current project, the effect on IL-1 release of a variety of drugs was studied. The drugs chosen affect several of the enzymes involved in arachidonic acid metabolism. The U937 cell line, which was used as an experimental model, was originally derived from a histiocytic lymphoma by Sundstrom & Nilsson (1976) and now grows in suspension culture. It has the characteristics of an immature monocyte and can be induced to differentiate into macrophage-like cells by agents such as lymphokines (Koren et al, 1979; Fischer et al, 1980; Amento et al, 1985), vitamin D (Amento et al, 1984), and PMA (Minta & Pambrun, 1985). When differentiated, the cells cease to grow and attach to the substrate. They increase their content of non-specific esterase, and their surface expression of complement and Fc receptors, and become reactive with monoclonal antibodies specific for mature macrophages. They also become active in phagocytosis and cytotoxicity assays.

5.2. Growth of U937 Cells

U937 cells were grown in suspension culture in an

atmosphere of 95% air/5% CO_2 in a medium of RPMI 1640 supplemented with 2 mmol/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% foetal calf serum. In an attempt to avoid having to use a CO_2 incubator, a comparison of growth rate was made with sealed flasks containing RPMI 1640 with 20 mmol/l Hepes buffer.

Cells were inoculated in 25 ml tissue culture flasks at a concentration of about 1.5×10^5 cells/ml. The flasks were placed either in a CO_2 incubator or a dry incubator, and allowed to grow without shaking for 7 days. The flasks were sampled each day and the cells counted with a haemocytometer.

Growth of the cells was clearly better in the CO_2 atmosphere. The cells entered a logarithmic phase of growth after a lag period of 2 days, with a doubling time of 30 hours. By 7 days, cell numbers reached 10^6 cells/ml, and they were still dividing rapidly. When the cells were transferred to Hepes buffer, the logarithmic phase was much shorter, and growth ceased when cell numbers reached 4×10^5 cells/ml, after which numbers decreased rapidly (figure 5.2). The doubling time was similar to that of the bicarbonate buffered cells during the brief logarithmic phase, but growth slowed markedly by the fourth day.

Although the growth rate of cells in bicarbonate buffer was adequate, it was noticed that the medium became very acid, and cell growth could not be sustained beyond 10^6 cells/ml. The original medium was supplemented with N-morpholinosulphonic acid buffer (12.5 mmol/l), 1 mmol/l pyruvate, and 1% non-essential amino acids. In this

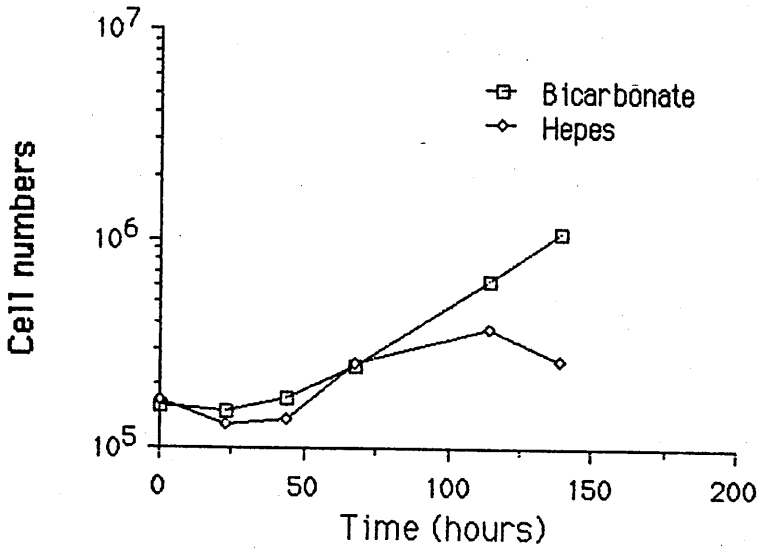


Figure 5.2. Growth rate of U937 cells in bicarbonate or Hepes buffered medium.

medium, cells remained viable without excessive alterations in pH at concentrations of up to 2 x 10^6 cells/ml.

5.3. Production of IL-1 from U937 Cells

The stimulus used for production of IL-1 was toxic shock toxin 1 (TSST-1). This toxin is the causative agent of toxic shock syndrome, and is produced by certain strains of Staphylococcus aureus (Poindexter & Schlievert, 1985). It has been reported to be a powerful stimulus of IL-1 release from peripheral blood monocytes (Ikejima et al, 1984) and from U937 cells (Knudsen et al, 1986).

5.3.1. Production of TSST-1

The strain of bacterium used was an isolate of Staphylococcus aureus from a vaginal swab of a patient with toxic shock syndrome. It was positive for TSST-1 as tested by the PHLS laboratory at Colindale. One ml of an overnight broth culture was used to inoculate 500 ml of Brain Heart Infusion (Oxoid Ltd, Basingstoke, Hampshire) in a 2 litre flanged flask. The flask was incubated for 24 hours at 150 rpm in an orbital incubator at 37°C. The bacteria were removed by centrifugation at 17000xg for 10 minutes, and the supernatant was passed through 0.2 μm filters. The culture of bacteria was carried out by Dr C McCartney of the Department of Bacteriology, Royal Infirmary, Glasgow.

5.3.2. Time Course of IL-1 Release

U937 cells were suspended in 1 ml cultures at 10^6 cells/ml. TSST-1 was added at a concentration of 2% (v/v). Incubation was continued up to 7 days, after which the cells were removed by centrifugation. The medium was dialysed against RPMI 1640 in Spectrapor 1 dialysis tubing (molecular weight cutoff 6-8000) for 48 hours with two changes, and the concentration of IL-1 was measured by the LAF assay.

The concentration of TSST-1 used proved to be suboptimal, and the maximum concentration of IL-1 which was released only produced a 2 to 3-fold increase in the assay counts compared to a PHA blank. However, there was some release of IL-1 in the first 24 hours of incubation (figure 5.3), followed by a continuing slow rate of production for periods of up to 7 days. A time of 24 hours was used in all experiments.

5.3.3. Optimum Concentration of TSST-1

Cells were incubated for 24 hours with concentrations of TSST-1 ranging from 0.5 to 10% (v/v). The culture medium was treated as above. As shown in figure 5.4, an increasing amount of IL-1 was released by the cells as the concentration of TSST-1 increased. Although a maximum value was not attained, concentrations of 10% were used in most further experiments. Addition of TSST-1 alone to thymocytes did not stimulate thymidine uptake.

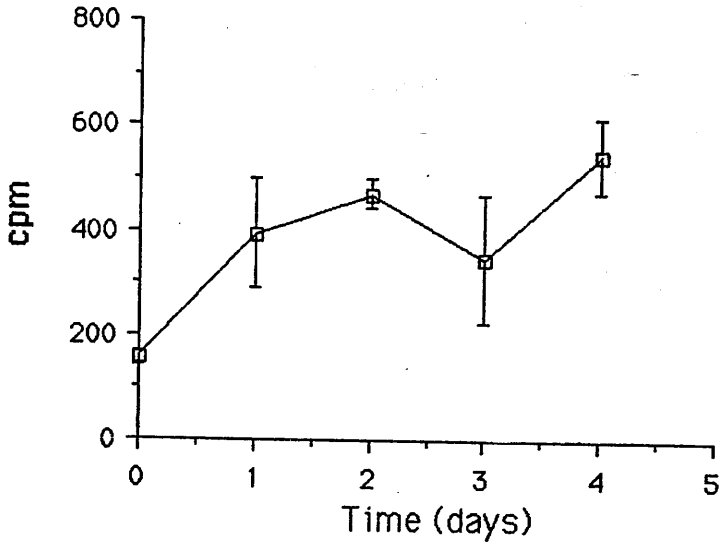


Figure 5.3. Time course of release of IL-1 from U937 cells in the presence of 2% TSST-1.

Results are expressed as mean ± 1SD.
n=3

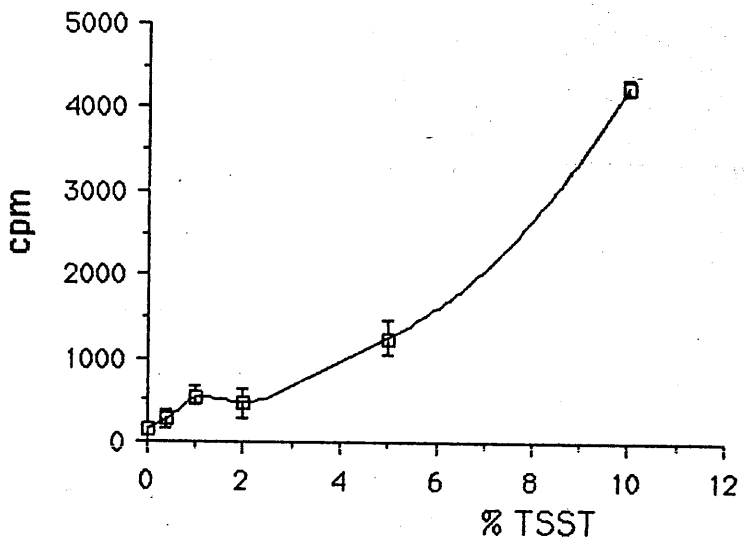


Figure 5.4. IL-1 production from U937 cells with different concentrations of TSST-1.

Results are expressed as mean ± 1SD.
n=3

5.3.4. Cell Numbers

The concentration of U937 cells per culture was varied from 10^6 to 5 x 10^6 cells/ml. The amount of IL-1 released was measured in medium containing 10% foetal calf serum or no serum. There was a substantial increase in production as the cell concentration was increased from 10^6 to 2 x 10^6 cells/ml, but little more was released if the cell concentration was further increased to 5 x 10^6 cells/ml (figure 5.5). If the data was expressed as a ratio of the number of cells per culture, there was a peak of IL-1 production at 2 x 10^6 cells/ml. When serum-free medium was used, much less IL-1 was released, though some was detectable, and the concentrations tended to decrease as cell numbers increased, possibly due to the release of inhibitory factors.

5.3.5. Serum Source

It was clear that the presence of serum was necessary for optimum production of IL-1. Recently, a serum substitute called Ultroser G has been marketed by LKB. This contains considerably less protein than serum, so would be less likely to contain inhibitory substances.

U937 cells were incubated at 10^6 cells/ml in the presence of foetal calf serum or Ultroser G. All samples were dialysed and filtered through Amicon ultrafiltration membranes with a cutoff of 30000 molecular weight (YM30) to remove high molecular weight inhibitors before assay.

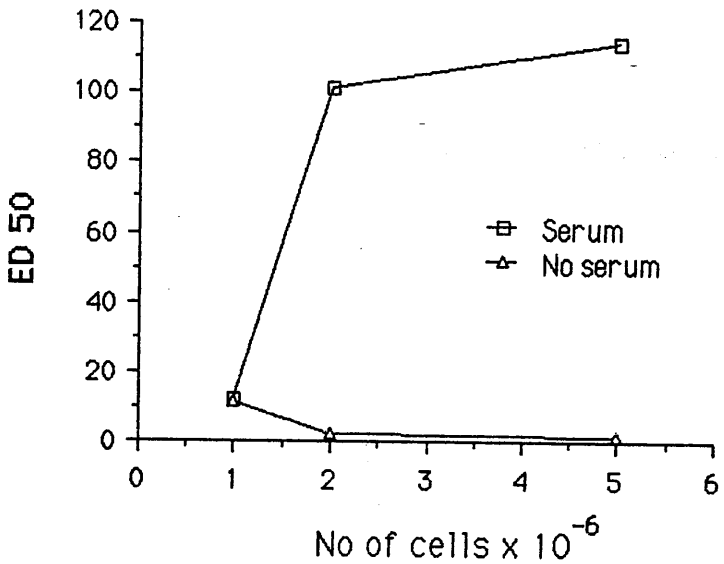


Figure 5.5. IL-1 released from U937 cells at different cell concentrations with and without foetal calf serum .

ED50: dilution of sample which gives half-maximal counts in the assay.

It was found that Ultroser G considerably enhanced the release of IL-1 from the cells in a concentration-dependent manner (figure 5.6). The use of 1% Ultroser G increased the production of IL-1 to that obtained from a peripheral blood monocyte preparation stimulated with 20 ng/ml LPS.

Although there was substantial production of IL-1, the cells grew poorly in Ultroser G (figure 5.7). It has been previously suggested that much IL-1 comes from damaged or dying cells, which would release intracellular IL-1 (Gery et al, 1981), and this observation would be consistent with that finding.

5.3.6. Drug Preparation

The drugs used were indomethacin (Thomas Morson Pharmaceuticals), a specific inhibitor of the cyclooxygenase enzyme (Randall et al, 1980); BW755C (Wellcome), 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride, which inhibits both the cyclooxygenase and lipoxygenase enzymes (Randall et al, 1980); Dazmegrel (Pfizer), a thromboxane synthetase inhibitor (Fischer et al, 1983); Iloprost (Schering), formerly known as ZK36374, which is a stable prostacyclin (PGI_2) analogue (Schroer et al, 1981); and PGI_2 (Wellcome) itself. The enzymes affected by these drugs are shown in figure 5.8.

Indomethacin and BW755C were dissolved in a minimum volume of ethanol and diluted into RPMI 1640. Ethanol in

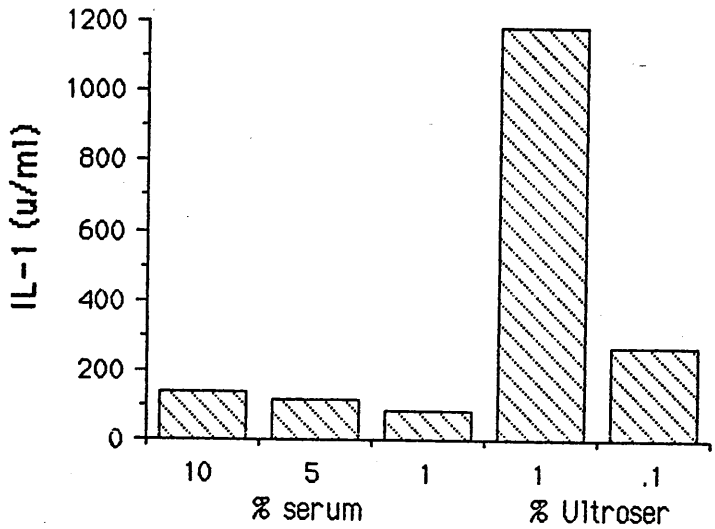


Figure 5.6. Production of IL-1 by U937 cells.
Comparison of serum with Ultroser G.

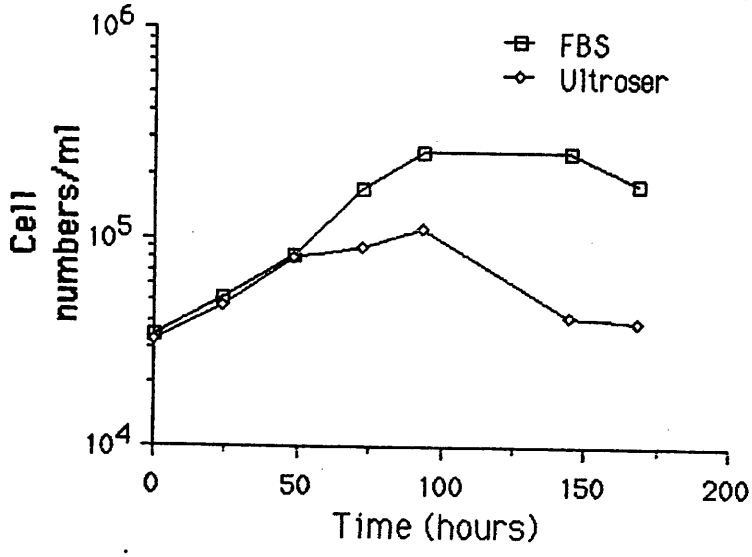


Figure 5.7. Growth of U937 cells in foetal calf serum or in Ultroser G.

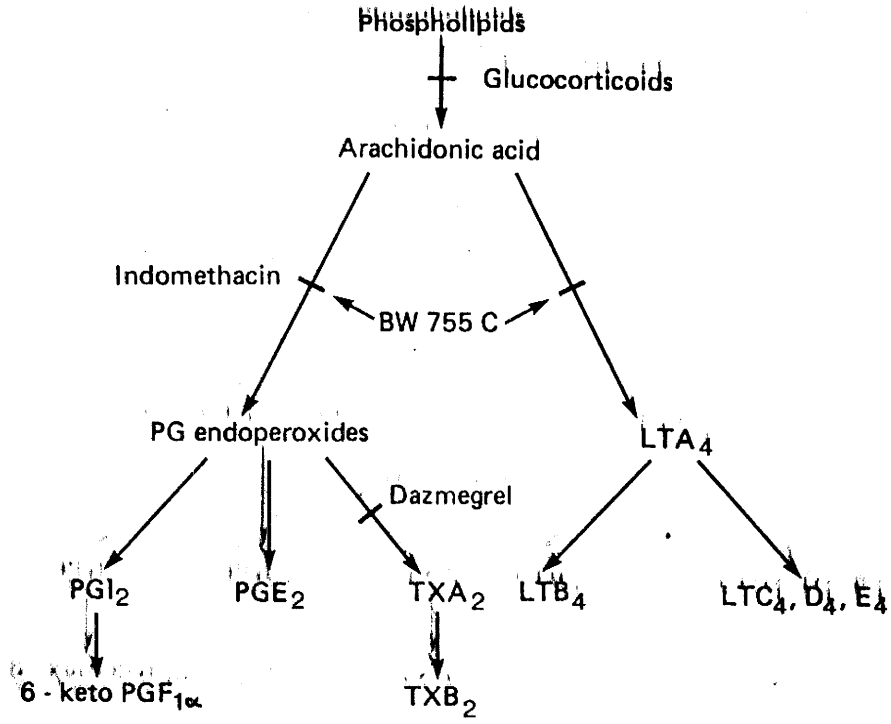


Figure 5.8. Pathways of arachidonic acid metabolism.

the final concentrations used had no effect on the IL-1 assay. Dazmegrel was dissolved in 0.1 mol/l NaOH, then diluted in RPMI 1640 and the pH adjusted to 7.2. Iloprost was diluted directly into RPMI 1640. PGI_2 was dissolved in the glycine buffer supplied, then diluted into RPMI 1640, the pH adjusted, and used immediately.

5.3.7. Induction of IL-1 from U937 Cells

Cells were centrifuged at 450xg for 5 minutes and resuspended in growth medium containing 100 U/ml of polymyxin B and 1% Ultroser G. They were then dispensed into 24-well plates at a concentration of 10^6 cells/ml, 1 ml per well. Appropriate concentrations of drugs were added to the wells in 100 μl aliquots and the cells incubated at 37°C in an atmosphere of 95% air/5% CO_2 for 1 hour. TSST-1 was added to a final concentration of 10% and incubation continued for a total of 24 hours. The cells were then removed by centrifugation and the medium dialysed and, for some experiments, passed through YM30 ultrafiltration membranes as above.

5.3.8. IL-1 Production from Peripheral Blood Cells

Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll-Hypaque (Ficoll-Paque, Pharmacia) and incubated at 10^7 cells/ml in RPMI 1640 containing 5% autologous plasma and 20 ng/ml E. coli endotoxin serotype 055:B5 (Sigma) for 24 hours. Cells were

removed by centrifugation and the medium was dialysed against RPMI 1640.

5.4. Results

5.4.1. Removal of Interfering Substances

Each sample was assayed in several dilutions, each dilution being tested in triplicate. This allowed testing of parallelism of the slopes of dilution curves between samples and standards, an essential precaution if interfering substances may be present. To test parallelism, the response variable (cpm) was transformed using a logit function and plotted against the logarithm of the sample dilution. It was found that this procedure effectively linearised the results. All dilution curves were tested against samples from cultures containing either U937 cells stimulated by TSST-1 or from PBMN cells stimulated with endotoxin.

While the slopes of dilution curves of IL-1 prepared either from PBMN cells or from U937 cells alone were similar, those from samples containing BW755C were more shallow (figure 5.9). Since it seemed possible that this effect was caused by macromolecular interfering substances, samples were passed through a YM30 ultrafiltration membrane. After this treatment, dilution curves from samples containing drugs became parallel to those from samples without drugs (figure 5.9). There was a loss of activity of 20-30% of the PBMN standard when it

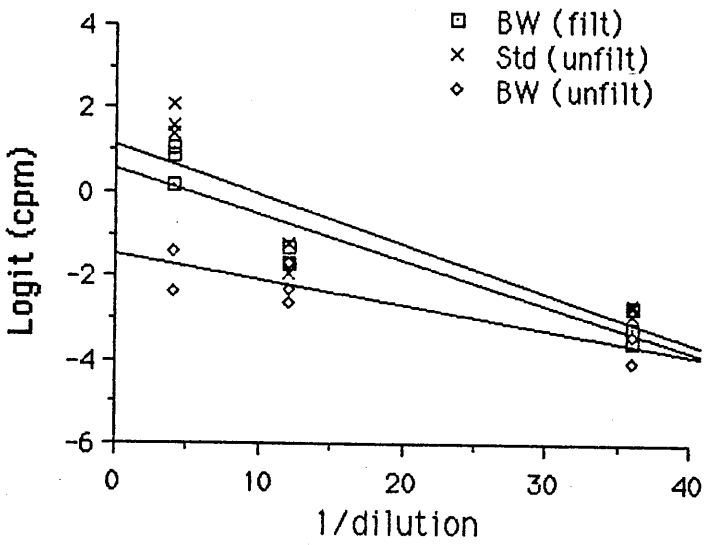


Figure 5.9. Dilution curves of IL-1 preparations, either unfiltered or passed through a YM30 ultrafiltration membrane.

BW, containing 50 µg/ml of BW755C in the induction medium.
Std, peripheral blood mononuclear cell standard.

was passed through the membrane, so all standards used were also passed through the ultrafiltration membrane.

To calculate units of IL-1 activity, a sample of PBMN-derived IL-1 was arbitrarily assigned a value of 100 U/ml. The concentration of each individual dilution of the sample was calculated by interpolation from the standard curve and the mean taken. The standard deviation of the individual values obtained for the various dilutions was taken to estimate the variance of the sample measurement. This procedure gave wider confidence limits than merely assessing the variability of triplicate estimations at a single dose, as the errors in dilution and in fitting the curve were included.

5.4.2. Effect of Drugs on IL-1 Production

The results are shown in table 5.1. Indomethacin did not affect IL-1 production at either 1 or 10 μg/ml. BW755C had no effect on release of IL-1 at concentrations of up to 100 μg/ml. Dazmegrel caused a marked suppression of IL-1 production at concentrations of 5 and 10 μg/ml to levels of 43% of control ($p<0.001$) and 23% of control ($p<0.001$) respectively. This drug did not affect the viability of the cells.

Iloprost, at concentrations of 1 and 10 ng/ml, caused an augmentation of IL-1 release by 22% and 59% ($p<0.05$; $p<0.001$). This effect disappeared at higher doses, and the drug became inhibitory at very high doses (47% of control at 500 ng/ml; $p<0.01$). Prostacyclin also augmented IL-1

Table 5.1.

Effect of Drugs on IL-1 Production by U937 Cells

Drug	(μg/ml)	IL-1 (U/ml)
Indomethacin	1	89 ± 7
	10	92 ± 15
BW755C	10	80 ± 29
	50	98 ± 13
	100	86 ± 28
Dazmegrel	1	114 ± 16
	5	43 ± 11***
	10	23 ± 16***
	(ng/ml)	
Iloprost	1	122 ± 17*
	10	159 ± 39***
	50	80 ± 9
	100	88 ± 17
	500	47 ± 22**
PGI_2	1	131 ± 35*
	10	63 ± 19*
	50	89 ± 17
	100	71 ± 13

Significantly different from control by t-test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

release, although a significant effect was only seen at 1 ng/ml (31% greater than control; $p<0.05$). Higher concentrations were either without effect or slightly inhibitory.

5.4. Discussion

TSST-1 proved to be an effective stimulus for IL-1 release from U937 cells. More IL-1 was produced in the presence of the serum substitute Ultroser G than foetal bovine serum. This may reflect the presence of inhibitors of IL-1 production in serum, or it is possible that Ultroser G may facilitate the release of intracellular IL-1, which is produced in large quantities in stimulated cells. This was not directly tested, but, if true, may be related to the lower viability of cells in serum-free medium.

Hirose et al (1985) claimed that sparse cultures of U937 cells produced more IL-1 per cell than dense cultures, and that a short-lived inhibitor of production was released. While cell concentrations as low as the ones used in his study were not tested here, a definite increase was observed in IL-1 production as cell concentrations were increased above 10^6 cells/ml, even when results were normalised to the numbers of cells.

There was some evidence that inhibitors of IL-1 action could also be produced by the U937 cells in the presence of the drugs tested. When BW755C was used, the slope of

the dilution curves of samples was more shallow than that of samples without the drug. This interference with the assay could be removed by ultrafiltration, suggesting the presence of a macromolecular inhibitor of IL-1 action. One explanation of this finding could be the residual presence of BW755C bound to serum proteins. It is also possible that the drug allows the synthesis of an IL-1 inhibitor to take place, which would suggest that a function of lipoxygenase metabolites could be to suppress the production of this inhibitor. There is, however, no direct evidence of an inhibitor being present in these studies. Amento et al (1984) found that U937 cells stimulated with lymphokines produced substantial quantities of inhibitory substances, far more than was observed in this study.

The effects of inhibiting the cyclooxygenase and lipoxygenase enzymes appear to depend on the cell system studied and on the stimulus used. Kunkel & Chensue (1985) found that the addition of indomethacin increased IL-1 production from LPS-stimulated peritoneal macrophages, but that addition of lipoxygenase inhibitors had no effect. On the other hand, Dinarello et al (1984c) did see inhibition on adding BW755C, a combined lipoxygenase and cyclooxygenase inhibitor, to peripheral blood monocytes, while cyclooxygenase inhibitors alone had no effect in their system.

In the present study, no effect was seen of inhibition of either the cyclooxygenase or the lipoxygenase enzyme. When this finding is coupled with the negative results of Kunkel & Chensue (1985) it must be doubted whether

lipoxygenase metabolites have a necessary role in stimulation of IL-1 production. Several groups of workers have shown that exogenous leukotrienes can stimulate IL-1 release from a variety of cells (Chensue & Kunkel, 1985; Polla et al, 1985; Rola-Pleszczynski & Lemaire, 1985), but this does not necessarily prove that intracellular leukotrienes have a similar role. It is known that there are cell surface receptors for leukotrienes and that they can act as calcium ionophores (Serhan et al, 1982). In addition, it is known that LPS and PMA, both powerful stimuli for IL-1 release, do not increase leukotriene production by macrophages (Bonney & Humes, 1984; Kunkel et al, 1986). A similar phenomenon to the one postulated here has been noted to occur during the activation of natural killer cells (Bray & Brahmi, 1986). While LTB_4 stimulated the cells when added exogenously, activation could not be prevented by blocking endogenous synthesis of leukotrienes.

Marked effects of altering the levels of thromboxanes and prostacyclin were noted. Inhibition of thromboxane synthesis substantially reduced the production of IL-1. This may represent an important role for TxA_2 in signal transduction. Activated macrophages preferentially maintain thromboxane synthesis in spite of a marked reduction in all other enzymes of arachidonic acid metabolism (Tripp et al, 1985). Thymic reticular cells are capable of synthesising thromboxanes even in the presence of indomethacin (Homo-Delarche et al, 1985). This could explain how inhibition of thromboxane synthesis

substantially reduced IL-1 production, while inhibition of cyclooxygenase did not. A role for thromboxane as a mediator of lymphocyte activation has also been suggested (Kelly et al, 1979).

An alternative explanation is possible for the effects of Dazmegrel. It is known that inhibition of thromboxane synthesis with this drug causes diversion of prostaglandin endoperoxides towards other metabolites, particularly PGE_2, PGI_2, and PGD_2 (Fischer et al, 1983). PGE_2 is known to inhibit IL-1 production (Kunkel et al, 1986), so this could be the reason for the effects seen. This has been suggested to be the mechanism of the inhibition of natural killer cell activity caused by inhibitors of thromboxane synthesis (Rola-Pleszczynski et al, 1985).

It was found that PGI_2 and its analogue Iloprost both caused increases in IL-1 production at very low doses, but that this effect disappeared at higher doses. PGI_2 has been reported to inhibit IL-1 production at micromolar concentrations (Kunkel et al, 1986), which would correspond to the dose levels found to be inhibitory in this study. These workers did not test levels as low as those found to be stimulatory. The mechanism of inhibition may be via stimulation of cAMP production, as the effect of PGE_2 is thought to be.

The stimulatory effect of PGI_2, if confirmed, could be an important signal for IL-1 production by endothelial cells. It is known that IL-1 is released by endothelial cells (Miossec et al, 1986) and that it is capable of stimulating the release of both PGE_2 and PGI_2 from them

(Albrightson et al, 1985; Rossi et al, 1985). Minor damage to endothelium could release small amounts of IL-1 which could then increase its own production in a feedback loop. Production would eventually be inhibited by higher concentrations of both PGI_2 and PGE_2. This could be an important prothrombotic mechanism, since IL-1 is known to increase the production of procoagulant activity by endothelium (Bevilacqua et al, 1984) and to inhibit protein C dependent anticoagulation (Nawroth et al, 1986).

The manipulation of arachidonic acid metabolites does seem to offer a means of altering IL-1 production, but interpretation of in vitro experiments is fraught with difficulties. It remains to be proved directly that BW755C does really cause production of an inhibitor. The contrasting effects of altering lipoxygenase activity found by different workers have not yet been satisfactorily explained, and this field has recently been complicated by the discovery of another major group of lipoxygenase metabolites, the lipoxins (Serhan et al, 1984) and by the claims of powerful effects of hydroperoxyacids on cellular functions (Bray & Brahmi, 1986).

The mechanisms of the effects of thromboxane synthetase inhibitors and of prostacyclin must be determined, to see whether manipulation of these metabolites could be of practical clinical use. The results of the present study using a continuously growing cell line need to be extended to monocytes and macrophages, since some of the effects seen could have been on the maturation of the cells rather

than on IL-1 production *per se*.

CHAPTER 6

PRODUCTION OF ANTISERA DIRECTED AGAINST SPECIFIC PEPTIDES SEQUENCES OF INTERLEUKIN 1

6.1. Antisera against Defined Peptides of IL-1.

The role of interleukin 1 in vivo is still uncertain. Although it has many reported activities in vitro and when it is injected into animals, it remains to be established that IL-1 is secreted into the circulation in sufficient quantities and in the correct time sequence to account for the response seen in intact animals to trauma or infection. It is possible that the major role of IL-1 is as a local hormone. The relative importance in vivo of IL-1 and TNF is also unknown. A major reason for this uncertainty is the lack of reliable assays for both hormones which can be used in body fluids. IL-1 has been detected in serum in various clinical conditions (Bendtzen et al, 1984; Cannon & Dinarello, 1985), but the presence of inhibitors has required extraction of IL-1 by gel filtration or by antibody extraction techniques before assay. Accordingly, absolute values of IL-1 are unreliable, and have been at the lower limit of the capabilities of the assays used.

There is a need for a sensitive, specific immunoassay, which would be less subject to interference and would be simpler to use than the bioassays currently employed. Until recently, antisera have not been readily available,

and insufficient pure IL-1 has existed to allow its use as immunogen or label in immunoassays. This is a common problem for many lymphokines and growth factors, which were only available in microgram amounts from tissue culture fluid until the advent of gene cloning techniques. It has often been necessary to process litres of culture medium to obtain enough for biochemical characterisation, and much work has, of necessity, been carried out using impure reagents. Recombinant material is still not available in sufficient quantities at a low enough cost to be useful for raising antisera.

Now that the primary amino acid sequence of an increasing number of proteins is becoming known as a result of the use of recombinant DNA techniques, an alternative approach is possible. Synthetic peptides corresponding to defined sections of the sequence can be prepared and used as immunogens. It has been shown that antisera raised against such peptides often bind to the intact molecule (Lerner, 1984).

When the sequence of the major pI 7 form of human IL-1, known as IL-1β, was announced (Auron et al, 1984; March et al, 1985), it was decided to try this approach.

All of the actual peptide synthesis work described here was carried out in the Faculty of Pharmacy, University of Lille. I performed all practical work, under the direction of Dr A. Tartar and Dr H. Gras-Masse.

6.1.1. Choice of Sequence

It is known that the N- and C-terminal amino acid sequences of proteins almost always yield antisera which will bind to the native molecule (Palfreyman et al, 1984). It is more difficult to choose internal sequences which are likely to yield useful antisera. Barlow et al (1986) have shown that, given the dimensions of a typical antibody binding site, most antigenic determinants of a globular protein will inevitably involve residues which are widely separated in the primary sequence. The only regions of a protein which may form sequences of contiguous amino acids likely to react with antisera will therefore be on exposed loops.

This is consistent with a great deal of previous work attempting to develop methodologies for selecting useful peptides. Kyte and Doolittle (1982) and Hopp and Woods (1981) used thermodynamic considerations of the relative hydrophobicity of individual amino acids to allow the prediction of the likelihood of particular short peptides being exposed on the surface of a protein. Janin (1979) studied proteins whose tertiary structure was known, and calculated the probabilities that particular amino acids would be found on the surface of a protein. These empirical indices can be used in a similar way to the hydrophobicity plot of Kyte and Doolittle.

Regions of high mobility within proteins are likely to react with anti-peptide antisera (Westhof et al, 1984), probably because they are able to deform to fit the

antibody binding site. The structural feature known as a β-turn tends to protrude from the bulk of the protein, and is therefore likely to yield useful antisera.

Predictions based on these criteria are only capable of picking one or two peptides with a reasonable probability of being useful from a molecule under investigation, and Palfreyman and his coworkers (1984) showed that there was only a 50% chance of an internal peptide selected by the algorithm of Hopp and Woods (1981) producing an antiserum which would react with the intact protein.

The mature form of IL-1β, running from amino acid 117 of the precursor molecule, was analysed by the methods of Hopp and Woods (1981), Kyte and Doolittle (1982), and Janin (1979). The hydrophobicity plot and a plot of predicted flexibility of the peptide chain are shown in figure 6.1. The secondary structure as predicted by the rules of Chou and Fasman (1974a,b) was also derived. It was confirmed that both the N- and C-terminal peptides were likely to be exposed, though the C-terminal peptide did show some hydrophobic characteristics at the extreme terminus, a region at which the algorithms become inaccurate. They were also predicted to be mobile. It was decided to synthesise two peptides, of 18 and 21 amino acids. It was hoped that these would include a sufficient length of the protein to provide useful secondary structure. The 18 amino acid peptide ran from amino acid number 117 to number 134 of the published sequence, and the 21 amino acid peptide ran from amino acid 249 to 269 (figure 6.2). It was hoped that the C-terminal peptide

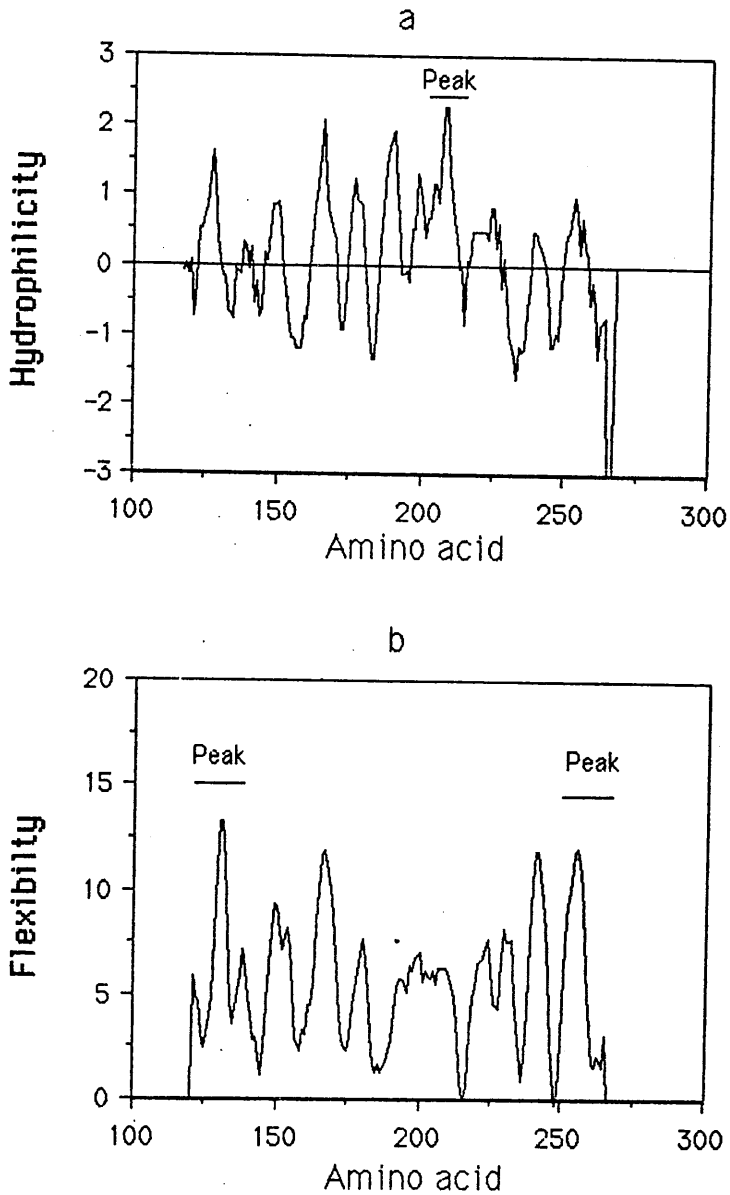


Figure 6.1. a. Hydrophobicity plot of IL-1, amino acids 117-269.
b. Flexibility plot of IL-1, amino acids 117-269.

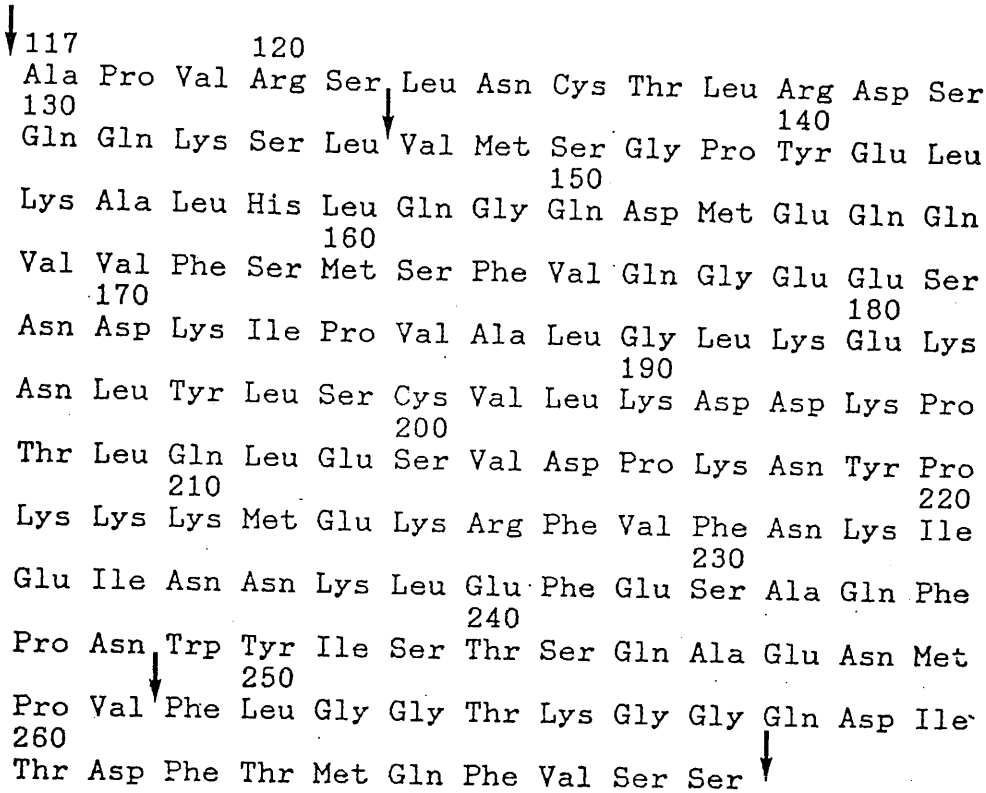


Figure 6.2. Amino acid sequence of IL-1. Peptides which were synthesised are marked with arrows.

would include a β-turn sequence. An internal peptide running from residue 197 to 210 was also selected by these algorithms. It was not synthesised, but it is interesting that Bayne et al (1986) did synthesise a peptide from this portion of the sequence, and corresponding antisera did react with the native molecule.

6.1.2. Synthesis of the Peptides

The solid phase peptide synthesis technique of Merrifield, as reviewed in Merrifield (1986) was used (figure 6.3). The C-terminal amino acid was coupled to a chloromethylated polystyrene resin, and amino acids added in sequence. The reactions were carried out in a cylindrical vessel approximately 15 cm long and 5 cm in diameter. This was fitted with a screw top, through which resin could be removed for testing, and with a sintered glass plug inside the base. Two glass tubes were inserted through this plug, and these served to introduce reagents and to remove waste. They were attached to a separating funnel fitted with a tap and to a vacuum pump connected to a waste trap respectively. The flow of reagents was controlled by a three-way tap into which the tubes fitted. The vessel was rotated in an oscillating arc at about 10 rpm by an electric motor attached to it by a clamp.

The N-terminal protecting group used was t-butyl oxycarbonyl (BOC), which was removed before each coupling with 40% trifluoroacetic acid (TFA) in dichloromethane. The TFA was neutralised before coupling with 5% diiso-

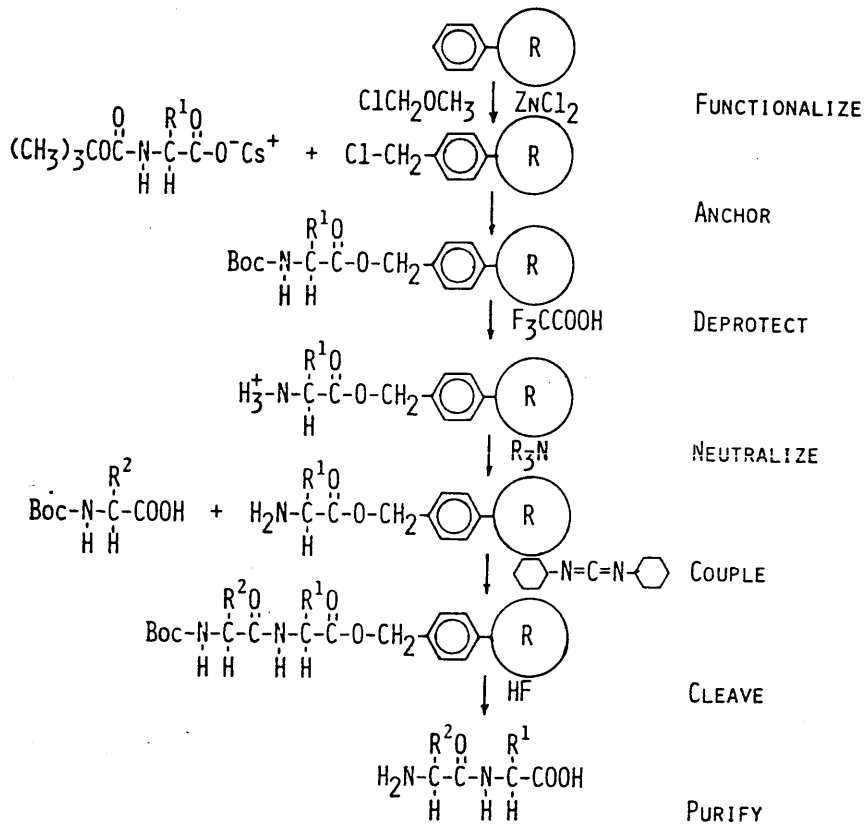


Figure 6.3. Scheme for solid phase peptide synthesis.

propylethanolamine (DIEA) in isopropanol (iPrOH). The protecting groups used for side chains were:

Arginine	Tosyl
Aspartic Acid	O-cyclohexyl
Cysteine	Acetamido
Lysine	Chlorobenzoxycarbonyl
Serine	O-benzoyl
Threonine	"

Coupling was mostly carried out using dicyclohexyl carbodiimide (DCCD) and hydroxybenzotriazole, though some amino acids were coupled as symmetric anhydrides. These reagents were used at 2.5 fold molar excess, which corresponded to 5 ml each of 126 mg/ml carbodiimide in dichloromethane and of 93.6 mg/ml hydroxybenzotriazole in dimethylformamide (DMF). Redistilled CH_2Cl_2 and spectroscopic grade DMF were used for coupling. To form symmetric anhydrides, the amino acid was reacted in a separate flask with DCCD, and the dicyclohexylurea formed was removed by filtration before the anhydride was used. To protect the methionine group in the C-terminal peptide from oxidation, ethane dithiol was added during each deprotection stage.

The sequence of reactions was as follows:

2x2 min	CH_2Cl_2	
1x2 min	TFA	Deprotection
1x25 min	TFA	

3x2 min	CH_2Cl_2	
2x2 min	iPrOH	
3x2 min	CH_2Cl_2	
3x3 min	DIEA	Neutralisation
5x2 min	CH_2Cl_2	
Coupling -	time variable	
3x2 min	CH_2Cl_2	
2x2 min	iPrOH	
3x2 min	CH_2Cl_2	

Testing for free amino groups was carried out using a ninhydrin spot test before and after each coupling. If the reaction was incomplete, a second coupling was carried out. If free amino groups were still present after the recoupling, they were blocked by acetylation with 5% acetic anhydride.

The total amount of N-terminal peptide synthesised was 1.76 g, which represented 70% of the theoretical yield. The amount of C-terminal peptide synthesised was 2.3 g. The completed peptides were removed from aliquots of the resin with liquid HF at 0°C in the presence of 10% cresol and 10% dimethylsulphide. This procedure removed all protecting groups except for the acetamido group protecting the cysteine residue.

The peptides were purified by gel filtration on Trisacryl 05 (LKB). The N-terminal peptide was purified in 5% acetic acid, but the C-terminal peptide proved to be sparingly soluble in aqueous solvents and was purified in dimethylformamide/acetic acid. Purity was checked by

reverse phase HPLC, using a Waters μ-bondapack column and a gradient of 0-80% acetonitrile in 10 mmol/l phosphate buffer, pH 2.0, and by TLC. Purity before gel filtration was at least 70% by HPLC (figure 6.4). Because not all of the peptide synthesised was cleaved from the resin, final figures for yields are not available.

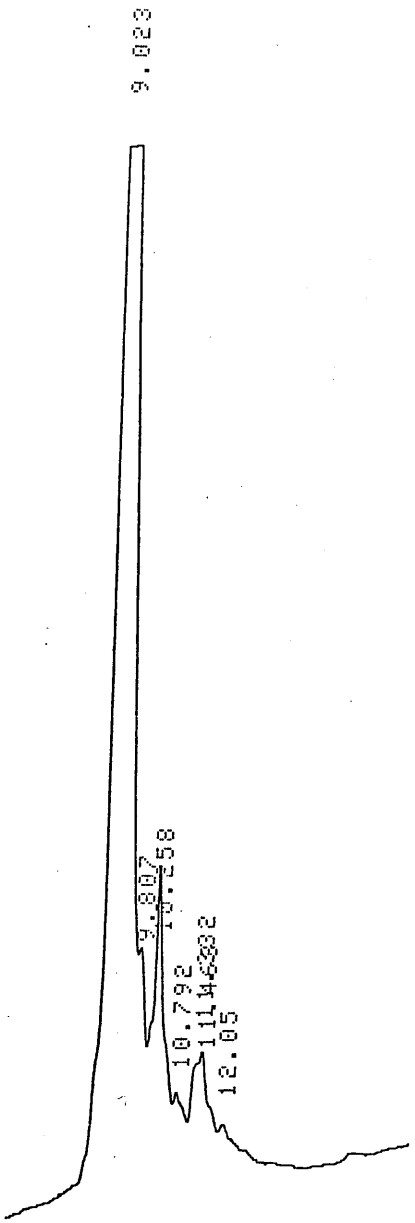
6.1.3. Preparation of Antisera

The peptides were coupled to keyhole limpet hemocyanin (KLH) with glutaraldehyde. The mixture was allowed to react for one week with continuous stirring in the dark and gradual addition of glutaraldehyde to a final concentration of 2.6 mmol/l, and then dialysed extensively against 10 mmol/l sodium phosphate, 150 mmol/l NaCl, pH 7.2.

Three rabbits were injected subcutaneously with 100 μg of each of the peptides coupled to KLH. The peptides were mixed with Freund's complete adjuvant at a ratio of 1:2 (v/v) and injected at multiple subcutaneous sites. After 4 weeks, the rabbits were given an identical booster injection, and samples were taken to test for antibody production. At 8 weeks, a further boost was made. Blood samples were taken at 6 weeks and 10 weeks.

6.1.4. Testing of Antisera

The production of antisera was tested by an indirect ELISA technique. Various concentrations of peptide or



CHROMATOPAC C-R3A
SAMPLE NO 0
REPORT NO 1192

FILE 0
METHOD 41

PKNO	TIME	AREA	MK	IDNO	CONC	NAME
1	9.023	199824			78.9269	
2	9.807	11510	V		4.5463	
3	10.258	24939	V		9.8504	
4	10.792	5023	V		1.9841	
5	11.332	4969	V		1.9627	
6	11.463	6045	V		2.3878	
7	12.05	866	V		0.3419	

Figure 6.4. HPLC profile of N-terminal peptide. 100 µl was run on a Waters µ-Bondapak column, with a 10 mmol/l phosphate buffer, pH 2.0, and a gradient of acetonitrile from 0 to 80%.

recombinant human IL-1. (kindly donated by Dr C.A. Dinarello, Boston) in 100 μl of 50 mmol/l carbonate buffer, pH 9.6 were adsorbed to microtitre wells (Nunc) overnight at 4^0C. The plates were washed three times with PBS (10 mmol/l phosphate, 150 mmol/l NaCl, 0.2% Tween 20, pH 7.2) and 100 μl of antiserum diluted in PBS with 25% foetal bovine serum were added. The plates were allowed to incubate for 2 hours at room temperature, washed again three times, and 100 μl of peroxidase-labelled goat anti-rabbit IgG (affinity purified, Sigma) were added at a concentration of 1:500 in PBS containing 1 mmol/l sodium citrate and 25% foetal bovine serum. After 2 hours, the plates were washed and substrate solution was added in 100 μl. This was o-phenylene diamine (2.2 mmol/l) and hydrogen peroxide (1.76 mmol/l) in a buffer of 126 mmol/l phosphate, 27 mmol/l citrate, pH 6.0. The colour reaction was allowed to proceed for 45 minutes in the dark, and was stopped with 100 μl of 4 mol/l H_2SO_4. The plates were read in a Titertek plate reader at 492 nm against an air blank.

Two of the three rabbits injected with each peptide produced high titre antisera after the first booster injection. There was no reaction with the irrelevant peptide. The titres were determined as the dilution of antiserum which gave twice the background absorbance when tested against 40 ng/ml of peptide or 250 ng/ml of IL-1. They are listed in table 6.1.

It can be seen that all antisera had titres of >1:10000, and that three of them (1 directed against the N-terminal peptide and two against the C-terminal peptide) had titres

Table 6.1

Titres of Anti-peptide Antisera

Antiserum	Titre	
	Peptide	IL-1
C-Terminal		
R1	>1:100000	1:120000
R3	1:25000	1:6400
R5	>1:100000	>1:100000
N-Terminal		
R2	>1:100000	1:300
R4	1:15000	<1:100
R6	1:30000	1:4000

Titres were calculated as the dilution which gave an absorbance value of twice the background when incubated with 40 ng/ml of peptide or 250 ng/ml of recombinant IL-1.

of >1:100000 when they were assayed against the immunising peptide. However, binding was less good when IL-1 was used as antigen. The titres of the antisera raised against the C-terminus were closely comparable with those assayed against the peptide, but there was a considerable discrepancy when those raised against the N-terminus were studied (figure 6.5). The titres of all antisera were several thousand times lower than had been expected from the binding to the peptide.

6.2. Discussion

From these results, it would appear that it is practical to raise antisera against defined peptides from the sequence of IL-1. The antisera against the C-terminal peptide, in particular, reacted well with recombinant IL-1. However, there was a problem with antisera against the N-terminal peptide.

The gross discrepancy of titres when the antisera were tested against IL-1 may have several different explanations. This may be a problem inherent in the structure of IL-1. The N-terminal peptide may take up a conformation in solution which does not correspond to its conformation in the native protein, or one or more of the dominant epitopes in the peptide may be hidden in the intact protein. However, the computer analysis of the protein indicated that the N-terminus was both hydrophilic and free to move, so this is an unlikely explanation.

Another possibility is that the chemical structure of

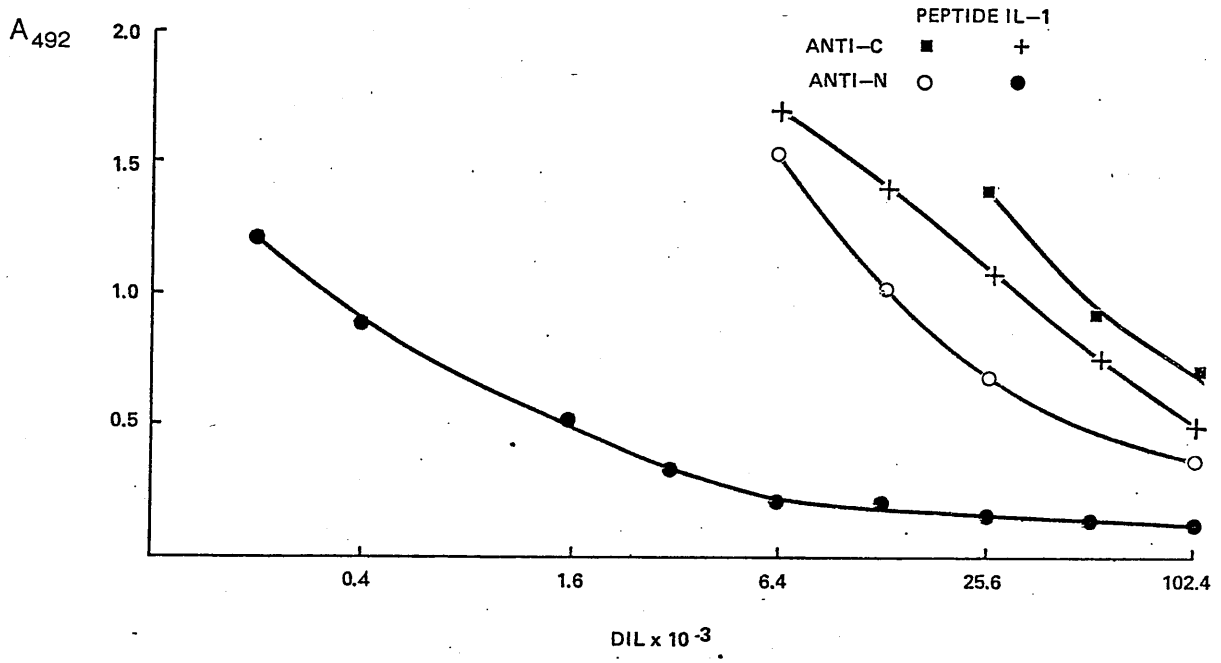


Figure 6.5. Antibody dilution curves of anti-peptide antisera. 250 ng of peptide or 40 ng of recombinant IL-1 were allowed to adsorb to microtitre wells, and serial dilutions of antisera were added. Visualisation was carried out using peroxidase-labelled anti-rabbit IgG, and o-phenylenediamine as chromophore.

the immunogen does not reflect that of the native peptide. The protecting group was not removed from the cysteine in the peptide, and this may have affected the population of antibodies produced. This is rarely of importance in such work, but it is possible that it may have had some relevance.

Since it is known (Dinarello et al, 1986b) that there is an extension of 5 amino acids on the N-terminus of this lot of recombinant material, the most likely explanation is that this could prevent binding of an antiserum directed against the N-terminus of the molecule. It will be necessary to study the binding of the antisera to native IL-1 and to recombinant material whose sequence does begin at residue 117.

These antisera are of high titre, and should prove valuable for use in an immunoassay. They should also be of value in immunohistopathology, as Bayne et al (1986) have demonstrated, and as extraction reagents for purification of IL-1. Preliminary evidence suggests that the antisera do not neutralise IL-1 activity, though they may remove activity if the immune complexes are precipitated. If this finding is confirmed, they will be less useful for functional studies on IL-1.

CHAPTER 7

GENERAL DISCUSSION

7.1. Factors which Control the Acute Phase Response

Some progress has now been made in understanding the factors which control the acute phase response to injury and infection. The changes in intermediary metabolism can be largely explained by increases in cortisol, catecholamines, and glucagon, and can be reproduced by infusions of these hormones (Bessey et al, 1984). The observed changes in metal concentrations in serum and in acute phase proteins cannot be explained by these hormones, however, and now appear to be consequences of secretion of certain members of a new group of protein hormones, known collectively as lymphokines or cytokines. The two which have been best characterised to date are interleukin 1 and tumour necrosis factor. Interleukin 1 exists in at least two forms, known as alpha and beta, though there have been no consistent differences reported in their biological activities.

Both IL-1 and TNF cause fever, induce an acute phase reaction, and have effects on connective tissue, inducing fibroblasts and chondrocytes to release enzymes and prostaglandins (Dinarello, 1986). They are both tumouricidal, though the mechanisms may differ, and have profound effects on vascular tissue. One of the few differences reported between IL-1 and TNF is the inability

of TNF to stimulate the immune system. Though its spectrum of action is so similar to that of IL-1, TNF acts through a separate receptor from the IL-1 receptor, which appears to respond to both forms of IL-1.

It has proved difficult to account for all the phenomena of the acute phase protein response by the actions of IL-1 or TNF. They have been shown to cause stimulation of the synthesis of acute phase proteins in murine, rat, and human systems, but the effect is always less than that associated with inflammation in vivo. It is likely that additional factors are required to initiate and maintain a full response.

One of these factors may be the hepatocyte stimulating factor described by Baumann and Koj. This factor has been claimed to stimulate the synthesis of all the acute phase proteins (Baumann et al, 1986), and has now been purified to homogeneity. In size and isoelectric point, the major form is very similar to TNF, but an amino acid sequence has not yet been reported.

7.2. Modification of the Acute Phase Response

There are various possible ways of modifying the acute phase response. They all probably act by altering the concentrations of hormones and lymphokines after injury or during the course of chronic inflammation, though there is still little evidence to support a primary role for any particular hormone.

7.2.1. Environmental Temperature

Increasing the environmental temperature has been shown to decrease the response to burns and to accidental injury. The mechanism of this finding is not known. Caldwell (1976) stressed the importance of relieving the abnormal heat loss caused by damage to the natural skin barrier, while Wilmore et al (1974a) demonstrated evidence for an internal resetting of heat production which could be countered to some extent by alterations in temperature. Interestingly, Kupper et al (1986) have shown that burn exudate fluid contains considerable quantities of IL-1, which may therefore be the agent involved in this resetting.

The reductions in metabolic rate and nitrogen excretion which they and other workers saw at higher temperatures were not reproduced in our studies. This seems likely to be a consequence of the lesser degree of injury of elective surgery as compared to the trauma of long bone fracture and the extreme metabolic upset caused by thermal injury. In our study, the catabolic hormones may not have been sufficiently elevated by the surgical procedure for any subsequent modification of the environmental conditions to have had a noticeable effect. We also failed to demonstrate any effect on the concentrations of acute phase proteins, in contrast to the study of Ballantyne & Fleck (1973), presumably for the same reason. The only significant changes which were noted were decreases in urine volume and in the excretion of cortisol and its

metabolites in the urine, which may have been a adaptive response to the change in temperature.

7.2.2. Treatment with Gold Compounds

Gold treatment may directly alter IL-1 release from monocytes (Gordon et al, 1985), and would therefore be expected to have a marked effect on acute phase proteins. Reductions were indeed observed in a number of proteins in patients with rheumatoid arthritis treated with either injectable or oral gold. The concentrations of C-reactive protein and haptoglobin changed to the greatest extent. These proteins have been previously reported to reflect the activity of the disease process (Amos et al, 1977; McConkey et al, 1979), and the findings of this study would support this conclusion. The concentrations of other acute phase proteins also decreased significantly, but the magnitude of the change was less, suggesting that they would be less suitable for the monitoring of disease. CRP and α_1-antichymotrypsin, on the other hand, appeared to be the most suitable proteins to measure to give an index of disease activity in untreated patients, as their levels were elevated in the majority of the patients studied before they commenced treatment.

Because of the wide variability between patients, it was considerably more difficult to demonstrate differences between patient groups at any particular time point. None of the acute phase proteins contributed markedly to any differences seen between treated and untreated groups, and

the tests which were most efficient in distinguishing patients treated with gold from those on placebo were shown to be IgA and pain score.

The reason for the great variation seen in both protein levels and in clinical scores is not known. Rheumatoid arthritis shows marked changes in disease activity over comparatively short time scales, which has always necessitated the use of large numbers of patients in drug trials. These changes may correspond to changes in IL-1 production or to secretion of IL-1 inhibitors. These are produced by several cell types, such as monocytes and B lymphocytes, and are known to affect the actions of IL-1 on the immune system and on connective tissue. Lotz et al (1986) have shown that monocytes from the synovial fluid of patients with rheumatoid arthritis secrete abnormally large quantities of IL-1 inhibitors, which may explain the reduced response of lymphocytes in synovial fluid to mitogens. Much further research will be needed to determine whether the large variations in the concentrations of acute phase proteins can be explained by changes in inhibitor production.

The effect of gold on IgA may reflect a direct inhibition of immunoglobulin production by cells involved in the primary inflammatory process, which is known to involve cells in mucous membranes throughout the body (Harris, 1982b). IgA is the major immunoglobulin involved in mucosal immunity, and so it could be anticipated that its production would be reduced if there were a direct action on the disease process. The large changes in pain

score, seen especially with the use of injectable gold, may also reflect changes taking place within the joint, possibly related to the inhibition of the secretion of IL-1 or TNF. Alternatively, phagocyte proteases may be involved. If neutrophils are incubated in vitro with gold, there is a stabilisation of their lysosomal membranes (Finkelstein et al, 1982), and this may directly affect joint pain by preventing the release of destructive enzymes.

7.2.3. Inhibition of IL-1 Production

The tacit assumption has been made in the above studies that inhibition of IL-1 and of the acute phase response is of benefit. This may not always be so. It has been shown that patients who are unable to make IL-1 have a poor prognosis (Keenan et al, 1982), though this may simply reflect the severity of their illness rather than being a consequence of deficiencies in the acute phase response. It has been suggested that administration of IL-1 may be of benefit in immunosuppressed or critically ill patients and that it may be useful as an antitumour agent, as TNF is already being used. In chronic inflammatory situations, however, there is little doubt that IL-1 production should be suppressed, if possible.

Various substances have been reported to inhibit IL-1 release. These include cortisol, catecholamines, prostaglandins, and materials such as cholera toxin which raise cAMP levels within cells. There may also be specific

lymphoid cells which inhibit IL-1 production. Release of IL-1 from within cells can be inhibited by blocking Ia molecules, but the significance of this observation is unknown.

In the experiments reported here, it was found that the monocyte cell line U937 did respond to inhibition of thromboxane synthesis with a reduction in IL-1 production. It was not established whether this was due to the effects on thromboxane per se, or to an indirect increase in prostaglandin production, but the effect was marked, and could have therapeutic implications. It was also found that prostacyclin in low concentrations stimulated IL-1 release, though it was inhibitory when higher concentrations were used. The reported effects of lipoxygenase inhibitors could not be confirmed.

One problem with this study is that an immature cell line was used to obtain sufficient numbers of cells for the experiments. It is therefore unclear whether the effects observed were on IL-1 production itself or on the differentiation of the cells into macrophages. It is possible to separate these two phases by using 1, 25-dihydroxyvitamin D_3, which only causes differentiation without stimulating IL-1 production. Addition of lipopolysaccharide will then cause IL-1 production. Alternatively, the study could be repeated using peripheral blood monocytes. Since a major proportion of IL-1 is retained within the cell, it could also be of interest to compare the effects of drugs on intracellular and extracellular IL-1.

7.3. Immunoassays for IL-1

Although the effects of IL-1 are impressive when it is injected into experimental animals or administered in vitro, it has not yet been definitively proved that it, in fact, acts as a circulating hormone in vivo. It has been extremely difficult to demonstrate the presence of IL-1 in human blood because of the presence of inhibitory factors, which are as yet incompletely characterised. Small quantities have been shown to be present in febrile patients and after aetiocholanolone injection (Cannon & Dinarello, 1984; Watters et al, 1986), but the methods used have not allowed accurate quantitation, and such useful information as the time course of IL-1 release into blood after a stimulus is not available in man. The availability of an immunoassay would be of great help in answering these questions.

Recently, an RIA for IL-1β has become available. It will be of interest to use it for measuring levels in tissue culture, though its sensitivity may not be high enough for blood levels. There is also apparently some interference with the method if blood is used (Dinarello, personal communication). Moreover, it is extremely expensive.

I have raised antibodies against peptides selected from the sequence of IL-1. These are of high titre, and it may be possible to develop an assay which is more sensitive than the current RIA, because of the theoretical advantage of the IRMA methodology. It will also be cheaper to use than presently available techniques. These antisera should

be useful both for developing immunoassays and for histochemistry. Bayne et al (1986) have shown that intracellular IL-1 can be detected with anti-peptide antisera, and immunostaining of histological sections with a battery of antisera against lymphokines and related antigens, such as IL-2, interferons, and DR antigens, should prove valuable in the diagnosis and study of inflammatory lesions. The antisera will also be valuable as reagents for the purification of IL-1.

Bioassays will, however, continue to be necessary because of the several forms of IL-1 which are known. Although IL-1β is the major form produced by monocytes, Kupper et al (1986) have claimed that keratinocytes produce primarily IL-1α, and that this could be the most important form after thermal injury. Additional molecular species of IL-1 probably exist, such as the B-cell derived form described by Rimsky et al (1986), which apparently has no homology with the known proteins.

Eventually, assays for IL-1α and β, TNF, and several possible species of HSF will be developed, and these will contribute to a full understanding of the changes in biochemistry and physiology seen after injury. Providing this data and interpreting the information obtained will be a continuing challenge for biochemists and clinicians.

APPENDIX - MATERIALS USED

a) Antisera and Standards

Anti-human C-reactive protein	Atlantic Antibodies, Scarborough, ME, USA.
	Dakopatts, Copenhagen, Denmark.
	Hoechst-Behring, Frankfurt, FRG.
	Immuno AG, Vienna, Austria.
	Kallestad Laboratories, Austin, TX, USA.
	Orion Diagnostika, Espoo, Finland.
	Seward Laboratories, London.
LN standard CRP	Hoechst-Behring, Frankfurt, FRG.
Anti-human α_1-antitrypsin	Atlantic Antibodies, Scarborough, ME, USA.
Anti-human α_1-acid glycoprotein	
Anti-human α_2-macroglobulin	
Anti-human haptoglobin	
Anti-human transferrin	
Calibrator 1	

Anti-human prealbumin Prealbumin standards	Seward Laboratories, London.
Anti-human α_1-antichymotrypsin Normal reference serum	Orion Diagnostika, Espoo, Finland.
Nor-Partigen RID plates CRP RBP LN reference serum	Hoechst-Behring, Frankfurt, FRG.
EMIT kit for C-reactive protein	Syva, Palo Alto, CA.

b) Chromatography gels

Activated CH-Sepharose Sepharose CL-6B Sephadex G-25M Sephacryl S300 PD10 disposable columns	Pharmacia, Uppsala, Sweden.
Trisacryl 05	LKB Produkter, Bromma, Sweden.

c) Tissue culture media

RPMI 1640 Foetal Calf Serum Penicillin/Streptomycin Non-essential amino acids	Flow Laboratories, Irvine, Scotland.

Sodium Pyruvate	
Ultroser G	LKB Produkter, Bromma, Sweden.
Ficoll-Paque	Pharmacia, Uppsala, Sweden.

d)Drugs

Indomethacin	Thomas Morson Pharmaceuticals (Division of Merck, Sharp, & Dohme Ltd) Hoddesdon, Herts, England.
BW755C Prostacyclin (Epoprostenol)	Wellcome Research Labs, Beckenham, Kent, England.
Dazmegrel	Pfizer Ltd, Sandwich, Kent, England.
Iloprost	Schering Pharmaceuticals, Burgess Hill, West Sussex, England.

e)Miscellaneous

Spectropor dialysis membranes	Spectrum Medical Industries Inc,

Los Angeles, CA, USA.

YM30 ultrafiltration membranes	Amicon Corporation, Danvers, MA, USA.

Other chemicals were obtained from BDH Chemicals Ltd, Poole, Dorset, England, and from Sigma London Chemical Company, Poole, Dorset, England.

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ADDENDA

The calorimetric measurements described in chapter 3 were carried out by the staff of the Surgical Metabolic Unit of Glasgow Royal Infirmary, particularly Mr A Sim and Mr N Harris. They also organised the collection of blood and urine samples. Urine nitrogen, 3-methylhistidine, creatinine, cortisol, and 17-hydroxycorticosteroids were analysed by members of staff of the Department of Pathological Biochemistry, Glasgow Royal Infirmary.

The measurement of clinical indices and ESR described in chapter 4 was carried out by the staff of the Centre for Rheumatic Diseases, Glasgow Royal Infirmary.

2.6.3.Validation of Interleukin 1 Assay

The LAF assay is technically simple, as it is easy to disaggregate thymi without the use of enzymes, and up to 10^8 cells can be obtained from one murine thymus. The response variable, incorporation of tritiated thymidine into DNA, is easy to measure, so the assay is suitable for the generation of large numbers of results with full confidence limits. However, there are specific problems with the use of the LAF assay.

Sterile technique is desirable, though not essential (Duff, G., personal communication), since a three day incubation is used. Several studies have documented the dependence of proliferation of thymocytes on temperature (Duff & Durum, 1985) and pH of the assay medium (Murphy et al, 1985). Conditions of incubation are therefore critical for reproducibility of assays. In my studies, considerable difficulty was found with the stability of the control of the concentration of CO_2 in the incubators, and an increase in CO_2 concentration of only 2% was found to damage assay performance severely.

The mice which were used were neither endotoxin-resistant nor tolerised to endotoxin. This was a theoretical difficulty because of the high sensitivity of macrophages to endotoxin and the possibility of macrophages being present in the thymocyte preparations,

but the levels of endotoxin in my reagents were less than 1 ng/ml (measured by the Limulus amoebocyte assay by Dr C McCartney, Department of Bacteriology, Glasgow Royal Infirmary). There was no interference at these levels. In case endotoxin might be present in samples, I included polymyxin B in the assay medium. This antibiotic binds and neutralises endotoxin.

The LAF assay depends on the generation of IL-2 by thymocytes in the presence of IL-1, so IL-2 will interfere in the assay. However, U937 cells cannot make IL-2, and peripheral blood cells were stimulated with endotoxin rather than with mitogens to avoid the release of IL-2. None was found in the supernatants by a specific IL-2 assay (carried out by Dr J Smith, Department of Medicine, Glasgow Royal Infirmary).

Because of the substantial variability which was found between individual triplicates (from 5 to 30%), and because of the possibility that interfering substances might be present which could alter the slope of the dose-response curve, all samples of IL-1 from the drug experiment described in chapter 5 were assayed in several dilutions. An estimate of precision was obtained from these dilution curves by the use of the full analysis of variance for bioassays (Finney, 1978). The samples were assayed in 4 dilutions with a dilution ratio of 1:3. Three replicates were carried out at each dilution.

Preliminary investigations of the shape of the dilution curves showed that they could be linearised by transforming both the dose and the counts per minute to logarithms. The samples were then analysed simultaneously with a standard preparation in a full analysis of variance. Sums of squares were derived for the difference of the means of the preparations, regression, parallelism of samples with the standard, and linearity of dilution curves. The assay was valid in terms of linearity, but the curve of one sample (BW755C, 50 ug/ml) was found not to be parallel to that of the standard. The estimated values and 95% fiducial limits are shown in table 2.12. The limits were frequently narrower than those found by linear interpolation, as they had originally been analysed, so this would appear to be the preferable method of data reduction. Because the limits derived by this method are not symmetrical about the mean, it is not possible to quote a meaningful coefficient of variation.

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TABLE 4.6

Auranofin study. Biochemical data.

Placebo group, medians and interquartile ranges.

Week	12	24
ESR	49 (26-79)	44 (29-72)
CRP	22 (12-52)	23 (13-44)
HP	2.4 (1.8-3.0)	2.0 (1.8-2.4)
AG	1.0 (0.9-1.5)	1.1 (0.9-1.6)
AT	2.2 (1.9-2.7)	2.0 (1.8-2.4)
ACT	1.2 (0.9-1.4)	1.1 (0.7-1.2)
IGA	3.3 (2.5-4.2)	3.6 (2.7-4.2)
IGG	14.8 (11.8-16.7)	14.9 (13.1-16.7)
IGM	1.5 (1.0-1.8)	1.4 (1.1-1.6)

ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, HP: haptoglobin, AG: α_1-acid glycoprotein, AT: α_1-antitrypsin, ACT: α_1-antichymotrypsin.
ESR in mm/hr, CRP in mg/l, all others in g/l.

TABLE 4.7

Auranofin Study. Biochemical data.

Auranofin group. Medians and interquartile ranges.

Week	12	24	36	48
ESR	44 (22-72)	40 (10-57)	45 (10-58)	30 (9-55)
CRP	23 (12-42)	12 (8-28)	13 (6-40)	11 (4-25)
HP	1.8 (1.3-2.5)	1.6 (0.9-2.4)	1.5 (1.0-2.8)	1.6 (1.1-2.0)
AG	1.1 (0.8-1.6)	1.0 (0.8-1.4)	0.9 (0.8-1.3)	1.2 (0.8-1.3)
AT	2.2 (1.8-2.7)	2.2 (1.6-2.3)	1.9 (1.8-2.3)	2.0 (1.6-2.3)
ACT	1.2 (0.9-1.4)	1.0 (0.8-1.2)	0.9 (0.8-1.2)	0.8 (0.7-1.0)
IGA	2.7 (2.2-4.0)	3.1 (2.1-3.9)	2.6 (2.2-3.4)	2.5 (2.1-3.6)
IGG	14.4 (12.3-15.7)	13.3 (10.8-14.0)	12.5 (10.8-13.9)	12.0 (10.2-14.1)
IGM	1.1 (0.8-1.6)	1.2 (0.8-1.6)	1.3 (0.9-1.6)	1.2 (0.8-1.8)

Abbreviations and units are as in table 4.6.

TABLE 4.8

Auranofin study. Biochemical data.

Thiomalate group. Medians and interquartile ranges.

Week	12	24	36	48
ESR	33 (14-55)	20 (5-52)	23 (5-45)	21 (8-43)
CRP	19 (9-42)	19 (5-32)	10 (6-31)	14 (5-41)
HP	1.7 (1.3-2.4)	1.3 (1.0-1.9)	1.2 (0.9-1.7)	1.3 (0.8-2.1)
AG	1.0 (0.7-1.2)	0.9 (0.8-1.1)	1.0 (0.8-1.2)	0.9 (0.9-1.3)
AT	2.4 (1.8-2.8)	2.0 (1.7-2.3)	1.9 (1.6-2.5)	2.0 (1.6-2.9)
ACT	1.0 (0.8-1.2)	0.8 (0.8-1.1)	0.9 (0.7-1.1)	1.0 (0.7-1.1)
IGA	2.6 (1.9-3.5)	2.3 (1.4-3.0)	2.2 (1.2-3.5)	2.2 (0.4-3.3)
IGG	13.4 (11.6-16.6)	12.5 (11.9-14.7)	11.5 (10.0-13.8)	11.6 (10.4-13.5)
IGM	1.1 (0.8-1.2)	0.9 (0.6-1.5)	1.0 (0.7-1.3)	1.0 (0.7-1.4)

Abbreviations and units are as in table 4.6.

TABLE 4.9

Auranofin study.

Percentages of week 0 values.

Placebo group, medians and interquartile ranges.

Week	12	24
ESR	92.7 (68.5-119.2)	100.0 (76.1-116.3)
CRP	105.8 (52.4-170.5)	120.0 (59.7-210.3)
HP	87.5 (74.3-127.3)	90.3 (67.9-119.8)
AG	88.9 (77.8-107.7)	92.6 (70.4-119.4)
AT	104.3 (79.2-116.7)	97.1 (78.8-111.1)
ACT	96.6 (84.6-110.4)	95.4 (85.4-121.5)
IGA	100.0 (79.2-115.6)	90.7 (82.9-117.4)
IGG	98.9 (92.7-110.4)	101.7 (89.0-105.9)
IGM	109.4 (85.4-128.9)	94.1 (86.7-113.3)

Abbreviations as in table 4.6. ESR in mm/hr, CRP in mg/l, all others in g/l.

TABLE 4.10

Auranofin study.

Percentages of week 0 values.

Auranofin group. Medians and interquartile ranges.

Week	12	24	36	48
ESR	84.0 (69.9-109.7)	78.1 (42.3-94.1)	72.7 (26.0-116.0)	58.8 (28.5-81.0)
CRP	105.6 (67.6-176.0)	60.3 (31.9-129.5)	107.1 (29.1-217.8)	52.6 (30.9-108.5)
HP	90.0 (71.0-113.6)	77.1 (49.3-90.3)	70.9 (40.0-121.2)	73.3 (52.9-91.7)
AG	88.9 (75.0-122.2)	80.0 (58.5-100.0)	90.0 (64.1-105.0)	78.3 (63.3-105.5)
AT	104.8 (83.3-115.8)	88.9 (75.3-105.6)	97.6 (75.6-117.3)	80.0 (64.2-109.8)
ACT	96.7 (88.0-113.5)	83.7 (70.9-108.2)	90.9 (72.0-108.8)	72.8 (61.9-87.6)
IGA	92.1 (77.5-102.4)	91.0 (72.0-101.2)	86.8 (68.0-98.5)	83.7 (67.9-108.2)
IGG	101.8 (89.3-107.0)	91.4 (82.9-99.0)	87.1 (76.1-95.1)	86.9 (76.0-99.2)
IGM	89.4 (73.6-104.7)	72.6 (63.2-92.3)	80.0 (66.3-106.5)	77.0 (65.6-97.8)

Abbreviations and units are as in table 4.6.

TABLE 4.11

Auranofin study.

Percentages of week 0 values.

Thiomalate group. Medians and interquartile ranges.

Week	12	24	36	48
ESR	65.1 (42.8-88.3)	44.4 (13.8-91.0)	42.8 (18.3-76.7)	40.5 (16.3-65.2)
CRP	69.4 (37.5-108.7)	49.5 (33.8-147.3)	55.4 (22.4-127.7)	43.5 (19.7-95.0)
HP	74.1 (50.0-106.2)	66.9 (53.6-103.9)	58.4 (37.3-118.0)	61.8 (43.2-105.2)
AG	72.7 (59.2-110.5)	72.7 (61.0-95.0)	83.3 (60.0-100.0)	81.8 (61.7-111.1)
AT	92.1 (80.1-124.1)	89.7 (74.4-101.6)	82.1 (70.5-120.2)	95.2 (70.9-131.2)
ACT	91.3 (79.7-101.4)	84.9 (67.8-96.6)	79.5 (64.8-100.0)	84.3 (62.0-103.8)
IGA	84.5 (67.0-97.5)	75.0 (56.9-88.2)	75.0 (56.7-87.2)	72.1 (32.1-92.4)
IGG	93.6 (85.4-103.7)	90.0 (78.8-100.2)	85.1 (71.6-92.7)	85.4 (73.5-92.9)
IGM	75.0 (69.7-105.6)	80.0 (60.4-97.4)	75.0 (68.4-93.3)	75.9 (58.7-100.0)

Abbreviations and units are as in table 4.6.