# CYTOKINE AND OTHER COMPONENTS OF THE INTEGRATED HOST RESPONSE TO INJURY

Robert John Baigrie

A thesis submitted for the degree of Doctor of Medicine

of the

University of Cape Town

Nuffield Department of Surgery
University of Oxford
Michaelmas 1991

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

This thesis is dedicated to a kind man, who also possessed the gift of gentle patient care - my late father Robert Diarmuid Hutchinson Baigrie MA FRCS(Ed). He would have recognised both the virtues and limitations of this work, and valued it all the more for having both.

On the interaction between surgical trauma and the host's response:

*"The cleaner and gentler the act of operation, the less the patient suffers, the smoother and quicker his convalescence, the more exquisite his healed wound"*

Lord Moynihan of Leeds (1902)

*"**Inflammation** is the response of vascularised tissues to injury and is characterised by the clinical signs first recorded by Aulus Cornelius Celsus: 'rubor, dolor, calor et tumor'.*

*It is the body's mechanism of rallying and amplifying the host's defences to combat injury. However, prolonged or extensive inflammation may lead to irreversible damage to the host"*

Anne S Hamblin (1991)

St Thomas' Hospital, London

*"While inflammation and immunity are frequently studied independently, in reality the two are inseparable. Most of the cells and molecules that defend the body are involved in both inflammation and immunity"*

Lloyd Old (1988)

"Discoverer" of tumour necrosis factor

Memorial Sloan-Kettering Cancer Center

## ABSTRACT

The aim of this thesis was to provide further insight into the sequence of events leading to the induction of the integrated host response to injury. This work has concentrated primarily on studies of mediators and components in the plasma of injured patients.

Only by studying frequent, serial samples, obtained from a clean and uniform model of major injury (20 abdominal aortic surgery patients), was interleukin-1 (IL-1) was shown to be detectable in plasma within two hours of injury. This preceded a rise in interleukin-6 (IL-6). Tumour necrosis factor (TNF) and interferon gamma (IFNγ) were not detected in this group, but TNF was present within a few hours of injury in a group of accidental trauma patients (n=6), usually preceding the peak in IL-6 levels. Together, these two findings have provided *in vivo* clinical evidence in keeping with the widely accepted *in vitro* finding that IL-1 and TNF induce the production of IL-6.

The systemic IL-1 and IL-6 response to surgical trauma was shown to increase with the severity of the surgical insult. Moreover an early, exaggerated IL-6 response was associated with the subsequent clinical development of major complications.

It has been proposed that following major surgery or injury, bowel-derived endotoxin induces inflammatory cytokines. This hypothesis was tested in eight patients undergoing abdominal aortic aneurysm surgery. Systemic and portal blood were serially sampled and assayed for endotoxin and its associated cytokines. Endotoxin levels were generally below the limit of detection (3.7pg/ml), TNF was not detected and IL-1 levels were similar in portal and systemic blood. However there was a significant difference ($p<0,0001$) between systemic and portal IL-6 levels, which increased markedly with time. These results implicate the colon as a major source of IL-6 and suggest that only a very low endotoxin concentration is required to induce cytokine production. Alternatively, endotoxin may evoke local production, within the ischaemic colon, of IL-6 without itself reaching detectable levels in the portal circulation.

The peripheral blood mononuclear cell (PBMC) is a well documented source of IL-1, TNF, IL-6 and IFNγ *in vitro*. Although TNF, IL-1 and IFNγ were not detected in the plasma of the aneurysm patients, there remained the possibility that these cells were secreting cytokines in concentrations that were too low to detect with ELISA, but which nevertheless could be important when these cells migrated to a site of inflammation. Using an uncommon technique, the reverse haemolytic plaque assay, an increase was demonstrated in IFNγ production by PBMC after injury, which persisted for more than a week. Production of IL-6 was not found, suggesting that PBMC are not a particularly important source of the high plasma levels of IL-6.

Recent evidence has pointed to glutamine as an important component of the host immune response, making it seem plausible that this amino acid might indeed be associated with the activity of cytokine mediators of the

host response. A remarkable correlation ($r = -0.95$, $p<0.0001$) between plasma IL-6 and glutamine was observed in six major surgery patients. The possibility of a significant physiological interaction between glutamine and IL-6 is discussed.

Thus, the theme running through this thesis begins with injury and endotoxin as the initiators of the host response, which is mediated in turn by the acute phase cytokines. These are induced in different quantities and in different organs, tissues and cells, depending on the initial insult, but a sequential pattern of production seems apparent. Consideration has been given to the role these cytokines might play in the pathogenesis of an inappropriate host response or their use as markers of this response.

The hypothesis is proposed that IL-6 functions as a circulating, quantitative signal of cell damage.

The present data provide information which may be of value in the management of injured patients, namely the quantification of the magnitude of injury and the prediction of its outcome. In principle, the use of cytokine assays, in particular IL-6, would appear to be a potentially useful adjunct to the clinical assessment of critically ill patients. High IL-6 levels appear to anticipate by at least 12 to 24 hours, clinical evidence of complications and assessment of CRP levels. There is the added benefit of IL-6 being an isolated measurement, therefore interpretable on its own, without the entry of other complex data into the data-base of an outcome scoring system.

## STUDY LOGISTICS, A STATEMENT OF ORIGINALITY AND ACKNOWLEDGEMENTS

The studies reported in this thesis were performed between March 1990 and September 1991, when the candidate was a Research Fellow and Clinical Lecturer in the Nuffield Department of Surgery at Oxford University. The work was performed under the supervision of Professor PJ Morris and Mr PM Lamont (Departmental Reader). The patients studied were those admitted under the clinical service of the Nuffield Department of Surgery. All the laboratory work was performed solely by the candidate in the departmental laboratories in the John Radcliffe Hospital in Oxford, with the following exceptions: (1) The initial tumour necrosis factor immunoassay undertaken by the candidate proved prohibitively expensive and, subsequently, a different assay was used which was performed in the Institute of Molecular Medicine at the John Radcliffe Hospital, under the supervision of Dr Dominic Kwiatkowski MRCP, (2) all the reverse haemolytic plaque assays, reported in Chapter 7, were carried out solely by the candidate in the laboratories of the Nuffield Department of Pathology, at the John Radcliffe Hospital, under the supervision of Dr C Lewis PhD, and (3) the glutamine assays were performed by Dr Mark Parry-Billings D Phil, of the Department of Biochemistry at the University of Oxford. The candidate was responsible for the collection and preparation of all samples. Dr Isla Halliday PhD (the Institute of Clinical Science at The Queen's University of Belfast) and Dr Sue Stephens PhD (Celltech Ltd, Slough), both generously processed endotoxin samples as part of the verification study in Chapter 6. The considerable technical help of all these associates is gratefully acknowledged.

No cytokine immunoassays nor endotoxin assays had previously been performed in the Nuffield Department of Surgery or at the John Radcliffe Hospital (with the exception of Dr Kwiatkowski's assay). These techniques were introduced to the department *ab initio* and performed unsupervised by the candidate. The haematological, biochemical and endocrine measurements were performed in the clinical laboratories of the departments of Haematology, Biochemistry and Immunology at the John Radcliffe Hospital, Radcliffe Infirmary and Churchill Hospital respectively. All data collection and statistical analysis were performed by the candidate, including trauma and APACHE II scoring, and measurement of plaque size and number in the reverse haemolytic plaque assays. The typing of the manuscript, and the preparation of figures and tables were performed solely by the candidate using an Apple Macintosh word-processor. Figure 2.1 is acknowledged to Scientific American.

I am indebted to the Medical Research Fund of the University of Oxford and the Oxfordshire Health Authority District Research Fund for their generous financial support. These studies were approved by the Central Oxford Research Ethical Committee.

I am very grateful to Professor Peter Morris for providing the inspiration, sowing the seeds and laying on the facilities for this research. Without the moral and intellectual support, the friendship and generosity of Peter Lamont, this project may well have foundered in the first few months. In good times he has kept my feet on the ground and in bad times he has dragged my head out of the ground. Amongst many other qualities, his profound good sense in the matter of researching and writing a thesis, will forever have my admiration and gratitude. I am grateful to Dr Maggie Dallman for her advice, for including me in her research group and

allowing me the use of one of her labs. The other surgeons in the department, Mr Jack Collin and Mr Derek Gray, and members of the Nuffield Department of Anaesthetics, in particular Professor Pierre Föex, all went to considerable trouble to assist my sometimes frantic sample collection and processing. Dr Helen Chapel was kind enough to provide early encouragement and later advice on the direction of the thesis. Numerous people, by their help and friendship, have made my time in the department easier and more pleasurable than it might have been and I am consequently grateful to Nick Aarons, Terry Denton and Mary Saunders, Brenda, Audrey and the other secretaries and Molly Harwood in Medical Illustration. Sanity was preserved (often only marginally) by sharing with Andrew Gordon and Rob Higgins, both the good days and those inevitable long dark days as we all laboured in our little lab. Johnny Butler shared both the frantic and the "dead-boring" hours at the "pump room bench" in theatres, where we both prepared endless samples, convinced we would never come to the end.

It remains only for me to thank the most important person in this research endeavour. Carolyn, only you and I know how insignificant has been your great contribution to the referencing and editing of this thesis, when compared to the patience you have shown, and the infinite lengths to which you have gone, in ensuring the emergence of a sane and happy little Baigrie family at the end of 1991. Thankyou.

## GLOSSARY OF ABBREVIATIONS

| | |
|---|---|
| ACTH | adrenocorticotrophic hormone |
| AIS | Abbreviated Injury Scale |
| APACHE | Acute Physiological And Chronic Health Evaluation |
| APP | acute phase protein(s) |
| APR | acute phase response |
| C | complement factor |
| °C | degrees Celsius |
| CNS | central nervous system |
| CRP | C reactive protein |
| DNA | deoxyribonucleic acid |
| EFW | endotoxin free water |
| EDTA | ethylene diamine tetra-acetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| E coli | Escherichia coli |
| EP | endogenous pyrogen |
| g | gram |
| IL- | interleukin- |
| Ig | immunoglobulin |
| ISS | Injury Severity Score |
| LEM | leucocyte endogenous mediator |
| LAF | lymphocyte activating factor |
| mg | milligram |
| ml | millilitre |
| M | Molar |
| mm | millimetre |
| µm | micrometre |
| µl | microlitre |
| NADH | nicotinamide adenine dinucleotide hydride |
| ng | nanogram |
| nm | nanometre |
| N | Normal |
| NK cell | natural killer cell |
| OD | optical density |
| PBMC | peripheral blood mononuclear cell |
| PG | prostaglandin |
| pg | picogram |
| PML | polymorphonuclear leucocyte(s) |
| rpm | revolutions per minute |

CONTENTS

# THESIS BACKGROUND AND HYPOTHESIS

## The research background

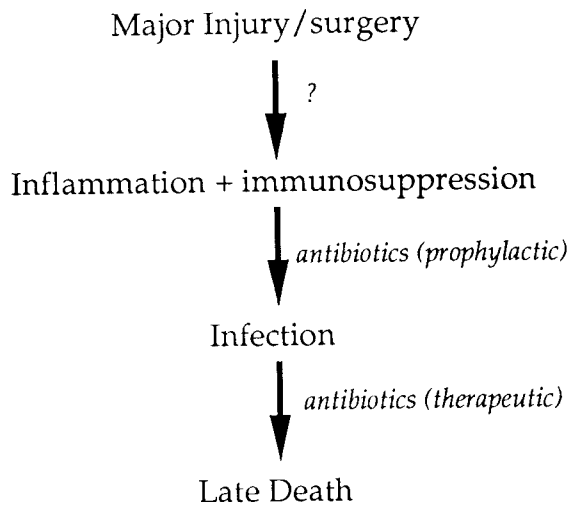



Figure i: Chain of events between major injury and late death.

Despite both careful attention to the principles of antisepsis and improved monitoring and therapeutics, sepsis remains a major cause of morbidity and mortality in patients after either major surgery or severe trauma. The chain of events leading from major injury to late death remains a persistent problem as a steadily increasing number of high risk patients undergo major surgery or are successfully resuscitated after major injury. Antibiotics, both prophylactic and therapeutic, have ameliorated the last two steps in the chain (Figure i), but the problem persists. There has been no substantial change in overall wound infection rates for over twenty years, with the level set at about five percent for all operative cases (Cruse PJE et al., 1980). This risk of sepsis occurs in the presence of a well documented acute phase response, which includes inflammatory events, as well as wide-ranging deficiencies in the immune response of patients subjected to trauma or major surgery. Impairment of humoral and cell-

mediated immunity has been correlated with the increased risk of sepsis in these patients (see Chapter 1).

Why only patient A, who has the same operation as patients B and C, goes on to develop sepsis is often not clear. Increasingly, therefore, the focus of research is shifting to the first step in the chain (Figure i) in an attempt to identify, understand and, finally, modify its mediators. An understanding of the underlying causes or "triggers" for these responses remain poorly defined, but it has become increasingly clear that they are largely mediated by cytokines, which are polypeptides produced by a wide variety of cells including macrophages and lymphocytes. The majority of reports on cytokine biology have concentrated on patients with either cancer or established infection. Less is known about the activity of cytokines in patients after surgery or trauma.

Published trauma studies have concentrated on the *in vitro* cytokine responses of cells isolated from trauma patients. Despite some impressive *in vitro* work, the clinical evaluation of cytokines has been scanty and inconsistent. Although cytokines had been variously and variably detected in body fluids of patients with a variety of diseases, at the start of this thesis there was only a single letter (Nijsten MWN et al., 1987) in the English literature of plasma cytokine levels after surgery or trauma. Indeed, two major text-books published in 1989 and 1990, reviewing the immune consequences of trauma with specific reference to cytokines, made virtually no reference to interleukin-6.

Considerable interest has also arisen in endotoxin and the mediation of its systemic effects via tumour necrosis factor and interleukin-1. However the detection in the clinical setting of all three of these factors has been

contradictory. As will be described in the Chapter 2, interleukin-1 went from being the most widely detected plasma cytokine in the first half of the last decade, to being virtually undetectable in plasma a few years later, after the advent of immunoassays and improved bioassays. Tumour necrosis factor was reported to predict morbidity and mortality, but these studies were usually based on a single measurement of the cytokine (for example at admission to an intensive care unit) or perhaps a daily measurement. Yet it was known that cytokines have a plasma half-life of only minutes. Moreover, in most reports, both the units of measurement used and the concentrations reported were widely disparate, partly as a consequence of the wide variety of bioassays or immunoassays being used. The detection of endotoxin was even more inconsistent, with statements such as "endotoxin is a normal constituent of portal blood" contrasting with "endotoxin is only detectable in very low concentrations in the portal blood of laparotomy patients" (see Chapter 6).

Finally, there has been renewed interest over the last two or three years in the acute phase response, after the realisation that interleukin-6, a pleiotrophic cytokine, is primarily responsible for the induction of acute phase protein synthesis. Allied to this has been renewed interest in glutamine which, while not being an acute phase *protein*, could perhaps be described as a "negative" acute phase *amino acid*. Recent glutamine research has stimulated clinical trials showing that its inclusion in enteral and parenteral feeds is of great benefit to both compromised patients and their gastrointestinal tract, an organ which is being increasingly shown to be of immunological importance. The possibility of a link between this amino acid and a polypeptide known to control acute phase protein synthesis (interleukin-6), was therefore investigated.

## The hypothesis

The sequelae to injury are dictated by the host's response to that injury. Cytokines are important mediators of the integrated host response. It was hoped that by studying major injury patients, with repeated sampling over a longitudinal time course, it would be possible to define more accurately any relationship that might exist between circulating cytokines, glutamine endotoxin and clinical progress. A better understanding of the kinetics and production of these factors was sought through sampling of both portal and systemic blood simultaneously.

The work presented in this thesis started with most of this hypothesis, the remainder evolving during the course of the research. It involves a series of studies performed in patients undergoing major or minor surgery as well as victims of major trauma. These studies were aimed at providing insight into the sequence of events leading to the induction of the integrated host response to injury.

# CHAPTER ONE

# THE INTEGRATED HOST RESPONSE

# CHAPTER ONE

## THE INTEGRATED HOST RESPONSE



### 1.1 A BRIEF HISTORY OF TRAUMA & SEPSIS

Archeological findings of trephined skulls and bones with healed fractures provide mute evidence of prehistoric man's efforts to care for the sick and injured. Through the ages, trauma and sepsis exacted a terrible toll in

human suffering and death as a result of misconceptions concerning the causes of infection and the effects of injury. While Homer described the nature of battle wounds and Hippocrates described healing as a naturally occurring response and Celsus described, in the first century, the classic features of inflammation, nonetheless little progress was made until the 18th century (Majno G, 1975). The underlying principles of invasive infection and tissue repair were neither understood nor applied to the care of wounds. Treatment such as cautery, phlebotomy, purgation and the application of filthy poultices all detracted from nature's own healing efforts and it is not suprising that a hunted animal, which sought simply shelter and rest when wounded, often fared better than the wounded hunter himself, especially if he was attended by the finest physicians in the town.

Although Ambrose Pare recognised in the sixteenth century, the value of natural healing when he wrote *"I dress the wound; God heals it"* (Packard FR, 1927), more than 200 years passed before John Hunter noted:

*There is a circumstance attending accidental injury which does not belong to disease, namely that the injury done, has in all cases a tendency to produce both the disposition and means of cure.*

John Hunter 1794

Thus Hunter recognised that inflammation is not a disease, but is in fact a remarkable process evoked within the host, following trauma or sepsis, to eliminate necrotic tissue, terminate infection and assist in tissue repair.

Nonetheless the cause and dissemination of infection remained unexplained and 92 percent of soldiers who underwent amputation of the

femur in the Crimean war died of "hospital gangrene" (Chenu JC, 1865). In the middle of the 19th century Pasteur, and later Koch, established the relationship between microorganisms and disease (King L, 1957) and Lister introduced, in the face of great ridicule, the concept of antisepsis to surgery (Lister J, 1870). The importance of the immunological response to infection was highlighted by the observations of Metchnikoff (Metchnikoff E, 1905) and Erlich (Bordet J, 1895) at the turn of the century, which demonstrated the important roles of phagocytic destruction of bacteria and humoral antibodies.

However, the most dramatic advance in the whole history of medicine has probably been the surgical revolution of the last one hundred years, made possible by the development of safe anaesthesia, blood transfusion and the discovery of sulphonamides and penicillin.

The pace quickened even further in the post-war decades with the development of organ transplantation, resulting in an explosion of interest in the field of immunology which has subsequently been carried into all specialties. In addition, surgeons continued to push their discipline to the limits with ever more extensive and demanding operations, which finally made clear the importance of the physiological and metabolic host responses and the need to support them, in order to enhance survival from major surgery, trauma and infection.

## 1.2 THE INTEGRATED HOST RESPONSE - A DEFINITION

We now know that the beneficial host response which Hunter recognised almost 200 years ago, has three main components: (1) the primary local response, (2) a non-specific systemic response and (3) a specific antibody response (Belfrage S, 1963). These can usefully be redefined as the

"Integrated Host Response" to injury consisting of a local and a systemic component, namely local inflammation and the systemic acute phase response. The latter includes immunological, metabolic, endocrine, neurological and haematological changes.

This particular description of the integrated host response is a distillation of several definitions. Many observations on the acute phase response have been made over the years and it has been defined in a number of ways. Koj and Gordon described the acute phase response as *"an early and unspecific but highly complex reaction of the animal organism to a variety of injuries, such as bacterial or parasitic infection, mechanical or thermal trauma, malignant growth or ischaemic necrosis"* (Koj A, 1985). They went on to say that *"It (the acute phase response) includes not only the local reaction but also neurological, endocrine and metabolic alterations which are expressed as fever, leucocytosis, increased secretion of certain hormones (among which cortisol is particularly important), changes in the concentration of some heavy metals in blood and liver, activation of the clotting, complement, kinin-forming and fibrinolytic pathways, negative nitrogen balance, transfer of amino acids from muscle to liver followed by drastic rearrangement of plasma protein synthesis".*

Dinarello, however, included certain immunological changes: *"Host responses to microbial infections, injury and inflammatory disease include dramatic changes in metabolic, haematologic and immunologic parameters that are often grouped together and called the acute phase response"* (Dinarello CA, 1984).

As a result of the complexity of these definitions some authors have concentrated on more specific changes that occur after injury, and

Whicher states that *"The acute phase response of inflammation comprises the increase in serum level of a family of liver derived proteins which appear to have important and diverse functions in the inflammatory process. This physiological response to injury is accompanied by a number of other systemic responses such as fever, leucocytosis and muscle proteolysis which are probably all mediated by a cytokine or family of cytokines, derived from macrophages and some tissue cells. known as interleukin 1"* (Whicher JT, 1985). Pepys emphasises the acute phase proteins response: *"Following most forms of tissue injury, infection or inflammation, the concentrations of a number of proteins increase, and then return to normal again as healing or recovery occurs"* (Pepys MB, 1981).

## 1.3 THE INTEGRATED HOST RESPONSE - MAIN FEATURES

These changes include elevation of body temperature, tachycardia, increased elaboration of pituitary and stress hormones, alterations in circulating leukocyte populations, increased hepatic synthesis of various proteins, and redistribution of iron and trace minerals within the body (Beisel W, 1975). A prolonged acute phase response, resulting from severe infection or injury, induces severe adverse effects which were first clarified by Cuthbertson in his classical nitrogen balance studies in post-traumatic patients (Cuthbertson DP, 1930). He demonstrated that the catabolic responses following injury are frequently out of proportion to any reduction in nitrogen intake. These responses were further refined as hypermetabolism, accelerated proteolysis and an erosion of lean body mass by Dr Francis Moore at Harvard University (Moore F, 1980).

## 1.4 FEVER

Fever is probably the oldest and most widely known manifestation of illness and the acute phase response. It is mentioned in the bible and Homer thought it a punishment from the Gods for bad conduct. In the humoral theory of disease purported by Hippocrates, fever was attributed to an excess of yellow bile which, like fire, was hot and dry (Atkins E, 1985). We now know that fever is regulated by specialised temperature sensitive neurones, localised in the anterior hypothalamus. The body's temperature "set-point" is increased in response to endogenous pyrogens, such as endotoxin and the acute phase cytokines (Chapter 2), whose actions involve intracerebral prostaglandins acting on the hypothalamus. Antipyretics, such as sodium salicylate, which inhibit prostaglandin synthesis from arachadonic acid, can attenuate endotoxin induced fevers with a concomitant decrease in cerebrospinal fluid prostaglandin levels (Bernheim HA et al., 1979). An increase in body temperature of 1°C gives rise to an increase in resting energy expenditure of about 13 percent.

## 1.5 ENDOCRINE CHANGES

The endocrine response to injury may begin before the injury itself, with awareness of approaching danger activating the hypothalamic defence area. This results in the secretion of a number of pituitary hormones, including adrenocorticotrophic hormone (ACTH), growth hormone and vasopressin, as well as the activation of the sympatho-adrenal system which also results in increased secretion of cortisol and adrenaline (Fleck A, 1978)(Frayn KN, 1986).

Once injury occurs, different stimuli will reinforce this stress response. These stimulators include nociceptive afferents from damaged areas, baroreceptors responding to hypotension and hypovolaemia and stimuli from osmo- and chemoreceptors responding to changes in blood

biochemistry. Hormones increasing after the injury include glucagon, insulin, ACTH, cortisol, the catecholamines, growth hormone, thyroxine and aldosterone (Kushner I, 1982).

The metabolic activities of the catecholamines augment cardiovascular function and are also calorigenic. Their metabolic responses are mediated by the second messenger, intracellular cyclic adenosine monophosphate (cAMP), and include hyperglycaemia, glycogenolysis, gluconeogenesis, lipolysis and suppression of insulin secretion (Wilmore DW et al., 1974). This initial response has been called the "ebb phase" and lasts typically around 12-24 hours. The ebb phase merges into a more prolonged period of catabolism known as the "flow phase", in which an increase in metabolic rate and breakdown of body tissue occurs (Cuthbertson DP, 1942).

## 1.6 TRACE ELEMENTS

The integrated host response gives rise to reproducible changes in the serum concentrations of iron, zinc and copper. The redistribution of iron and zinc into the liver is associated with new synthesis of the liver proteins ferritin and metallothionein, respectively. The benefits or drawbacks of provision of additional supplements of trace metals, remain controversial (Mertz W, 1981).

## 1.7 THE IMMUNE RESPONSE

A primary factor in the development of sepsis after major injury is depression of the host immune response. The fact that both humoral and cellular immunity are depressed after surgery and trauma, was rarely given any clinical consideration until it was highlighted in a Lancet editorial of October 1974 (Anon, 1974). Yet seven years previously, Riddle

and Berenbaum had noted that T lymphocytes, from postoperative patients, exhibited depressed proliferation in response to mitogen stimulation (Riddle PR et al., 1967). Anaesthesia and glucocorticoid release were the favoured explanations and, for the next few years, research in this area focused on the effects of anaesthesia. In 1969 Arturson et al followed serum immunoglobulins for several weeks in burns patients and recorded a fall in all types of immunoglobulin, thought to be related to protein loss from the burn wound (Arturson G et al., 1969). In 1974, Cooper et al also reported that the humoral response was impaired after surgery and noted that anaesthesia contributed very little to this response (Cooper AJ et al., 1974).

The first attempt to study the effect of a standard major surgical procedure on the immune response of healthy patients was carried out in 1975 by Slade et al who chose as their model, kidney donor patients undergoing nephrectomy. All parameters measured (T, B and total lymphocyte counts, mitogen blastogenic response, mixed leucocyte activity and antigen skin responses) fell as a result of surgery, except serum immunoglobulins which were unaltered. All, except skin responses, were near normal by the fifth postoperative day (Slade MS et al., 1975). The results of these and numerous other studies meant that, by the end of the decade, it was widely recognised that PML, macrophage and reticuloendothelial function are decreased after burns, mechanical trauma and surgery.

In a symposium in 1979, designed to draw together the results of the previous two decades research in this field, Howard noted that in many studies the same interesting observation had been made, namely that lymphocyte and neutrophil function differed, depending on whether the cells were tested in the patient's own serum or pooled donor serum

(Howard RJ, 1979). Invariably decreased responsiveness occured in the patient's own plasma but was not always evident in pooled plasma (Park SK et al., 1971)(Alexander JW et al., 1968). Furthermore the serum from burnt patients inhibited chemotaxis of normal polymorphonuclear leucocytes (Fikrig SM et al., 1977). The explanation for this was thought to lie in the reduced opsonic activity observed in blood from postoperative patients (Saba TM, 1975). However, during the next decade the increasing understanding of cytokines and other soluble mediators introduced a complexity, far beyond simple opsonic activity, to the control of the immune response (see Chapter 2).

Both specific and non-specific immune defences are impaired after trauma, and the remainder of this section aims to provide a synopsis of current knowledge.

### 1.7.1 Alterations in immune cells

Leucocytosis: As early as 1920, Cannon noted a consistent early leucocytosis after injury (Cannon W, 1919). A peripheral leucocytosis after thermal, non-thermal and surgical injury is now well documented, and results from an increased release of granulocytes from bone marrow storage pools, followed by an increased production of precursors. A mild leucocytosis can also be seen in the response to adrenalin released in situations of mild stress and it is apparent that there are two pools of granulocytes in the vascular compartment. The first is the circulating pool and the second is adherent to the vasculature - the "marginated pool". Demargination of the white cells from the vessel walls, provoked by adrenaline and cortisol, will cause an apparent leucocytosis. The opposite effect can be seen in endotoxic shock, where a leucopaenia may be observed. Here the bone marrow releases large numbers of neutrophils,

but a large number become marginated, resulting in a fall of circulating white cells (Athens JW et al., 1961).

Polymorphonuclear leucocytes: The three phases of polymorphonuclear leucocyte (PML) function are chemotaxis, phagocytosis and intracellular killing. Chemotaxis has been extensively researched in trauma and burns patients and all studies have noted a chemotactic deficit, although onset varied between days one and five (Fikrig SM et al., 1977)(Warden GD, 1981). Furthermore the serum from burned patients inhibited chemotaxis of normal PML (Warden GD, 1981).

While Alexander noted an increased rate of phagocytosis with decreased intracellular killing in burned patients (Alexander JW et al., 1968), Balch studied patients after severe battle trauma and found decreased phagocytosis of bacteria (Balch HH, 1955). While these workers used direct bactericidal assays, other measures of microbicidal function have been used to assess various parameters of the oxidative reactions causing microbial death, including oxygen consumption (Heck EL et al., 1975) and chemiluminescence (Howes RM et al., 1976). These and other newer assays have served to confirm the findings of the bactericidal assays whilst also providing insight into the nature of the microbicidal defect. Therefore, although there are a few contrary reports, the overwhelming impression is of decreased PML function following burns, mechanical trauma and operation (Howard RJ, 1979).

Lymphocytes themselves are probably most usefully studied after fractionation. The development of monoclonal antibodies such as the cluster of differentiation (CD) series, allowed more accurate definition of the functional subsets. This was greatly facilitated by the availability of

automated counting using the fluorescence activated cell sorter (FACS) which allows complete objectivity and a large sample size. The total number of circulating lymphocytes and of the subpopulations CD3 (pan T cell), CD4 (helper T cell), CD8 (suppressor/cytotoxic T cell) and natural killer cells have been noted to fall after injury (Lennard TWJ et al., 1985)(Grob P et al., 1988)(Bauer AR et al., 1978). However, the suppressor cell response has been inconsistently reported, with (1) an increased response (Hansbrough JF et al., 1984)(McIrvine AJ et al., 1982), (2) a decreased response that did not reach significance (Rodrick Ml et al., 1986)(O'Mahoney JB et al., 1985) and (3) a significantly decreased response (Lennard TWJ et al., 1985)(Faist E et al., 1986), noted.

O'Mahoney, who had reported a decrease, was also an author of the earlier McIrvine paper which reported an increase. In the later paper he described an artifactual finding to explain the contradictory results. In the 1982 study, cells stained with monoclonal antibodies were identified by fluorescence microscopy and this suggested an increase in CD8 cells. Following the introduction of flow cytometry, which selected cells by size prior to counting them, it became apparent that some of the CD8 positive cells were not T cells. These were the large granular lymphocytes, monocytes and immature PML which were binding to the monoclonal antibodies and hence masquerading as T cells.

Thus, with regard to alterations in lymphocyte subgroups following injury, there remains inconsistency in results of the CD8 population response. On the other hand, the fall in the CD4 population has been a consistent finding in all these studies.

However the significance of all these studies is now open to question, because it depends on the extent to which the phenotypic markers, detected by the monoclonal antibodies, reflect the function of the T cells which they identify. It is now clear that the correlation between phenotype and function is not as close as was originally proposed. In particular, a small subset of CD4 positive cells has been found to be of importance in the generation of suppressor cells (Thomas Y et al., 1982) and it is now clear that both the two main T cell subsets are heterogenous, both in phenotype and function (Takada S et al., 1983).

Monocyte/macrophages: In recent years the macrophage has emerged as a key cell in immune dynamics after trauma. Macrophages play a central role in cell-mediated immunity, both in antigen recognition as antigen presenting cells and also in the effector limb as inflammatory, tumoricidal and phagocytic cells. These cells elaborate cytokine mediators including tumour necrosis factor (TNF), interleukin-1 (IL-1) and interleukin-6 (IL-6), as well as complement components, plasminogen activator and tissue thromboplastin (Unanue ER et al., 1980). An interesting concept describes two general classes of macrophage: "facilitatory" and "inhibitory" (Miller SE et al., 1982). The former are characterised by (1) interaction with T helper cells, (2) the presence of the immune response antigen (Ia antigen), (3) production of cytokines and (4) inhibition by prostaglandin $E_2$ ($PGE_2$) and by T suppressor cells.

Inhibitory macrophages are characterised by (1) interactions with T suppressor cells, (2) absence of the Ia antigen and (3) activation by and production of $PGE_2$. In particular the production of $PGE_2$ by macrophages may represent an important immunosuppressive mechanism because these cells are increased after injury (Faist E et al., 1987) and $PGE_2$ is capable

of suppressing various immune functions. These include augmentation of T suppressor cell proliferation and inhibition of T helper and B cell proliferation, as well as the inhibition of cytokine production. Several studies have documented the beneficial effects of cyclooxygenase inhibitors, which inhibit prostaglandin synthesis, in animal trauma models and trauma patients (Hansbrough JF et al., 1986)(Faist E et al., 1990).

Originally it was thought trauma, burn injury or intra-abdominal infection produced an immediate reduction in the circulating numbers of monocytes (Bauer AR et al., 1978), but more recent studies using monoclonal antibodies have shown a significant elevation of circulating macrophages following injury (Faist E et al., 1988). This group have also shown that inhibitory macrophages are dominant after injury resulting in immunocompetence. During the post-traumatic course the macrophagic $PGE_2$ output was significantly elevated (upto eightfold on days five to seven) compared to controls, a finding that was originally reported in burn trauma by Miller (Miller CL, 1981). They also showed a reduction in IL-1 and interferon gamma (IFN$\gamma$) production during this time, although Rodrick et al have shown increased IL-1 production and marked interleukin-2 (IL-2) suppression in non-thermal injuries (Rodrick Ml et al., 1986). Macrophage chemotaxis (McCabe WP et al., 1973), phagocytosis and respiratory burst activity are also reduced in injured patients (Miller CL, 1981).

These findings all indicate a severe depression of cell-mediated immunity leading to a down-regulation of the immune response.

Mitogen responsiveness: Following the initial description of Riddle and Berenbaum of a postoperative depression of the lymphocyte proliferative

response to the extract of red beans *phaseolis vulgaris* - phyto-haemagglutinin (Riddle PR et al., 1967), mitogens have been widely used to study a variety of surgical patients. The assay measures the proliferative response (percentage uptake of tritiated thymidine) of an individual's T cells when stimulated with the mitogen. The mitogen response reflects the interaction of macrophages and T cells during T cell proliferation, a response which Constantian showed in 1978 to be mediated by a serum polypeptide (it would now be called a cytokine) which activated suppressor T cells (Constantian MB, 1978). In addition T cells show decreased mixed lymphocyte responses (Sakai H et al., 1974).

Studies of burns patients have demonstrated a significant correlation between mitogen responsiveness and a patient's ability to life-threatening sepsis, with all patients succumbing to fatal sepsis demonstrating a virtual absence of phytohaemagglutinin response (Miller SE et al., 1982). This and other studies have, therefore, verified the early observations of Riddle and Berenbaum (Miller CL, 1981)(O'Mahoney JB et al., 1984).

Natural Killer cells(NK cells): These cells require no antigen presentation and are not restricted by their major histocompatibility complex (Heberman RB, 1984). Using a monoclonal antibody (Leu-7) and flow cytometry, the number of circulating NK cells has been shown to fall after major surgery and trauma (Lennard TWJ et al., 1985)(Grob P et al., 1988). These cells have been shown to have a wide range of cytotoxic activity against a number of tumour cell lines and one of their probable roles in the circulation is to act against circulating tumour cells. A fall in number of NK cells could therefore be expected to reduce resistance to circulating tumour cells. This was supported by a study of hind limb amputation in mice, which suppressed NK activity in tumour-free mice. Amputation of

non tumour-bearing limbs in tumour-bearing mice also suppressed NK activity and led to a significant increase in the number of metastases (Pollock RE et al., 1984).

### 1.7.2 Alterations in humoral immunity

Serum immunoglobulin response to injury has received only scattered attention, and the few studies done of immunoglobulins A, G and M (IgA, IgG and IgM) levels have yielded varying results. Elective surgery and trauma have been shown to be associated with reduced levels of IgM and IgG respectively (Gauperaa T et al., 1985)(Alexander JW et al., 1979), but Bjornson found no effect on IgG following trauma (Bjornson AB et al., 1978b). However he and others noted reduced IgG levels after burn injury (Bjornson AB et al., 1977)(Alexander JW et al., 1979). Arturson et al followed serum IgG, IgM, IgE, IgD and IgA for several weeks in burns patients and recorded an initial fall in all types of immunoglobulin, thought to be related to protein loss from the burn wound (Arturson G et al., 1969). Serum IgM returned to normal after one week, IgA, IgG and IgE after two weeks and IgD after one month.

Hershman et al set out to document overall IgA, IgG and IgM levels and establish their patterns in severely injured and burns patients, during the initial two weeks after injury. They found that all three classes were reduced in all patient groups on postoperative day one with IgG affected most. Levels in the "uneventful recovery group" returned to mid-normal by two weeks. The "infected nonthermal group" showed a supranormal IgA, IgG and IgM response by seven days which, in the case of IgG, persisted to two weeks. In the "infected burns group" the IgG response was the most clearcut, with sharply reduced levels at all times. Humoral immunity is also altered, as reflected by reduced serum immunoglobulins

after severe trauma (Hershman MJ et al., 1988a). In a study of patients, over the first four days following varying grades of surgical trauma as well as major mechanical trauma, IgA, IgG and IgM fell in all patient groups, reaching their lowest level within 24 hours and staying low until the end of the study. The drop was most marked in the major trauma group (Grob P et al., 1988).

These immunoglobulin changes could, at least partly, be caused by compartmental shifts, because B cells sampled after trauma are able to produce normal quantities of immunoglobulins, although they cannot be stimulated to produce specific antibodies (Nohr CW et al., 1984). Moreover, B lymphocyte levels, as determined with monoclonal antibodies and flow cytometry, remain relatively normal (Lennard TWJ et al., 1985)(Grob P et al., 1988). The second explanation is a defect of terminal B cell maturation, with switching from IgM to IgG production attributable to macrophage induced suppression (Ertel W et al., 1987).

### 1.7.3 Complement

The complement (C') system is a complex, integrated cascade of serum proteins that is important in both chemotaxis and opsonisation. It is activated along the classical pathway, consisting of C1, C4 and C2, by interaction with antigen-antibody complexes resulting in the activation of C3. Activation along the alternative pathway bypasses these early components and is initiated directly, after activation of C3 by damaged tissues or bacterial polysaccharides.

A number of investigators have reported profound abnormalities of complement levels and functions with burns. In 1966 Arturson noted that levels of C1-4 fell by upto 20 percent after lethal burns (Arturson G et al.,

1966). Bjornson demonstrated reduction in components of both the classic and alternate pathways after thermal injury (Bjornson AB et al., 1978a), but two years later the same group reported rather different findings. They found that functional activity of the alternative complement pathway and the concentration of properdin were decreased during eight weeks post-burn in septic and non-septic patients. This compared with reduced classical pathway activity only in septic patients, and only in the initial post-burn period (Bjornson AB et al., 1980). Heideman suggested that burn injury might cause a direct activation of complement via the alternative pathway either by aggregating serum proteins or by thermal alteration of host tissues (Heideman M, 1979).

Increased levels of C3a and C5a have been reported in burned patients as a result of activation of the alternative pathway by anaphylotoxins (Gelfand JA et al., 1983). While mean classical pathway titres were 49 percent below the normal mean, they found the alternative pathway titre to be reduced by more than 90 percent and provided evidence supporting complement activation, not simply protein loss, as the cause of this depletion. An early fall in C3, C4 and C factor B was seen after surgical injury resulting from complement activation, consumption and compartmental shifts, but by 48 hours levels were recovering (Grob P et al., 1988).

During the early 1970s, several studies attempted to utilise changes in complement components as indices of the effect of trauma on the host defence and as prognostic indicators (Robin M et al., 1975), but the results were inconsistent (Leon C et al., 1982) and interest in this area has fallen away.

### 1.7.4 Anergy

Various studies had suggested that preoperative anergy was associated with an increased risk of sepsis and it was claimed that persistent anergy would predict development of sepsis (Maclean LD et al., 1975). However further research has dampened these hopes and, whereas all studies show an association between anergy and sepsis, in many cases it appears that sepsis precedes the development of anergic reactions. In addition, preoperative testing was found to be no more successful at predicting the subsequent development of sepsis than hypoalbuminaemia and less successful than clinical judgement in predicting sepsis and mortality following surgery for gastrointestinal malignancy (Ottow RT et al., 1984).

Many studies are open to criticism on the basis of poorly matched study groups, for example, a study of skin testing in 46 preoperative patients claimed to show that the incidence of death and sepsis was greater in the anergic group. However, not only did the results fail to reach significance, but the anergic group was significantly older and had a greater incidence of malignant disease (Christou NV et al., 1984). In a British study of 166 patients about to undergo major elective abdominal surgery, 11 percent were found to be anergic and of these 21 percent had major septic complications compared to 8 percent of the normally reactive group, but the difference was not statistically significant. They concluded *"preoperative delayed hypersensitivity testing is valueless either for warning or reassurance about the occurrence of postoperative complications"* (Ausobsky JR et al., 1982).

It seems probable therefore, that delayed hypersensitivity skin testing will find a role as a research tool, able to identify a group of patients likely to have an impairment of the immune system, for example Nohr et al have

shown that anergic patients do not produce antibody normally in response to tetanus toxoid (Nohr CW et al., 1984). However skin tests do not predict the development of sepsis reliably and widespread clinical use seems unlikely to be useful.

### 1.7.5 Summary

After severe trauma, delayed type hypersensitivity responses, allograft rejection, T cell proliferation in response to mitogens, mixed lymphocyte responses and T-dependent antibody responses are all diminished. These are all hallmarks of a general T cell defect. In addition defects in macrophage and neutrophil function are pronounced, whereas B cell activity appears to be relatively unaffected (Table 1.1).

Table 1.1 Acquired immune cell abnormalities in patients suffering trauma, burn injury or intra-abdominal infection.

| Cell system | Function or characteristic | Acquired abnormality |
|---|---|---|
| Neutrophils | circulating cell number | increased |
| | spontaneous migration | depressed |
| | chemotaxis and phagocytosis | depressed |
| | bactericidal activity | depressed |
| | lysosomal enzyme levels | depressed |
| | chemiluminescence | depressed |
| | dye reduction | depressed |
| Monocytes | circulating cell number | increased |
| | chemotaxis | depressed |
| | microbicidal activity | depressed |
| | facilitatory class | decreased |
| | inhibitory class | increased |
| | mitogen responsiveness | decreased |
| T lymphocytes | circulating cell number | decreased |
| | mitogen responsiveness | decreased |
| | migration | decreased |
| | IL-1 production | decreased |
| | IFN gamma production | decreased |
| | CD 4/CD 8 cell ratio | decreased |
| NK cells | circulating number | decreased |
| | tumoricidal activity | decreased |

## 1.8 THE ACUTE PHASE PROTEIN RESPONSE

For a long time it has been recognised that the concentrations of plasma proteins alter after trauma. In 1935 Cuthbertson and Tompsett reported that after both surgical and accidental trauma, a fall in the plasma concentration of the albumin moiety is observed with a marked rise in the globulin fraction (Cuthbertson DP et al., 1935). They also noticed that fibrinogen was often raised under these conditions. Five years earlier, Tillet and Francis (Tillet WS et al., 1930) had observed that acute phase sera from individuals with lobar pneumonia possessed the capacity to precipitate a non-protein somatic fraction known as fraction C, derived from pneumococci. Once the pneumonia resolved, this reaction was no longer demonstrable. MacLeod and Avery characterised the reactive substance in the acute phase sera as a protein and named it C-reactive protein (CRP) (Macleod CM et al., 1941a)(Macleod CM et al., 1941b). CRP has been found to be undetectable in normal plasma, but to increase dramatically during infection of various types.

Electrophoresis of plasma after trauma shows a decrease in the albumin band and an increase in the alpha-1 and alpha-2 globulin bands (Sunderman FW Jr, 1964) thus giving rise to the term acute phase protein. Although it was known that many of the acute phase proteins were alpha-globulins (Hoch-Ligeti C et al., 1953), electrophoretic studies could not differentiate between the many plasma proteins contained in the regions. The study of individual proteins was made possible by the introduction of immunological methods (Mancini G et al., 1965)(Laurell CB, 1966)(Minchin-Clarke HG et al., 1971), and it became apparent that a considerable number of proteins increase during an acute phase response.

Koj has reviewed in detail the protein changes after trauma in humans and has classified the proteins into five groups on the basis of their change in concentration during an acute phase response (Koj A, 1983)(Koj A, 1985). The first group is the "very strong" or "spectacular" acute phase proteins which usually increase in concentration by 20-1000 fold after trauma and includes CRP. The second group is the "strong" acute phase proteins which usually increase by 2-5 fold after trauma, and includes alpha-1-acid glycoprotein and fibrinogen. The third or "weak" group, increase by 30-60 percent after trauma and include caeruloplasmin and the complement factor C3. The fourth group comprises the "neutral" proteins which show no regular changes in concentration after trauma. This group includes alpha-2-macroglobulin and the immunoglobulins. The fifth and last group consists of "negative" or synthesis inhibited acute phase proteins, and includes those whose concentrations usually decrease by 30-60 percent after trauma. This group includes albumin and transferrin.

A prefix of "positive" or "negative" is often added to describe the direction in which the concentration changes. In addition, Fleck noted that there was evidence that the increases in the acute phase proteins are controlled by similar mediators, but different mediators and mechanisms operate to decrease the concentration of the "negative" acute phase proteins (Fleck A et al., 1985).

Thus the acute phase proteins are a family of approximately 30 plasma proteins produced in increased amounts by the liver in inflammation. Extra-hepatic expression of several acute phase proteins has been demonstrated, particularly in macrophage rich tissues, and the regulation of macrophage protein production may be independent of hepatic expression of the same gene. Serum amyloid A (SAA) is an example of

this phenomenon. Several cytokines, in particular IL-6, IL-1 and TNF induce their synthesis. These cytokines are discussed in Chapter 2. The increased plasma concentration of these proteins probably reflects an important physiological mechanism providing an increased supply of the proteins to the site of tissue injury, where they either modulate the inflammatory response or replenish the proteins that are rapidly consumed in the inflammatory process.

They can be grouped according to their role at the site of inflammation, for example there are scavengers (haptoglobin), inflammatory mediators (complement components) and protease inhibitors (alpha 1 antitrypsin). Others are involved in both the repair of tissue damage and the removal of products caused by tissue damage. For example CRP is an opsonin, binding onto foreign particles, and, once bound, the complex can activate complement and enhance phagocytosis. CRP also binds onto nucleosomes released from damaged cells and facilitates their removal preventing auto-antibody production. Haemoglobin, released into plasma after haemolysis, is bound by haptoglobin, and haemopexin will bind free haem groups. These complexes are rapidly cleared from the plasma. Caeruloplasmin has been found to scavenge superoxide ions and transports copper for tissue repair (Powanda MC et al., 1981), while alpha 1 acute globulin may be involved in tissue repair and modulation of phagocytic cells. Thus, although the acute phase protein response is a non specific response, many of the proteins involved play an important role in the initial control of tissue damage.

## 1.9 MEASUREMENT OF THE ACUTE PHASE PROTEIN RESPONSE

The use of measurements of cytokine production to predict outcome and monitor progress is currently under evaluation in a wide variety of

clinical settings. In most of these studies the results have been set against the more established markers of the acute phase response, namely the acute phase proteins. Marano et al looked at serum TNF in critically ill burns patients and concluded that this cytokine correlated with infection and mortality (Marano MA et al., 1990). Nijsten et al noted that IL-6 displayed a genuine relationship to the generation of fever which they called "the hallmark of the acute phase response", noting also, that this cytokine increased earlier than the acute phase proteins (Nijsten MWN et al., 1987).

The measurement of acute phase proteins can be used to diagnose the presence of inflammation and to monitor changes in inflammatory activity. Acute phase proteins can be measured either directly, or indirectly via the erythrocyte sedimentation rate (ESR) and plasma viscosity. The ESR has long been the gold standard against which other markers of inflammation have been measured. It is a measure of the rate at which red cells sediment in a column of blood and depends upon the weight, size and charge on the cells. The negative charge (zeta potential), holding healthy cells in suspension by mutual repulsion, can be neutralised if the cells are coated with positively charged polymeric plasma proteins. Amongst the plasma proteins, fibrinogen, alpha 2-macroglobulin and the immunoglobulins all have this ability. However the ESR is not specific for the acute phase response because it is altered by anaemia, blood transfusion and changes in erythrocyte morphology such as microcytosis, as well as by polyclonal and monoclonal increases in immunoglobulins. It is also altered following blood transfusion. Plasma viscosity has been developed more recently to obviate the effects of red cell changes, but this technique is also influenced by the same group of plasma proteins that affect the ESR and its role requires further evaluation.

The plasma concentrations of the different proteins vary considerably because of differential induction of synthesis by cytokines and differences in metabolism. The latter is influenced by molecular size and catabolic rate, both at the site of inflammation and in the circulation. Those that are rapidly consumed include the complement components, fibrinogen and haptoglobin which results in their being unsuitable for use as indices of inflammatory activity.

In assessing the relative values of the different acute phase proteins for practical use as non-specific indicators of disease, Crockson et al suggested that the ideal protein would be normally absent in plasma, have a wide range of quantitative response and a rapid rate of rise and fall (Crockson RA et al., 1966). They suggested CRP could satisfy these criteria. As early as 1957 Yocum and Doerner recommended its use in preference to the ESR, having found it to be always elevated in bacterial infections, acute rheumatic fever, myocardial infarction and widespread malignant disease (Yocum RS et al., 1957). They also found that CRP was commonly, though not consistently raised in rheumatoid arthritis, viral infections and acute tuberculosis. Hedlund also found that CRP was a good marker of disease and found that it was elevated in every instance where an increase in body temperature was associated with inflammation (Hedlund P, 1961). After measuring CRP, fibrinogen, haptoglobin, ESR and the alpha-globulin region of a protein electrophoretic strip in various diseases, Belfrage concluded that CRP gave the best indication of disease activity (Belfrage S, 1963).

Since then, the popularity of CRP as an indicator of disease has grown, largely because it has continued over the years to fulfil the criteria

suggested by Crockson et al for a useful non-specific indicator of disease. More recently Stahl compared the CRP response in patients undergoing surgery of increasing magnitude and his results suggested that peak levels of CRP were proportional to the degree of tissue injury. However he also confirmed that this peak level was not reached until 48 hours, which implies that CRP has little value in the most critical postoperative or post-trauma period, namely the first 24 hours (Stahl WM, 1987).

However upon closer scrutiny, Stahl's findings are perhaps not as clear as he suggests, because his results refer to mean values for the different groups, yet within each group there is a wide range of peak values making it difficult to conclude anything about tissue damage for any individual patient. This difficulty was emphasised by Colley and Myers et al who performed similar studies in surgical patients. They noted that the degree of trauma did not appear to influence the time course nor the magnitude of the CRP response to surgery. A maximal response was obtained with herniorraphy and was of the same magnitude as the concentrations reached with partial gastrectomy and cholecystectomy. Moreover the peak CRP concentration showed considerable variation between patients, for example after herniorraphy, CRP ranged from 38-196mg/l. They concluded that this variability made the single estimation of CRP unreliable as an indicator of the severity of an acute injury (Colley CM et al., 1983).

## 1.10 CONCLUSION

While the survival advantage of some components of the "acute-phase response" is unclear, there is evidence to support the proposal that certain components of the "acute-phase response" confer benefit on the host. For example, elevation of body temperature has been related to increased survival following bacterial infection in invertebrates (Kluger MJ et al.,

1979), and artificially induced hyperpyrexia (fever therapy) was a mainstay of treatment of certain bacterial infections, e.g., typhoid, in past centuries. Moreover, T and B cell activity are enhanced by fever (Jampel HD et al., 1983). Conversely, since the rate of chemical reactions *in vitro* doubles with every 10°C rise in temperature, pyrexia may well contribute to the catabolic state of the critically ill host. Similarly, following injury or sepsis, systemic hypotension and diminished intravascular volume frequently occur and are associated with diminished perfusion of vital organs.

Activation of the sympathetic nervous system and adrenal medullary hormones, in such circumstances, may be essential for restoration of adequate blood flow to vital organs. However, chronically elevated circulating concentrations of catecholamines and glucocorticoids are associated with adverse metabolic alterations, including hyperglycaemia, increased peripheral insulin resistance and accelerated proteolysis (Bessey P et al., 1984). Death following critical illness has also been related to the extent of depletion of catecholamine stores from autonomic ganglia (Goodall M et al., 1965).

Certain other components of the "acute-phase response" have more obvious benefits to the host. Alterations in circulating white cell populations represent a well recognised component of the integrated immunological response of the host to invasion, and failure of this response to occur, for example following certain types of chemotherapy for malignant disease, is associated with greatly increased mortality following bacterial and other infections (Talcott J et al., 1988). Similarly, the marked decrease in circulating iron concentrations associated with host invasion by Gram-negative organisms enhances survival since replication of such

organisms is inhibited when the availability of iron is reduced (Powanda M, 1977).

The acute phase responses that occur in infected or injured animals have been strongly conserved throughout evolution, suggesting that they are indeed important for host survival. Nonetheless, it is far from clear that all components of the response are invariably beneficial to the host. An increased understanding of the reasons why these responses occur, and of the mediators involved in the induction of such changes, is important if future strategies to modify the course of critical illness in human beings are to be developed.

# CHAPTER TWO

# MEDIATORS OF THE INTEGRATED HOST RESPONSE

# CHAPTER TWO

## MEDIATORS OF THE INTEGRATED HOST RESPONSE



### 2.1 INTRODUCTION: A mediator cascade

The host's responses to trauma or infection are orchestrated by a range of mediators activated by pain, tissue damage, infection or organ failure. As the patient progresses from shock to the acute phase and on to convalescence, so the mediators change. There is an enormous variety of these mediators ranging from those which act only locally at their site of production, to those which evoke responses affecting the whole body, of which the neuro-endocrine hormones are an obvious example. Various cytokines cover this whole spectrum of action (Figure 2.1).

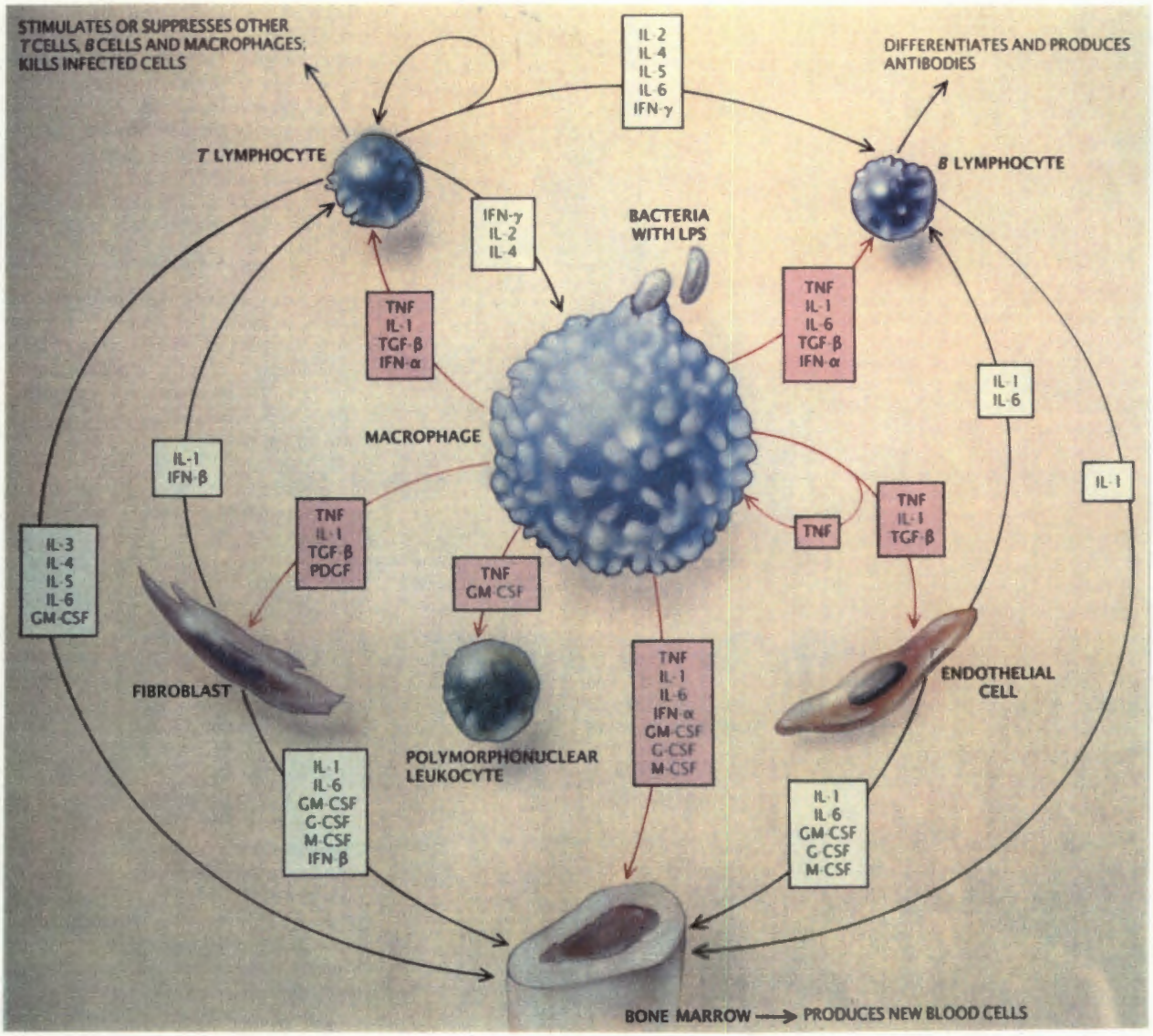



Figure 2.1

*Cytokines constitute the molecular language of inflammation and immunity*

*They form a complex network of overlapping and interacting signals, which together propagate the mediator cascade and orchestrate the integrated host response*

### 2.1.1 Pain and neuro-endocrine mediators

Pain serves at least two major functions, it warns of tissue injury and it indirectly activates neuro-endocrine mediators. Afferent impulses from sensory end-organs of skin, parietal peritoneum, bone, and other peripheral tissues are transmitted via somatic nerves, whereas impulses from most visceral end-organs are carried by sympathetic nerves (White JC et al., 1952).

The paraventricular and medial ganglia of the hypothalamus are activated as impulses are transmitted to the sensory cortex, where the sensations of pain are recognised. Stimulated hypothalamic ganglia induce autonomic nervous activity (Davies CL et al., 1984). Hypovolaemia, hypotension, and hypoxia, recognised by baroreceptors and chemoreceptors in the brain, great vessels, atria and carotid bodies, also induce autonomic nervous system activity (Hume DM et al., 1959). In addition to activation of paraventricular hypothalamic nuclei, these same stimuli induce the cells of the median eminence to secrete releasing hormone into capillary blood of the pituitary portal system which perfuses the anterior pituitary. In response to these releasing hormones, specialised cells secrete a variety of hormones including adrenocorticotrophic hormone (ACTH). Both neural and hormonal feedback systems modulate the neuro-pituitary-adrenal responses (Eiseman B et al., 1977)(Gann DS et al., 1977).

The use of post-operative epidural anaesthesia was shown to attenuate the neuro-endocrine and catabolic changes following major abdominal surgery (Kehlet H et al., 1979) and interruption of pain sensation, by denervation or cord section, inhibits both catecholamine and ACTH secretion (Hume DM et al., 1959). Thus initial studies suggested that these neuro-afferent signals from the injury site were primarily responsible for

the pain, fever, cardiovascular changes and activation of the pituitary/adrenal axis.

However, other investigators denervated superficial wounds or utilised spinal anaesthesia to block nervous afferent impulses and found that both manipulations had little effect on hypermetabolic responses (Wilmore D et al., 1975). Moreover, neither the responses of the sympathetic nervous system nor the anterior pituitary to bacteraemia or endotoxinaemia, are affected by cord section. This evidence, therefore, suggested that immunological or other cellular mediators are capable of stimulating the sympathetic and pituitary mechanisms of hormone secretion and mediating post-traumatic responses.

Another complex series of hormonal responses to hypotension, hypovolaemia, and inadequate perfusion involve release of vasopressin from the posterior pituitary, renin from the kidney and aldosterone from the adrenal cortex. Insulin is the principal hormone which promotes energy storage and protein synthesis. In post-traumatic or septic patients, the blood insulin concentration may be high or low. In septic or severely injured patients high blood glucose concentrations have been observed despite elevated blood insulin concentrations. Insulin resistance has been postulated (Kahn CR, 1978) as a cause of this phenomenon. On the other hand, the probable mechanism of the hypoinsulinaemia observed in shock, is intense sympathetic activity. Several hormones antagonise the actions of insulin by inducing glycolysis, gluconeogenesis, and lipolysis. Also known as counter-regulatory hormones, they include glucagon, cathecholamines and glucocorticoids.

### 2.1.2 Other non-cytokine mediators

In addition to those which act either locally or systemically, mediators may play both a local role in establishing the inflammatory response at the site of injury and also have a systemic effect. Histamine, prostaglandins, leukotrienes and products of the classical cascades of coagulation-fibrinolysis, kallikrein-kinin and complement are good examples of this type of mediator. In addition to their direct effects, these components stimulate a wide variety of cells involved in host defence such as mast cells, fibroblasts, neutrophils, monocytes and macrophages. Subsequently these cells, in addition to their direct activities (phagocytosis, tissue repair and the release of lysosomal and other chemicals such as histamine), secrete polypeptide mediators which initiate the systemic acute phase response. Thus mediation of the integrated host response can be conceptualised as a tiered response which is illustrated in Figure 2.2.

## 2.2 MEDIATOR STUDIES: Animal versus human

The laboratory animal has been essential in medical research and has enabled increased understanding of the mechanisms of human disease. Additionally, studies involving lethality to the recipient of administered agents can clearly only be performed using this approach. However, studies in laboratory animals of the mediators of the integrated host response to infection and injury are often unsatisfactory for several reasons.

1) There is a marked inter-species variation in the susceptibility of animals to the effects of agents such as endotoxin or cytokines. Laboratory rodents are much less susceptible to these agents than human beings (Wolff S, 1973).

2) Small animal models are intrinsically limited in the extent to which accurate haemodynamic and other measurements can be made, and the

extent to which blood can be sampled. These issues have been extensively reviewed elsewhere (Appelgren K et al., 1986).

3) Most of the recombinant cytokines currently available for research are identical to human cytokines. It has been shown that there is a marked inter-species specificity not only in relationship to the biological effects of these agents, but also in their cytokine-receptor interactions (Smith R et al., 1986). Similarly, most of the assay systems available to measure cytokines will only measure human-derived moieties.

4) Ethical considerations also limit animal experimentation and this applies particularly in the United Kingdom. In general, a major insult may not be inflicted upon a conscious animal and it must be sacrificed before reversal of anaesthesia. Therefore burn and multiple injury animal models cannot be easily created and can only be studied for a very short period after insult.

5) Finally, many studies *are* amenable to human research and the value of performing them first in animals is often debatable.

For these reasons, several groups of investigators have focused their studies on human subjects, albeit with the considerable limitations that this approach sometimes necessitates. For these reasons also, all work contained in this thesis describes human studies.

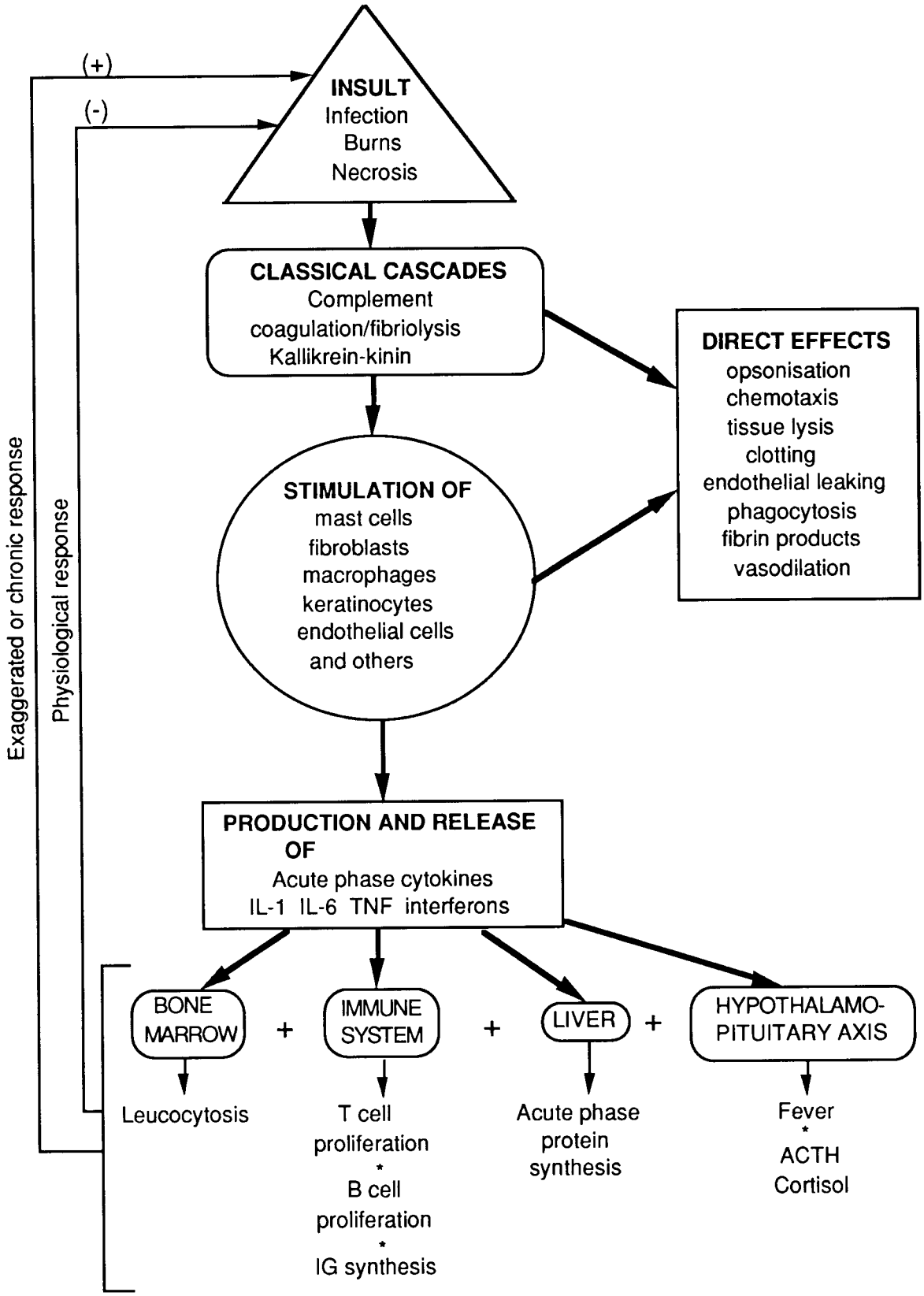


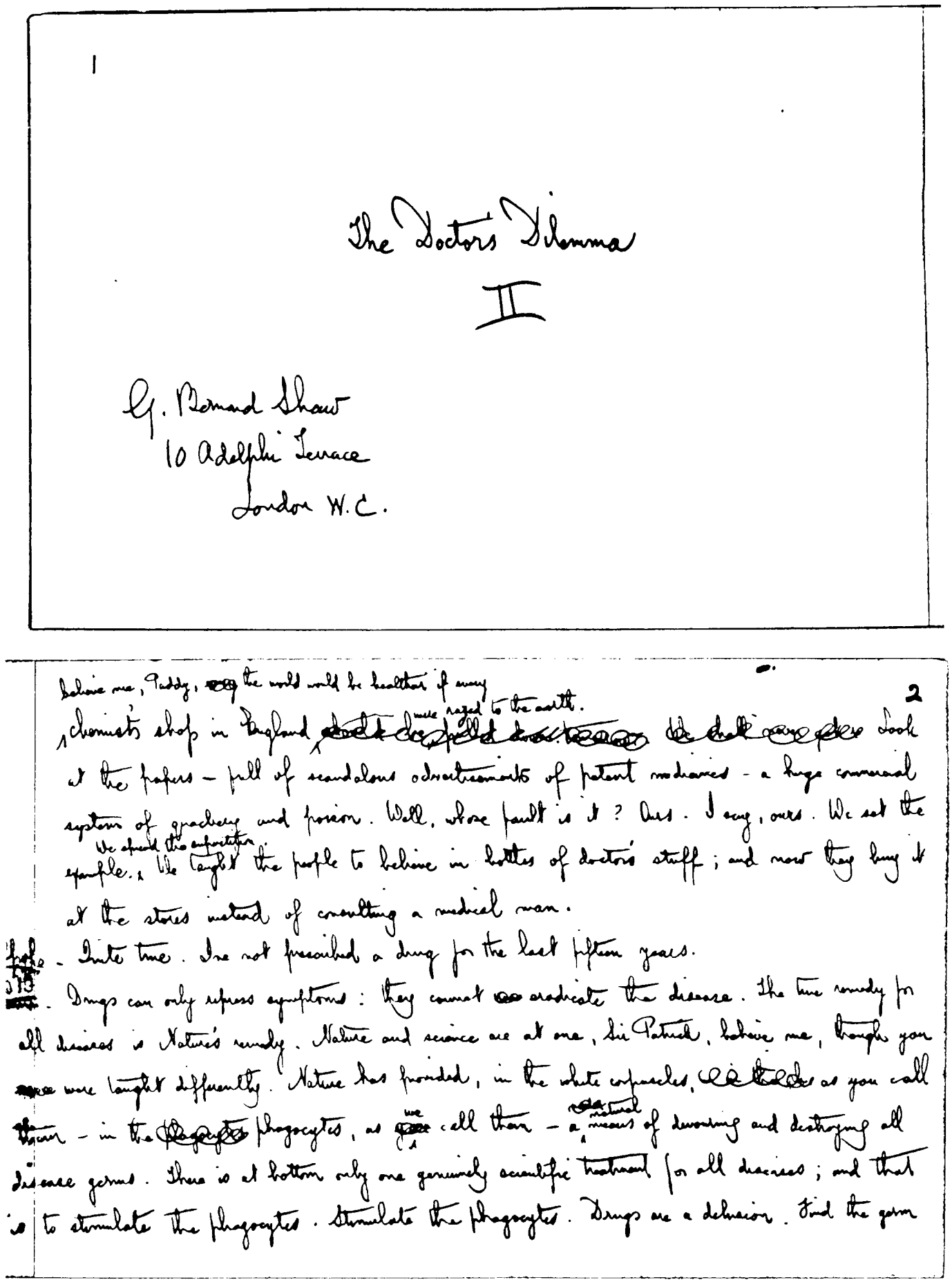

Figure 2.2 Mediation of the integrated host response - a cascade phenomenon.

## 2.3 LYMPHOKINES TO MONOKINES TO CYTOKINES

### 2.3.1 A brief history

The classical concept enunciated by Metchnikoff at the turn of the century , placed phagocytosis and digestion by the mononuclear-phagocyte system as the key to host defence (Metchnikoff E, 1905). Sir Almoth Wright, following up Metchnikoff's work at St Mary's Hospital in London, coined the termed "opsonin" when he observed that "the white corpuscles or phagocytes only attack and devour disease germs...when we butter the germs with a natural sauce (opsonin)". The enthusiasm this doctrine aroused even reached the theatre stage, where in George Bernard Shaw's "The Doctor's dilemma" performed in 1906, Sir Ralph Bloomfield Bonnington declared "There is at bottom only one genuinely scientific treatment for all diseases; and that is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion." (Figure 2.3) (Shaw GB, 1906). Shaw was an acquaintance of Sir Almoth Wright. However the discovery that macrophages have another important defensive function, namely the production of inflammatory mediators, resulted from some fascinating studies in the late 1970s.

The first study took advantage of a spontaneous mutation in a strain of mice. They developed near immunity to the effects of Gram-negative organisms and their endotoxins. When these mice were irradiated and given bone marrow from normal mice, sensitivity to the effects of endotoxin, including death, was conferred (Michalek S et al., 1980). Furthermore, the infusion into the resistant strain of serum from endotoxin treated mice, also resulted in a syndrome similar to endotoxaemia (Kawakami M et al., 1982). Thus endotoxin was mediating its effects via a serum factor synthesised by a bone marrow cell. At about the



1

The Doctor's Dilemma

II

G. Bernard Shaw
10 Adelphi Terrace
London W.C.

believe me, Paddy, the world would be healthier if many
chemist's shop in England were razed to the earth. 2
at the papers – full of scandalous advertisements of patent medicines – a huge commercial
system of gracherey and poison. Well, whose fault is it? Ours. I say, ours. We set the
example. We taught the people to believe in bottles of doctor's stuff; and now they buy it
at the stores instead of consulting a medical man.
– Quite true. I've not prescribed a drug for the last fifteen years.
– Drugs can only repress symptoms: they cannot eradicate the disease. The true remedy for
all diseases is Nature's remedy. Nature and science are at one, Sir Patrick, believe me, though you
were taught differently. Nature has provided, in the white corpuscles, as you call
them – in the phagocytes, as we call them – a natural means of devouring and destroying all
disease germs. There is at bottom only one genuinely scientific treatment for all diseases; and that
is to stimulate the phagocytes. Stimulate the phagocytes. Drugs are a delusion. Find the germ

Figure 2.3 A copy of the original draft of "The doctor's dilemma" written by George Bernard Shaw in 1905-6.

same time, Vogel was studying congenitally athymic or B lymphocyte depleted strains of mice, showing that neither T nor B cells affected the mediation of endotoxicity (Vogel S et al., 1979a)(Vogel S et al., 1979b), thus implicating the monocyte/macrophage. The circle was completed when the sensitivity of these animals to endotoxin, was shown to be enhanced by the administration of macrophage stimulants like glucan, zymosan or bacillus Calmette Geurin (BCG) (Di Luzio N et al., 1980). The existence of monocyte/macrophage-derived serum factor, a so-called second messenger, was therefore confirmed.

It had now become clear that macrophages and other cells of the reticuloendothelial system, in addition to clearing bacteria, endotoxins and other noxious agents from the circulation, produced circulating factors which modified the host response to injury or infection. It is interesting to reflect that while Metchnikoff and others at the beginning of the twentieth century were expounding the importance of phagocytosis as the key mechanism of host defence, Walter Cannon was recording clinical observations about shock at the battlefront, that hinted at the concept of inflammatory mediators.

> *No doubt hemorrhage may have an important part in the development of this state and infection likewise is important; but there are recorded cases in which both hemorrhage and infection were absent or slight and in which, nevertheless, shock was observed. These facts of observation have led to the view that beside hemorrhage and infection there is an unknown factor at work.*
>
> Walter Cannon 1919

Walter B Cannon was part of a team of British and American basic scientists and clinicians sent to France in 1916 to study front-line casualties as soon as possible after they were wounded. Problems that were observed at the front were recreated in controlled experiments in established laboratories in England and America. Much was learnt about the "state of shock" and the findings were finally collated by Dr Cannon in the classical monograph *Traumatic Shock*, published in 1923. Interestingly, Cannon himself did not favour the term shock, which was introduced in error to the English language by an unknown translator of Le Dran's 1743 treatise on gunshot wounds, who used it to translate the French "choc" which was used in the context of an impact, a blow or a jolt. Cannon and others felt the word was not informative and he suggested "exaemia", but none of the many suggestions have ever replaced the short and convenient term "shock".

The concept of soluble mediators became more clearly defined as a result of the classic work of Sir Henry Dale who investigated the role of histamine in acute inflammation (Dale HH, 1929)(Dale HH, 1933).

He noted

> *"The discovery in artificial extract from an organ or tissue of a substance which on artificial injection produces a pharmaco-dynamic effect, provides only a first item of presumptive evidence in support of a theory that the action of this substance plays a part in normal inflammation."*
>
> Sir Henry Dale 1933

and suggested

> *"What was needed was a system of criteria or postulates (comparable to Koch's celebrated postulates dealing with the*

*aetiological significance of a microorganism), representing conditions which must be fulfilled before physiological significance was attributed to the effects produced by the artificial application of an organ extract."*

Sir Henry Dale 1929

Over the course of the next 50 years many mediators would be described, but in the rush to describe immune mediators and mediators of intercellular communication, Dale's sage observations would be ignored. Numerous soluble factors were described purely on the basis of a pharmacodynamic effect resulting from an artificial injection of an artificial extract such as culture supernatant, into a laboratory animal.

In 1943, during investigations into mechanisms of fever, Menkin isolated a substance from inflammatory exudates that caused fever when injected intravenously into rabbits (Menkin V, 1943). This substance was called pyrexin. However the possible presence of endotoxin contamination was a criticism of the early work on pyrexin until several years later, when Beeson extracted a fever-promoting substance, which was distinct from endotoxin, from suspensions of rabbit leucocytes containing predominantly polymorphonuclear leucocytes (Beeson PB, 1948). This fever promoting substance became known as endogenous pyrogen (EP) (Wood WB Jr, 1958). Endogenous pyrogen has been shown to be released from the leucocytes of many species, both mammalian and non-mammalian.

Then came the discovery that cell-free soluble factors in culture supernatants of sensitised lymphocytes incubated with antigen, could (1) cause macrophage migration inhibition (Bloom BR et al., 1966), (2)

produce skin lesions similar to delayed type hypersensitivity (Bennett B et al., 1968) and (3) be mitogenic for lymphocytes (Kasakura S et al., 1970). These findings suggested that molecular mediators were involved in cellular immune responses. More and more crude culture supernatants were shown to influence the *in vitro* behaviour of a wide variety of target cells in many different ways, leading to the view that many cellular immune interactions were regulated by soluble factors.

The term "lymphokine" was introduced in 1969 and was chosen to emphasize the origin of these soluble factors (lymphocytes) and also their role in the physiology of the immune system (kinesis) (Dumonde DC et al., 1969). However during the 1970s the term became more widely used to describe the large number of biological activities assumed to be the properties of factors in the culture supernatants. Thus lymphokines were named on the basis of the activity they produced *in vitro* and their names abbreviated to acronyms. Similar activities were found in body fluids such as serum and urine and terms such as "monokine", "cytokine" and "interleukin" were used in an attempt to clarify these factors on the basis of their cellular origin or function.

In the early 1970s, Kampschimdt et al and Wannemacher et al described a leucocyte derived polypeptide called leucocytic endogenous mediator (LEM) which could promote most aspects of the acute phase response in animals after intravenous or intraperitoneal injection (Kampschmidt RF et al., 1973)(Wannemacher RW Jr et al., 1975). This mediator was shown to promote fever, granulocytosis, hypoferraemia, hypozincaemia, hyper-cupraemia and induce the synthesis of several acute phase proteins in rats and rabbits. Leucocyte endogenous mediator was shown to have a molecular weight of 14 000 and an isoelectric point of pI 7-7.6, and

therefore to be similar, if not identical, with EP (Kampschmidt RF, 1981). Meanwhile significant advances were made in studies of mediators involved in humoral and cell mediated immunity. Gery et al described a soluble product necessary for T lymphocyte activation, which was released by activated macrophages. This factor was called lymphocyte activating factor (LAF) (Gery I et al., 1972a)(Gery I et al., 1972b).

Not surprisingly, scepticism arose about the very existence of lymphokines, because the large number of activities, their disparate sources and their lack of chemical characterisation were enough to suggest that the whole concept was based on artefact. However the advent of gene cloning and the development of improved protein purification and sequencing techniques, resulted in a rapid improvement in the characterisation of lymphokine structure. From this it became clear that a number of biological activities were actually different effects of the same substance and eventually an attempt was made to clarify the field at an International Lymphokine Workshop in 1979, where more than 100 factors with bioassay based names were reviewed. A number of factors were found to be identical, for example, LAF, LEM, EP, B cell Differentiation Factor, B cell Activating Factor and Mitogenic Protein were all shown to be one and the same and the term interleukin-1 was agreed to describe them all (Aarden LA et al., 1979). Dinarello described IL-1 as a single molecule or a family of closely related molecules, because the preparations used were not pure and probably contained many contaminants (Dinarello CA, 1984).

Originally it was assumed that the vast majority of these polypeptide mediators originated from macrophages and lymphocytes, but in the last few years it has become increasingly apparent that a wide variety of cells

are able to produce these substances which, in turn, act on many different cell types. For this reason there is growing acceptance of the term "cytokine" rather than "lymphokine" or "monokine". Two important developments of the past few years have helped clarify the role of these factors in the immune response: (1) the availability of recombinant cytokines has enabled the activities of the individual polypeptides to be more accurately defined and (2) the development of monoclonal antibodies against individual cytokines (often several different monoclonal antibodies against different epitopes of the same cytokine) has improved the specificity of both cytokine bioassays and immunoassays and also facilitated the purification of these peptides.

### 2.3.2 Cytokines - a definition

Cytokines, which are polypeptides of 100-200 amino acids, are released by haemopoietic and non-haemopoietic cells and have multiple actions on multiple target cells. They consist of the interleukins (IL-1 to IL-10), the interferons, the tumour necrosis factors and the colony stimulating factors and these cytokines play essential roles in normal immune and inflammatory responses. They act as intercellular mediators not only between different leucocytes but also between leucocytes and other cells of the body.

Of the various cytokines, several share the ability to stimulate or augment cell proliferation and these act locally in an autocrine or paracrine fashion to recruit and activate cells at sites of inflammation.

Other cytokines, notably IL-1, TNF and IL-6, are able to initiate the synthesis of new proteins in a variety of cells and induce the production of inflammatory metabolites. They are biologically similar and considerable

interest has focused on them as mediators of systemic acute phase responses. They are sometimes collectively known as "the acute phase cytokines". Injecting experimental animals with either IL-1 or TNF results in fever, hypozincaemia, hypoferraemia, increased hepatic acute phase protein synthesis and other manifestations of the integrated host response. Injection of IL-6 induces fever and hepatic acute phase protein synthesis.

### 2.3.3 Interleukin-1

Structure and synthesis: Two forms of IL-1 have been cloned, IL-1α and β which correspond to the previously described acidic (pI=5) and neutral (pI=7) forms. Although they are distinct gene products with only limited amino acid homology (30 per cent) (March CJ et al., 1985), they share the same biological properties and recognise the same receptor on the surprisingly large variety of target cells including T and B lymphocytes, neutrophils, macrophages, fibroblasts, chondrocytes, epithelial, endothelial, muscle cells and even some neurons (Dinarello CA, 1989b).

Interleukin-1 is synthesised predominantly by blood monocytes and tissue macrophages, but is also produced by a variety of other cell types including endothelial cells, keratinocytes, neutrophils, Langerhans cells, smooth muscle cells, renal mesangial cells, astrocytes, microglia and B lymphocytes. The amount of IL-1 secreted depends upon the cell type and the conditions of stimulation, but the monocyte/macrophage appears to be the cell best equipped to secrete IL-1 (Auron PE et al., 1987).

Antigens may induce IL-1 production either directly by involvement of cell to cell contact, or indirectly by releasing other cytokines from activated T cells, particularly IL-2, interferon gamma (IFNγ) and the colony stimulating factors, which themselves stimulate IL-1 production.

Antigenic stimuli that result in IL-1 production include viruses, bacteria, and directly acting cytokines, for example, IL-1 itself, TNF, granulocyte-macrophage colony stimulating factor and IFNγ. Factors known to downgrade IL-1 production include corticosteroids, IL-4, $PGE_2$, fever and malnutrition.

Both forms are synthesised as 31 kilodalton precursor peptides and both are also readily degraded to their 17 kilodalton form. Neither has a signal peptide sequence, resulting in a considerable amount of synthesised IL-1 remaining cell-associated, with IL-1α being predominantly membrane bound and 80 per cent of IL-1β being retained intracellularly in precursor form. However, human monocytes stimulated with endotoxin produce approximately 10-fold more IL-1β than IL-1α. Therefore, a substantial amount of IL-1β is, in fact, released from cells while IL-1α remains cell associated (Dinarello CA et al., 1989a). Cell death and lysis results in sufficient protease activity to process, spontaneously, the IL-1β precursor into its lower molecular weight active form.

Biological effects: Both forms are equipotent and have circulating half-lives of 8-10 minutes (Newton RC et al., 1988). Interleukin-1 augments T, B and natural killer cell responses, including the production of other cytokines (Shalaby MR et al., 1989). It induces the slow wave sleep pattern (Shoham S et al., 1987) and anorexia (Oomura Y, 1988) seen during illness, and attenuates the perception of pain after injury or inflammation by increasing the release of beta endorphins and the number of opiate-like receptors in the brain (Wiedermann CJ, 1989). It evokes systemic acute phase responses including fever, by stimulating a local release of prostaglandins in the anterior hypothalamus (Walter JS et al., 1989), hepatic acute phase protein synthesis (see Chapter 1), hypoferraemia,

hypozincaemia (Goldblum SE et al., 1987) and increased levels of hormones including glucocorticosteroids and insulin. It promotes myelopoiesis by inducing colony stimulating factors resulting in a leucocytosis (Ulich TR et al., 1987). In addition to its inflammatory mediating and immuno-enhancing effects, IL-1 is also a growth promoter of several types of cells, including fibroblasts and vascular smooth muscle cells (Schmidt J et al., 1984). Further properties of this cytokine are listed in Table 2.1.

This pluripotent peptide exerts important beneficial influences during injury but inappropriate synthesis of IL-1 maybe harmful. At high dose it induces hypotension and a "shock-like" state (Okusawa S et al., 1988). Chronic release of IL-1 has also been implicated in the pathogenesis of rheumatoid arthritis, atherosclerosis and cancer cachexia.

Technical considerations: Much early work reporting the presence of IL-1 in various samples has been discounted. In the past, preparations of IL-1 were often contaminated with lipopolysaccharide which confused the specificity of any response observed. In addition, early bioassays relied on the ability of this protein to stimulate proliferation of lymphocytes. However numerous more recently discovered cytokines are also mitogenic for T cell blastogenesis, moreover these cytokines may also induce IL-1 production. It is not suprising, therefore, that recent studies using sensitive immunoassays (radioimmunoassays and ELISA), have failed to confirm the frequent appearance of IL-1 in the circulation of individuals with inflammation that was reported in the original bioassays (Fong Y et al., 1990a).

IL 1 receptor antagonist (IL-1ra): The discovery of this cytokine, first reported in 1985 (Arend W et al., 1985) but only recently purified

(Hannum CH et al., 1990) and cloned (Eisenberg SP et al., 1990) by the same group in Colorado, may have important clinical consequences. It is produced by both monocytes and tissue macrophages, indeed the same population of human monocytes produces both the agonists IL-1α and IL-1β as well as IL-1ra. This fact supports the view that IL-1ra is a physiologically significant regulator of IL-1 and that an appreciation of its properties will be essential to understand the function of IL-1 (Hannum CH et al., 1990). However, what stimuli induce and whether other cells produce IL-1ra has yet to be determined.

Interleukin-1 receptor antagonist appears to be a pure receptor antagonist, not activating target cells. Target cells are highly sensitive to IL-1, therefore excess amounts of IL-1ra are required to flood the system and block IL-1 activity (Arend WP et al., 1990a). Nonetheless there have been several studies suggesting that IL-1ra may be important in regulating the inflammatory response. Administration of endotoxin to human volunteers led to measurable IL-1ra activity in plasma (Spinas GA et al., 1990), while a Swedish study reported a reduction in the lethality of endotoxin-induced shock in rabbits (Ohlsson K et al., 1990). Similarly, inflammatory responses in mice, including the IL-6 response, were attenuated by IL-1ra (Gershenwald JE et al., 1990).

The discovery of IL-1ra will facilitate a better understanding of the biological actions of IL-1, even though its own role in normal physiology remains uncertain. However, its discovery and these early studies have led to the assumption that this molecule may function *in vivo* to regulate the pleiotropic extracellular effects of IL-1 in physiological or pathological processes (Arend WP, 1990b).

### 2.3.4 Tumour necrosis factor (cachectin)

As its names suggest, this cytokine's role in inflammation was discovered after its metabolic and anti-cancer properties. During the 1890s, Dr William Coley (Figure 2.4), an assistant surgeon at The Hospital for Ruptured and Crippled (now the Memorial Sloan-Kettering Hospital) in New York, deliberately infected tumour-bearing patients with various killed bacterial preparations (Coley's toxins) and demonstrated that local tumour regression was obtained with these agents (Coley W, 1893)(Coley W, 1906).

*"I innoculated a case of recurrent sarcoma of the neck and tonsil...which at operation had proved too extensive to remove. Five decigrammes of a fresh culture of streptococcus erysipelatis were injected into the tumour substance...Within an hour he had severe pain, nausea, vomiting and a chill...his temperature rose to 105°...and the erysipelas ran the usual course. At the end of ten days the tumour began to break down..and discharged. ...At the end of two weeks the neck tumour had disappeared...The patient continued well for eight years."*

William Coley 1906

Coley and others believed that cancer was caused by a parasite. This was because microbiologists claimed to be able to demonstrate the constant presence of protozoa in a very large number of cancer specimens. Therefore Coley explained his results thus: *"...The toxic products of the erysipelas streptococci ...bring about such changes in the blood-serum as to destroy the parasite of cancer."*

Figure 2.4 William Coley.

Subsequent work made it clear that the active agents in Coley's preparations were bacterial endotoxins (Shear M et al., 1943). In 1934, the American Medical Association listed Coley's toxins as the only recognised systemic treatment for cancer (Merz B, 1986). However the results had been inconsistent, and radiotherapy and chemotherapy soon supplanted Coley's approach.

During the 1930s and 1940s researchers confirmed that injection of live or killed Gram-negative bacteria could cause haemorrhagic necrosis of mouse tumours and lipopolysaccharide or endotoxin was identified as the

active component of Gram-negative bacteria. Dr Lloyd Old and colleagues at the Memorial Sloan-Kettering Hospital, New York noted that blood from endotoxin treated mice induced haemorrhagic necrosis in tumours of other animals and was highly toxic to cancer cells *in vitro*. They correctly concluded that the mice had produced large amounts of an anti-tumour factor which was responsible for the cancer killing and called it tumour necrosis factor (Carswell E et al., 1975). Subsequently, this protein was sequenced and found to be a protein with a molecular weight of 17 000 kilodaltons (Aggarwal B et al., 1985).

Over the same period, Beutler and Cerami and others at the Rockefeller University in New York, had found a factor produced by activated macrophages that inhibited lipoprotein lipase, an enzyme essential for the normal storage of fat, and which was suppressed in infected cachectic mammals (Kawakami M et al., 1982). They called this factor "cachectin". However when this protein was isolated and characterized, its amino acid sequence proved indistinguishable from that of tumour necrosis factor (Beutler B et al., 1985).

Synthesis and structure: Tumour necrosis factor, a product of stimulated monocytes and macrophages, is also produced by lymphocytes, endothelial cells and keratinocytes. It has a molecular weight of 17 kilodaltons and a pI of 5.3 (Aggarwal B et al., 1985). Tumour necrosis factor is translated as a 233 amino acid precursor which undergoes proteolysis to become the active, 157 amino acid TNF polypeptide (Beutler B et al., 1987). It has a circulating half-life of 14 to 18 minutes (Blick M et al., 1987). Lymphotoxin, a structurally related polypeptide sometimes known as TNFβ, is produced in smaller quantities by activated T cells.

Biological properties: These are remarkably similar to IL-1. Tumour necrosis factor modulates immune function by activating neutrophils and lymphocytes and induces the synthesis and release of immunostimulatory polypeptides like IL-1 and IL-6 from both immune and accessory cells (Shalaby MR et al., 1989)(Dinarello CA et al., 1986). In addition, TNF initiates neutrophil margination, transendothelial migration and degranulation (Shalaby MR et al., 1985). Nearly every non-immunological biological property of IL-1 has been observed with TNF including fever, mediated by this cytokine's ability to stimulate hypothalamic prostaglandin synthesis (Dinarello CA et al., 1986). Both molecules induce fibroblast proliferation and collagen synthesis as well as bone and cartilage resorption (Kohase M et al., 1986) (Table 2.1).

Hepatic amino acid uptake (Warren RS et al., 1987), acute phase protein synthesis and complement component synthesis are increased, while albumin synthesis is decreased by TNF (Moldawer LL et al., 1987). This polypeptide exerts a direct effect on carbohydrate metabolism in muscle cells and may represent an early signal for anaerobic glycolysis within somatic tissues (Lee DM et al., 1987), while further catabolism is induced via muscle protein wasting (Flores EA et al., 1989). Thus from a metabolic point of view, TNF is primarily catabolic while nonetheless shifting the metabolic processes of the liver to anabolism.

Tumour necrosis factor produces hypotension, leukopaenia and local tissue necrosis (Tracey K et al., 1986) and is more potent than IL-1 in producing shock (Okusawa S et al., 1988) which is probably a reflection of its effects on the vascular endothelium. The acute, exaggerated secretion of TNF is thought to be pathogenic in the cardiovascular collapse, shock and death associated with severe endotoxaemia or infection. Pathological

findings, similar to those seen in septic shock, are seen in animals infused with high doses of TNF, including adrenal necrosis, pulmonary congestion and caecal necrosis (Tracey K et al., 1986). However, the most convincing evidence for the role of endotoxin in endotoxic shock was seen when a TNF antiserum was shown to be protective against endotoxin infusion in rabbits (Mathison JC et al., 1988) and a prophylactic monoclonal antibody protected baboons against the consequences of a lethal dose of bacteria. Furthermore this blockade attenuated the appearance of IL-1 and IL-6 providing evidence for the synergism of these cytokines in mediating the acute phase response (Fong Y et al., 1989a). Further properties of this cytokine are listed in Table 2.1.

Technical considerations: Depending on the assay used, TNF is or is not detectable in the plasma of healthy individuals. This is probably because some assays measure both free TNF and TNF already bound to its antibody, while others measure only the free (and therefore, it is thought, clinically relevant) form. The TNF assay used in this thesis is of the latter type. In addition, studies as recently as 1989, have been devalued because heparin was used as the anticoagulant and this has been shown to stimulate TNF release (Freeman R et al., 1990). Finally, and for reasons that are not known, high levels of TNF appear to be a feature of some healthy people.

### 2.3.5 Interleukin-6

Interleukin-6 is probably unique among cytokines, because it was cloned inadvertently, in 1980, long before the discovery of its major biological activities (Weissenbach J et al., 1980). It was called interferon-b2 because it showed some anti-viral activity. During the next ten years a variety of other teams were independently involved in trying to identify various

growth and differentiation factors, so that by 1988 these groups, all of whom assumed they were involved in the identification of unrelated factors, came to the unsettling conclusion that they had cloned or purified the same protein. The primary amino acid sequence is the ultimate criterion used to assign an interleukin number to a substance and the term IL-6 was proposed when the nucleotide sequences of all these factors were determined and found to be identical (Van Damme J et al., 1987)(Poupart P et al., 1987). The term 'Interleukin-6' therefore replaced approximately 10 different names and resulted in a remarkable simplification of cytokine nomenclature:

IFNb2 = 26K = BSF-2 = IL-HPI = HGF = HSF = CDF = **IL-6**.

Structure and synthesis: This 212 amino acid polypeptide has a molecular weight of 21-28 kilodaltons depending on its cellular source and includes a hydrophobic signal sequence (Hirano T et al., 1986). The gene for human IL-6 has been mapped to chromosome 7 (Sehgal PB et al., 1987). Interleukin-6 is produced by an extraordinary variety of cells including fibroblasts, hepatocytes and kupfer cells, microglial cells, astrocytes, endothelial cells, keratinocytes, monocytes/macrophages, T and B cells, anterior pituitary folliculostellate cells, mast cells, endometrial stroma cells, pancreatic beta cells and some tumour cell lines (Wolvekamp MCJ et al., 1990). However accessory cells are undoubtedly the major source of IL-6 in freshly isolated cell preparations (Aarden LA et al., 1987). The plasma clearance of IL-6 is biphasic with a rapid first phase followed by a slower, 55 minute, disappearance phase.

Biological activity: The improved discrimination of cytokine activities, resulting from the advent of cytokine immunoassays and improvements

in bioassays, has demonstrated that several activities, formerly ascribed to IL-1, are in fact the result of the combined action of IL-1 and IL-6 or even IL-6 alone (Figure 2.5). For example, the original IL-1 assay system of murine thymocyte costimulation partially responds to IL-6, resulting in many actions of IL-6 being originally ascribed to IL-1. However, while many of the functions of IL-6 are overlapping or synergistic with IL-1 and TNF (Table 2.1), it is of singular importance in the induction of hepatic acute phase protein synthesis.

For many years researchers have attempted to identify the mediators of the hepatic acute phase response. Experiments *in vivo* could not provide unequivocal answers because of the possibility of second messengers produced by other cells, so hepatoma cell lines became the established investigation model. Although IL-1 and TNF were reported to induce this response in certain cell lines, neither was capable of inducing the full spectrum of proteins, in particular, C reactive protein. The missing cytokine turned out to be IL-6 in the guise of hepatocyte stimulating factor (HSF), originally described in 1983 (Gauldie J et al., 1987). The recognition that IL-6 is the most potent mediator of APP synthesis has been confirmed by the observations that it induces acute phase proteins *in vivo* (Geiger T et al., 1988) and is the only cytokine capable of inducing the full spectrum of acute phase protein synthesis in human hepatocytes (Castell JV et al., 1989).

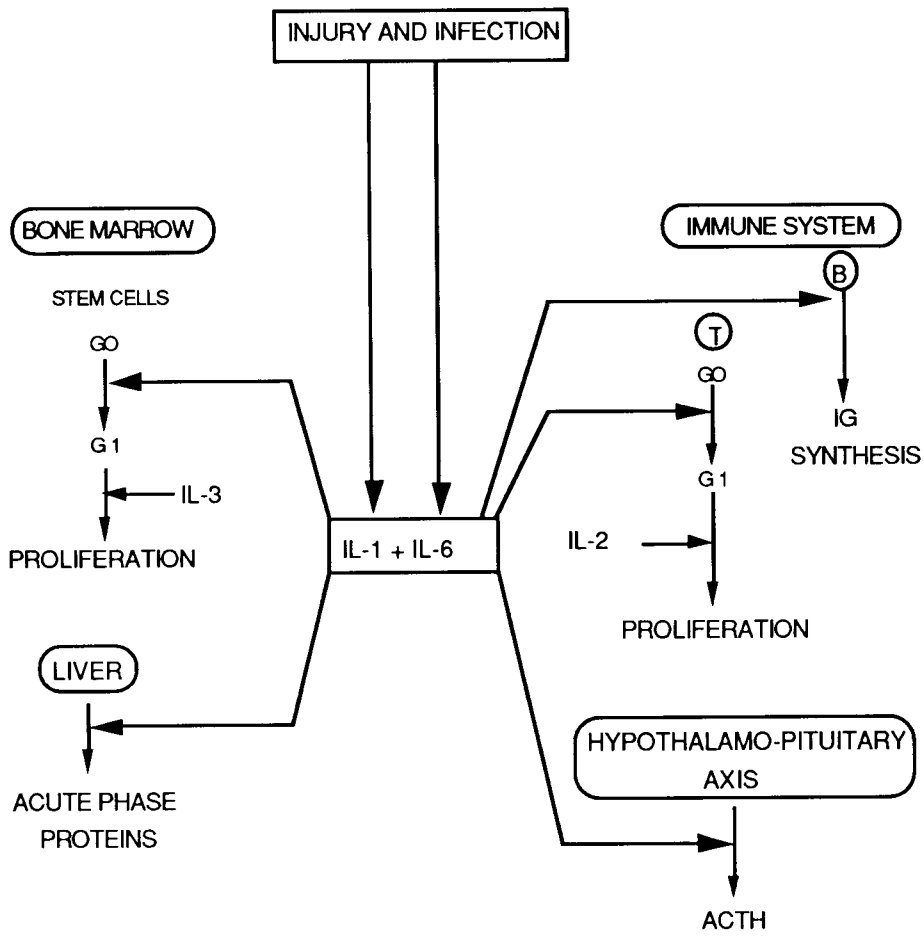



Figure 2.5 The coordinated and synergistic response to insult of IL-1 and IL-6 (after van Snick 1990).

Like TNF this cytokine appears to be rapidly released into the circulation in response to injury and can be detected within 60 minutes of an endotoxin infusion (Michie HR et al., 1988a). However, unlike IL-1 and TNF, IL-6 does not induce IL-1 or TNF. In fact it suppresses endotoxin- and TNF-induced IL-1 production (Dinarello C, 1988).

Thus IL-6 appears to be a weak inflammatory peptide when compared to IL-1 and TNF, but with a spectrum of activities that include haematological and neurological influences. Its multiple effects all contribute to a co-ordinated response by the body to injury and, unlike IL-1 and TNF, it seems incapable of inducing adverse effects, even when

administered in high dose. Evidence that IL-6 adversely influences cardiovascular stability or cellular integrity has not been found, so that the major effects of this protein appear to be beneficial to the host by enhancing immune function and acute phase protein synthesis. If one adds that injury or infection are promptly followed by a massive induction of IL-6 and that essentially every cell is capable of making IL-6, it becomes clear that this cytokine is tailored to function as an "SOS signal" (van Snick J, 1990). The high titres of IL-6 associated with inflammation and infection may result from complex amplification mechanisms involving IL-1 and TNF, which both induce strong IL-6 responses *in vivo* (Jablons DM et al., 1989)(Shalaby MR et al., 1989).

In conclusion, while IL-6 appears to be one of the most pleiotrophic cytokines yet discovered, the claim of some IL-6 investigators that *"it has the capacity to cause clinical abnormalities, directly or indirectly, by non-physiological over-production during the course of several diseases"* (Wolvekamp MCJ et al., 1990), remains unproven and perhaps unlikely in the face of current evidence.

Table 2.1 A comparison of the biological properties of IL-1, TNF and IL-6.

| BIOLOGICAL PROPERTIES | IL-1 | TNF | IL-6 |
|---|---|---|---|
| **Metabolic** | | | |
| acute phase protein synthesis | + | + | ++ |
| decreased plasma Iron/Zinc | + | + | - |
| decreased albumin synthesis | + | + | + |
| increased insulin production | + | + | - |
| inhibition of lipoprotein lipase | + | ++ | - |
| increased corticosteroid synthesis | + | + | + |
| lactic acidosis | + | + | - |
| **Central nervous system** | | | |
| fever | + | + | + |
| increased ACTH production | + | + | + |
| decreased REM sleep | + | + | - |
| increased slow wave sleep | + | + | - |
| anorexia | + | + | - |
| CNS prostaglandin synthesis | + | + | + |
| **Haematologic and vascular wall** | | | |
| haemodynamic shock | + | ++ | - |
| stem cell (haemopoietin-1) activity | + | + | + |
| endothelial activation | + | + | - |
| radioprotection | + | + | ± |
| non-specific resistance to infection | + | + | + |
| neutrophilia | + | + | + |
| increased cardiac output | + | + | - |

| | | | |
|---|---|---|---|
| **Immunological** | | | |
| induction of IL-1 and TNF | + | + | - |
| induction of IL-6 | + | + | - |
| T/B cell activation | ++ | + | + |
| activation of NK cells | ++ | + | - |
| **Local non-immune effects** | | | |
| neutrophil degranulation | + | + | - |
| bone resorption | + | + | - |
| keratinocyte proliferation | + | + | - |
| collagen synthesis | + | + | - |
| chemotaxis | + | + | - |
| increased prostaglandin synthesis | + | + | + |
| tumour cytotoxicity | + | ++ | - |
| fibroblast proliferation | + | + | - |

### 2.3.6 Interleukin-2

This cytokine was discovered through its activity as a T cell growth factor (Morgan DA et al., 1976). It is produced by mitogen or antigen stimulated T lymphocytes, has two distinct polypeptide chains and a molecular weight of 15 kilodaltons (Smith KA, 1988). Unlike IL-1, TNF and IL-6 which are pleiotrophic, IL-2 acts predominantly as an immunostimulant. Besides being a growth factor for mature T cells and thymocytes, IL-2 enhances cell-mediated T cell cytotoxicity and stimulates natural killer cell activity (Rosenberg SA et al., 1988). It is, therefore, able to promote host defences against infection and its administration to mice has been shown to protect them against lethal doses of intraperitoneal *E coli* administration (Goronzy J et al., 1989).

Because IL-2 is immunostimulatory, antibodies against its receptors are potentially immunosuppressive and therefore may be useful in organ transplantation, or protective in conditions of aberrant immune function such as autoimmune diabetes mellitus or systemic lupus erythematosus. Additionally, it could be a useful agent in restoring immune function to immunocompromised patients, including trauma or septic patients.

This cytokine does not appear to have any active role in the integrated host response and its direct role in mediating detrimental changes in any clinical disease remains to be demonstrated (Fong Y et al., 1990a).

### 2.3.7 Interferon gamma

Three distinct classes of interferon ($\alpha$,$\beta$ and $\gamma$) are recognised. Although they all have anti-viral activity in common, IFN$\gamma$ differs from the other interferons both in structure and function. Interferon gamma (IFN$\gamma$) is produced during an immune response by antigen-specific T cells and natural killer (NK) cells, recruited by IL-2 (Green JA et al., 1969).

This glycoprotein has pronounced immunoregulatory effects, including activation of macrophages to enhanced phagocytosis and tumour killing capability, activation and growth enhancement of cytolytic T cells and NK cells, induction of class II MHC antigen on many cells including macrophages and the proliferation of B-cells and induction of their immunoglobulin secretion (Trinchieri G et al., 1985). Production of IFN$\gamma$ is triggered by microbial antigens (Kelly CD et al., 1987) or IL-2 (Kasahara T et al., 1983) and increased transcription for IFN$\gamma$ can be detected within six hours. Release of this protein peaks at approximately 48-72 hours (Kelly

CD et al., 1987), but may continue for seven to eight days (Green JA et al., 1969).

*In vitro* studies have demonstrated a decrease in interferon gamma (IFNγ) production by peripheral blood mononuclear cells (PBMC) isolated from major injury patients (Faist E et al., 1988)(D Livingstone et al., 1988). However the bioassays and immunoassays used in these studies measured only the bulk release of cytokines by entire populations of cells in culture after lengthy mitogen stimulation. Work presented in Chapter 8 will suggest that the spontaneous release of IFNγ from PBMC is, in fact, augmented in the post-surgical patient.

Studies in which IFNγ has been administered to injured (thermal and non-thermal) animals have suggested an improvement in outcome after a bacterial challenge (Hershman MJ et al., 1988b)(M Malangoni et al., 1989), and preliminary results from a multicentre clinical trial have suggested some reduction in septic complications in certain subgroups of major trauma patients given IFNγ therapy (Polk H C, Presentation of preliminary data from a multicentre trial of interferon gamma therapy in major injury patients: at the 2nd International Congress on the Immune consequences of Trauma, Shock and Sepsis; Munich, March 1991). Interferon gamma release could therefore be interpreted as a beneficial component of the host's immune response to injury, and a possible target for immunotherapy to augment the patient's own endogenous release of IFNγ.

## 2.4 CONCLUSION - Cytokine interactions (Figures 2.1, 2.5 and 2.6)

This chapter began with an outline of the cascade of inflammatory mediators involved in the integrated host response, then some of the cytokines most central to the theme of this thesis were introduced. It is therefore appropriate that it should end with a re-emphasis of the complex interaction of these mediators.

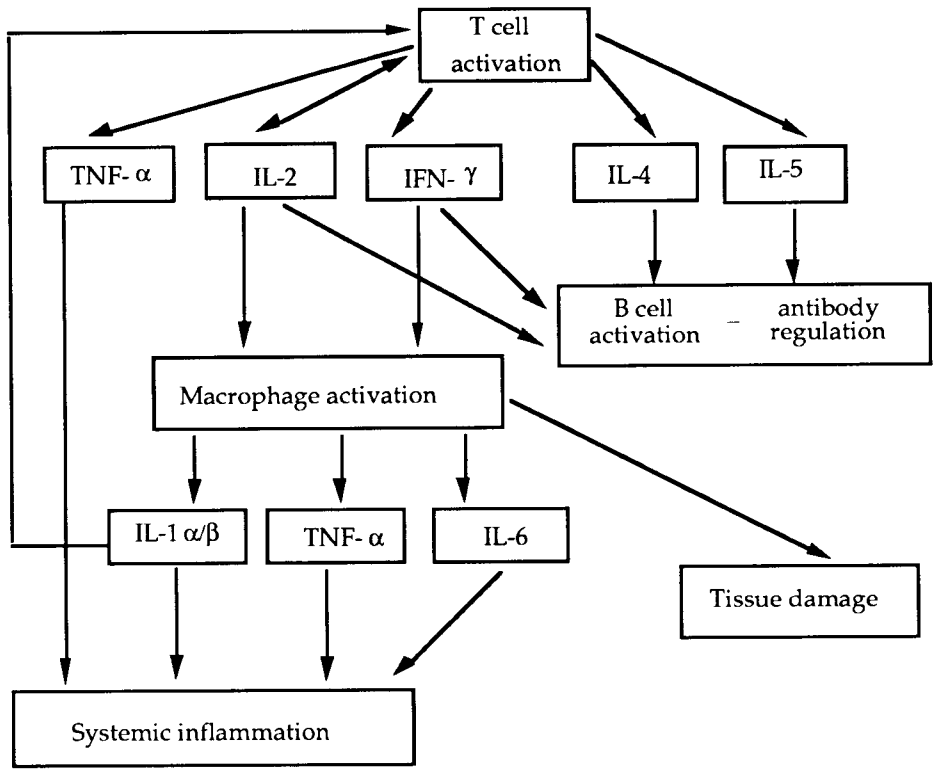



Figure 2.6 Cytokine interactions and synergism (courtesy of Dr Salmon, University of Birmingham).

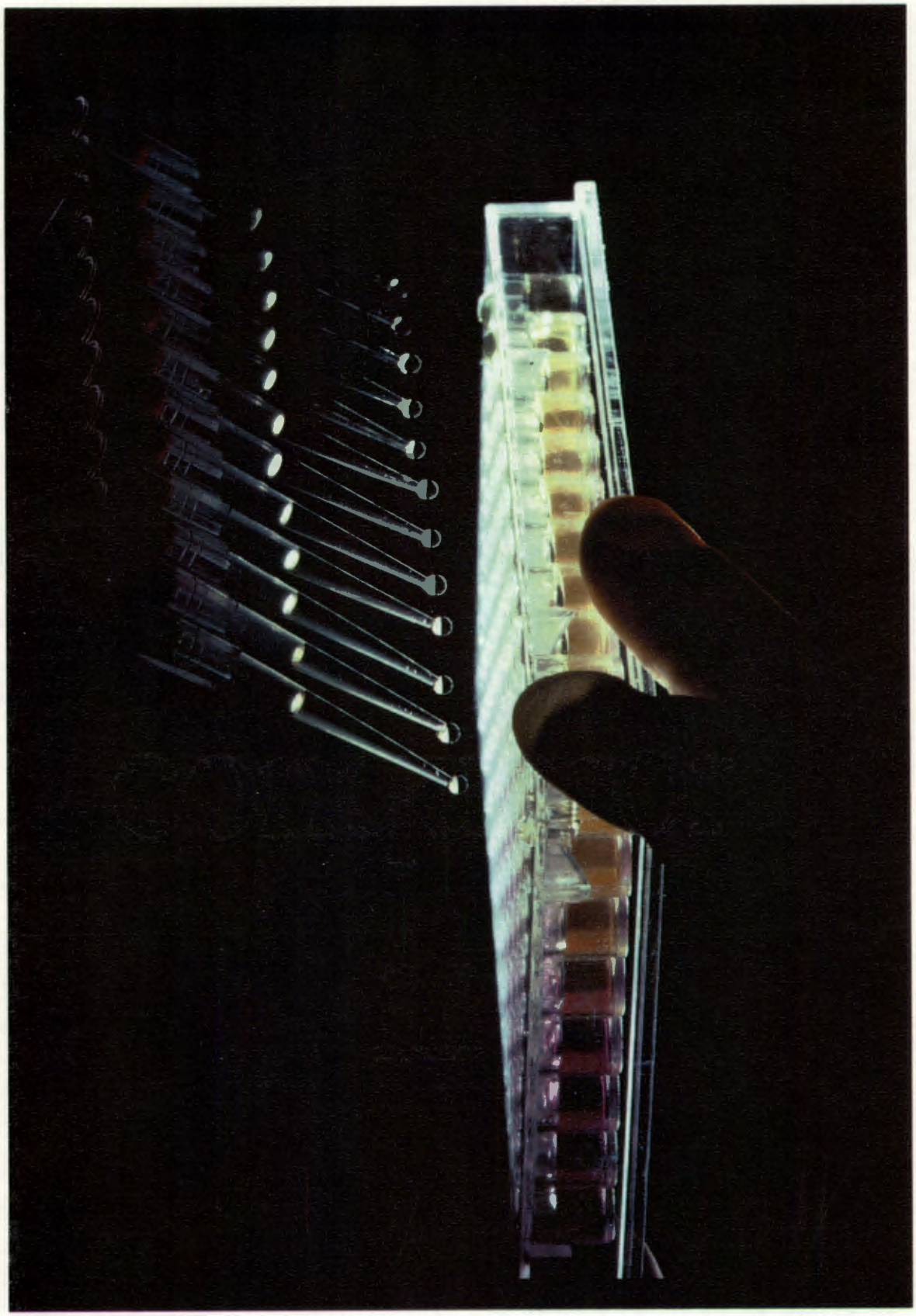
Cytokines constitute the molecular language of inflammation and immunity, and as such they form a complex network of overlapping and interacting signals, which together propagate the mediator cascade and orchestrate the integrated host response. They can elicit the release of non-cytokine mediators, for example, TNF elicits the release of the counter-regulatory hormones and prostaglandins, and along with IL-1 and IL-6, stimulates the release of adrenal and pituitary hormones. They also

exhibit synergy with non-cytokine endogenous mediators, which also helps propagate the mediator cascade, for example TNF enhances the glucagon-mediated uptake of amino acids (Warren RS et al., 1987).

Finally, cytokines exhibit synergy among themselves; IL-6 induces IL-2 receptor expression, IFNγ potentiates interleukin-4 induced proliferation of human B-cells and the synergism of IL-1, TNF and IL-6 has been frequently mentioned (Figures 2.5 and 2.6). These three cytokines, probably more than any others, are capable of initiating a powerful cytokine amplification cascade.

# CHAPTER THREE

# MATERIALS AND METHODS



Frontispiece (Materials and methods)

The 96 well polystyrene plate was central to most of the assays described in this chapter

# CHAPTER THREE

# MATERIALS AND METHODS



## 3.1 PATIENTS

All subjects studied were managed in the surgical wards, operating theatres and intensive therapy unit at the John Radcliffe Hospital in Oxford. Three groups were recruited: (1) patients admitted for elective aortic surgery for either aneurysmal or occlusive disease, (2) patients admitted for elective inguinal hernia repair, (3) victims of major trauma admitted through the Accident and Emergency department. Patients in the

first two groups gave informed consent for the studies, all of which were approved by the Central Oxford Research Ethics Committee.

Twenty eight patients (25 men and three women), aged between 59 and 83 years, undergoing elective aortic aneurysm repair were studied in the work reported in chapters 4, 6, 7 and 8. No patient had liver disease, inflammatory or ischaemic bowel disease. Preoperative bowel preparation was not performed, but all patients received a prophylactic dose of cefuroxime (1.5gram) at induction. Operative and post-operative details were recorded, including the durations of operation, aortic clamping and ventilation, as well as blood transfusion volume, post-operative temperatures, leucocyte count, APACHE II scores and duration of admission. APACHE II scoring was performed on the basis of the initial and worst physiological parameters during the first 24 hours post-operatively.

Five patients (four men and one woman), aged between 50 and 75 years, undergoing elective inguinal hernia repair, were studied.

The victims of major trauma were all admitted via the Accident and Emergency Department of the John Radcliffe Hospital, between January 1991 and July 1991. It is department policy to transfer to the Regional Neurosurgical Unit at the Radcliffe Infirmary, those with major head injuries and relatively minor involvement of other systems. These patients were therefore excluded. Severely injured patients with minor head injuries, or those with both major head injuries and major injuries of other systems were included. This last group were managed on the intensive care unit of the John Radcliffe Hospital. All major burns patients are admitted directly to the Regional Burns Unit in Aylesbury and therefore thermally injured patients were not available for inclusion.

Using these criteria, six patients were studied. At admission, patients were assigned an Injury Severity Score (ISS) using the Abbreviated Injury Scale (AIS) scoring system (Greenspan L et al., 1985).

### 3.2 ANAESTHESIA AND SURGERY

Standard anaesthetic techniques were used for the first three patient groups. Two anaesthetists were involved in the operative management of the first group, one of whom anaesthetised more than 80 percent (%) of the patients. Only one anaesthetist was responsible for all patients in the second group, while two were involved in the operative management of the third group. By necessity, there was no uniformity of these techniques for patients in the fourth group.

Four different consultant vascular surgeons performed all operations in the first group, although two performed more than 80 percent of them. The operations all involved replacement of the abdominal aorta with a dacron tube or bifurcated graft to the iliac or femoral arteries. Two surgeons performed all the cardiac surgery. Surgery in the other two groups was performed by several surgeons.

### 3.3 PLASMA PREPARATION

Five ml of venous blood was collected in standard disposable pyrogen free sterile syringes and added to pyrogen free glass tubes containing EDTA (0.34M) (Becton-Dickinson) and the proteinase inhibitor aprotinin (20µl/ml) (Sigma). Samples were immediately microfuged, in the operating suite, at 11 000g. Aliquots of plasma were stored at -70°C. The sample preparation time was less than 5 minutes.

During the course of the study two alterations were made to the sampling method. The first followed a comparison of results obtained when blood

was collected into tubes with and without aprotinin. This showed no variation in cytokine levels and thereafter aprotinin was not used. The second alteration resulted from a comparison between venous and arterial blood. Synchronous venous and arterial samples were drawn at various time points during the sampling of the first ten patients. It was observed that there was no significant difference between the levels of cytokines measured in 30 synchronous samples. While venous blood continued to be sampled whenever possible, there were occasions when the central venous catheter was not accessible because of complex anaesthetic monitoring and on these occasions arterial blood was sampled.

A different method was used to prepare plasma for endotoxin analysis (section 3.8).

### 3.4 CORTISOL

This hormone was measured by radioimmunoassay, after plasma extraction with dichloromethane.

### 3.5 C-REACTIVE PROTEIN

This was measured using flow nephelometry (Array Protein System, Beckman Diagnostics) with a minimum detectable level of 6mg/l.

### 3.6 FULL BLOOD COUNT

These were performed by a H-6000 coulter counter on blood anticoagulated with ethylene diamine tetra-acetic acid (EDTA).

## 3.7 CYTOKINES

The wide range of cytokine levels reported in clinical studies in the literature is evidence of the absence of standardisation of both bio- and immunoassays. This makes it difficult to compare different studies in quantitative terms, with the result that a comparison of kinetics or positives and negatives is often all that is possible. With large profits at stake there has been, without doubt, an unseemly rush by some biotechnology companies to present their commercial immunoassays to researchers. The result has been that some of the assays have been inadequately tested for specificity, and standardisation has been insufficiently rigourous.

For this reason several weeks were spent at the beginning of this research experimenting with the products of several well-known companies. Intra- and interassay variation was checked using in house plasma samples. Because of the potentially large order it was possible to obtain 'pilot' kits free of charge or at nominal expense. The assays were only selected after this scrutiny and, moreover, it was possible to virtually elimate batch-to-batch variability by bulk ordering or booking from a particular batch. The biotechnology companies all had their headquarters close to Oxford and it was not difficult to elicit their cooperation in holding stock from particular batches.

### 3.7.1 INTERLEUKIN - 6

**Principle of the assay**

This assay employs the quantitative "sandwich" enzyme immunoassay technique. Samples are pipetted into the wells of a microtitre plate coated with a monoclonal antibody specific for IL-6. Any IL-6 will be bound by the immobilised antibody. After washing away any unbound sample proteins,

an enzyme-linked polyclonal antibody, specific for IL-6, is added to the wells. This, in turn, binds to any IL-6 which was bound during the first incubation. Following a wash to remove the excess enzyme-linked polyclonal antibody, a substrate solution is added to the wells and colour develops in proportion to the amount of IL-6 bound in the initial step. Along with the samples to be tested, a series of wells is prepared using known concentrations of the IL-6 standard and a standard curve is prepared by plotting the optical density (OD) versus the known concentration of IL-6 in the standard wells. The concentration of the IL-6 in the unknown samples is determined from their OD, using this standard curve.

**Materials**

Storage: all materials were stored at 2-8°C.

IL-6 microtitre plate: a 96 well polystyrene microtitre plate coated with a mouse anti IL-6 monoclonal antibody.

IL-6 conjugate: Polyclonal antibody against IL-6, conjugated to horse-radish peroxidase, with preservative.

IL-6 standard: Recombinant human IL-6 (10ng) in a buffered protein base with preservative, lyophilised.

Assay Diluent : 6ml of a buffered protein base with preservative.

Calibrator Diluent : 21ml of bovine serum with preservative.

Wash buffer concentrate: 21ml of a 25-fold concentrated solution of buffered surfactant with preservative.

Substrate solution(a): 12.5ml of stabilised hydrogen peroxide.

Substrate solution (b): 12.5ml of stabilised chromogen.
(tetramethylbenzydine).

Stop solution: 6ml of 2N sulphuric acid.

Note: This enzyme linked immunosorbance assay (Quantikine™) for IL-6 was developed by R & D Systems Inc, 614 McKinley Place, Minneapolis, MN 55413. The materials listed were supplied by this company.

**Preparation of reagents**

Wash buffer: The concentrate was diluted 25 fold with doubly distilled water to prepare 500ml of wash buffer.

Substrate solution: Solutions (a) and (b) were mixed together in equal volumes and used within 15 minutes of preparation.

Interleukin-6 standard: The lyophilised standard was reconstituted with 5 ml of calibrator diluent producing a reconstituted stock solution of 2000pg/ml.

**Preparation of the standard curve**

The stock solution was serially diluted with calibrator diluent in reagent tubes. The resulting standards were 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml and 0pg/ml. The stock solution was used for the highest point on the standard curve and calibrator diluent for the lowest point.

A fresh standard curve was made up for each assay.

**Assay procedure**

(1) Assay diluent (50µl) was added to each well on the plate prior to adding the sample or standard.

(2) The standard or sample (200µl) was added to each well and the plate incubated for 2 hours at room temperature. All samples and standards were assayed in duplicate.

(3) After the incubation, all wells were emptied by inverting the plate. Each well was washed with the wash buffer, using a wash bottle. Three

washes were performed. At the end of each wash the plate was inverted and forcefully blotted against clean tissue paper to ensure removal of all liquid.

(4) After the last wash IL-6 conjugate (200µl) was added to each well and the plate incubated for a further 2 hours at room temperature.

(5) The washing procedure was repeated as in (3).

(6) Substrate solution (200µl) was added to each well and the plate incubated for 20 minutes at room temperature.

(7) Stop solution (50µl) was added to each well and mixed thoroughly.

**Spectrophotometry**

The OD of each well was determined within 30 minutes of completing the assay. The spectrophotometer used was an Anthos Reader 2001. This filter photometer combines an optomechanical system with micro-computer electronics and is able to read a microtitre plate within 5 seconds. The data reduction programme was adjusted for use with this assay. Samples were read at a wavelength of 450nm with wavelength correction set to 540nm. The data reduction programme calculated the average of the duplicate readings and subtracted the zero standard OD from the sample optical density. After drawing a linear standard curve using a log/log transformation, the sample values were calculated from the standard curve.

Assay sensitivity: The minimum sensitivity was determined by adding 2 standard deviations to the mean OD value of 20 zero-standard replicates and calculating the corresponding concentration from the standard curve. Using this method, the minimum detectable IL-6 level was 3.5pg/ml.

Interassay variation (% CV): less than 10%.

### 3.7.2 INTERLEUKIN-1

**Principle of the assay**

The plasma cytokine concentration was measured using a modified ELISA assay called EASIA (Enzyme Amplified Sensitivity Immuno-Assay). It is based on an oligoclonal system in which several monoclonal antibodies, directed against different distinct epitopes of IL-1, are used. This avoids hyperspecificity and allows high sensitivity of the assay with an extended standard range and a short incubation time.

The assay is performed on a 96 well microtitre plate. Each well is coated with "catcher" monoclonal antibodies. The standards or samples containing IL-1 react with these antibodies and also with the added monoclonal antibodies labelled with horse radish peroxidase. An incubation period allows the formation of a "sandwich" of IL-1 on the microtitre plate before it is washed to remove unbound enzyme labelled antibodies. The substrate solution (tetramethylbenzydine) is added and after a short incubation, the reaction stopped with sulphuric acid.

**Materials**

IL-1 Standard: Recombinant human IL-1 in a buffered human serum solution; lyophilised.

Calibrator Diluent: 8ml of human serum and preservative.

IL-1 conjugate: anti IL-1 monoclonal antibody conjugated to horseradish peroxidase.

Wash buffer: 10ml of 20% Tween.

Substrate solution: chromogen tetramethylbenzydine.

Substrate solution buffer: Hydrogen peroxide in acetate/substrate buffer 21ml.

Stop solution: 6 ml of sulphuric acid (1.8 N).

Note: This enzyme amplified sensitivity immunoassay (EASIA™) for IL-1 was developed by Medgenix Diagnostics, PO Box 42, 1160, Brussels. The materials listed were supplied by this company.

**Preparation of reagents**

IL-1 Standard: Reconstituted in 2ml of double distilled water.

Calibrator diluent: Reconstituted with 8ml of double distilled water.

Wash buffer solution: 2ml of the 20% Tween solution was made up to 400ml with double distilled water.

Substrate solution: 0.2ml of the chromogen tetramethylbenzydine was pipetted into 21ml of substrate buffer. This was performed within 15 minutes of use.

**Preparation of the Standard Curve**

A standard curve was created by a serial dilution of the IL-1 standard concentrate using the human serum calibrator diluent. The resulting standard curve series was 150pg/ml, 100pg/ml, 75pg/ml, 50pg/ml, 33pg/ml, 16pg/ml and 0pg/ml.

**Assay Procedure:**

(1) Each standard or sample (200µl) was pipetted into the appropriate wells. The assay was performed in duplicate.

(2) Anti IL-1 horse-radish peroxidase conjugate (50µl) was pipetted into each well and the plate allowed to incubate for 2 hours at room temperature on a horizontal shaker set at 400rpm.

(3) After the incubation, all wells were emptied by inverting the plate. Each well was then washed with the wash buffer, using a wash bottle. Three washes were performed. At the end of each wash the plate was

inverted and forcefully blotted against clean tissue paper to ensure removal of all liquid.

(4) Freshly prepared substrate solution (200µl) was dispensed into each well and the plate incubated for 15 minutes at room temperature on a horizontal shaker set at 400 rpm.

(5) The stop reagent (50µl) was pipetted into each well and mixed thoroughly.

**Spectrophotometry**

This was performed with the same equipment used for the IL-6 samples (see section 3.7.1). Samples were read at a wavelength of 450nm with wavelength correction set to 630nm and a linear standard curve was drawn using a linear/linear transformation.

Assay sensitivity: The minimum sensitivity was determined by adding 2 standard deviations to the mean optical density value of 20 zero-standard replicates and calculating the corresponding concentration from the standard curve. Using this method, the minimum detectable IL-1 level was 2pg/ml.

Interassay variation (% CV): less than 5%.

### 3.7.3 TUMOUR NECROSIS FACTOR

Tumour necrosis factor (TNF) was measured using a sandwich type ELISA. Microtitre plates were coated with murine monoclonal antibody to human TNF (Rockefeller University: SDW 18.1.1) in carbonate buffer (pH 9.0). This antibody has been tested against TNFβ, IL-1α, IL-1β, IL-6 and IFNγ and shown to be specific for TNFα. of After washing plates with

phosphate-buffered saline plus 'Tween 20' (PBS-T), duplicate samples (plasma diluent) were incubated in the wells. The following reagents were then applied successively, with PBS-T washes before each step: (1) ammonium-sulphate-precipitated rabbit anti-serum to recombinant human TNF (rhTNF) with 2% normal goat serum in PBS-T, (2) affinity-purified, alkaline-phosphate-conjugated goat antibodies to rabbit IgG (adsorbed to mouse and human immunoglobulin, supplied by American Qualex) with 2% human serum in PBS-T, and (3) p-nitrophenylphosphate in diethanolamine buffer.

Optical densities at 405nm were determined by automated dual-beam ELISA and TNF concentrations in the samples were determined by reference to a serially diluted rhTNF standard (Amersham International, UK) included on each plate. Data were analysed with the software package SPSS/PC + v3.1. The detection limit of the assay was 10pg/ml. Both plasma samples 'spiked' with TNF and clinical samples known to contain TNF, have been used to determine intra- and interassay variations (%CV) of approximately 8%.

### 3.7.4 INTERFERON GAMMA

Interferon gamma was measured using a modified ELISA (Medgenix Diagnostics, High Wycombe, Bucks, England). The principles and utilisation of this assay are identical to the description for the IL-1 immunoassay (section 3.72). Random samples were also independently assayed using a different ELISA system at the National Institute for Biological Standards and Controls, Hertfordshire, England (lower limit of detection=10pg/ml).

## 3.8 ENDOTOXIN

**Principal of the assay**

Gram-negative bacterial endotoxins catalyse the activation of an enzyme system in the circulating amoebocytes of the horseshoe crab, "Limulus polyphemus". The terminal enzyme reacts with a clottable protein resulting in a gel formation (Figure 3.1). By using the clot forming capability of the limulus amoebocyte lysate (LAL) it has been possible to obtain a semi-quantitative determination of endotoxin.

It has been shown that the terminal enzyme also splits certain chromogenic substrates, for example peptide-pNA, to form a coloured product which can be measured photometrically. The amount of enzyme needed to obtain suitable hydrolysis of a chromogenic substrate is less than the amount needed to form a clot. In addition, the intensity of the yellow colour is linearly proportional to the amount of endotoxin present. For these reasons the chromogenic assay has replaced the gel formation assay in almost all laboratories where an attempt is being made to quantify endotoxin.

The blood from the horseshoe crab is taken directly from the heart using a syringe (the crab is then returned to the water alive). The blood cells are centrifuged down and washed. They next undergo ultra-sound lysis, followed by chloroform extraction to destroy inhibitory components. All factors involved in the chain of reactions except endotoxin (the trigger) and the peptide-pNA substrate are present in the LAL reagent.

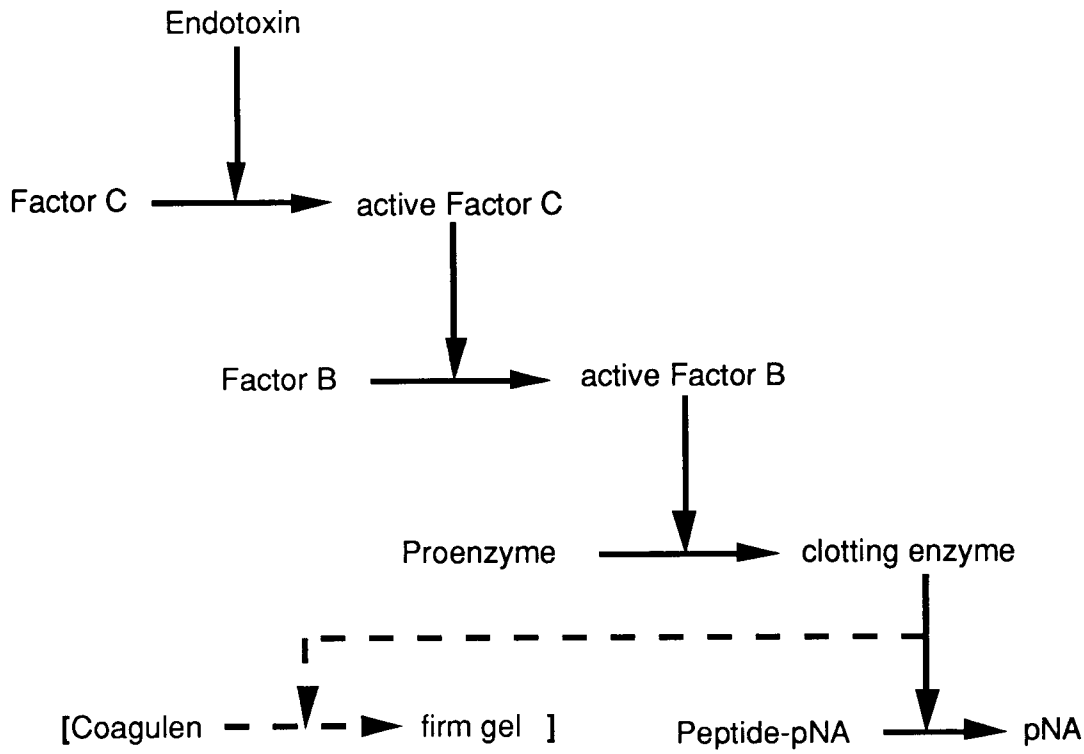



Figure 3.1 The principal of the LAL test: the chain of enzyme reactions in the limulus amoebocyte lysate which is initiated by endotoxin.

**Materials and their preparation**

Endotoxin: Freeze dried in albumen. Reconstituted using endotoxin free water (EFW) to a standard concentration of 1000pg/ml (1 Endotoxin Unit=83pg/ml). Stored at 4°C.

Limulus amoebocyte lysate (LAL): Freeze dried. Reconstituted with 1.4ml EFW at room temperature 10-15 minutes prior to use to ensure complete dissolution (loss of activity begins after this time). Avoiding vortexing, the LAL solution was swirled gently to mix. Stored at -20°C for up to one month.

Chromogenic substrate (Ac-Ile-Glu-Gly-Arg-pNA HCl) (Kabi S-2423): Freeze dried (9mg) with mannitol. Reconstituted with 6.6ml of EFW to a concentration of 2.0mmol/l. The solution is stable for 1 month at 2-8°C provided that no contamination occurs.

Substrate-buffer solution: Sterile, endotoxin free Tris buffer (50 mmol/l, pH 9.0).

Microtitre plate: 96-well, flat bottomed plate (Sterilin) (catalogue No: N29AR).

Note: All analyses were done with the Coatest Endotoxin kit (Kabi Diagnostica, Molndal, Sweden). The materials listed were supplied by this company.

**Sample preparation:** Blood was aspirated into a pyrogen free syringe and immediately transferred to a sterile endotoxin-free heparinised (15 units per ml) polypropylene skirted screw top tube (Astra). The heparin (Evans Laboratories) used was also endotoxin-free and this was confirmed by testing each new batch on the LAL assay. After mixing, the blood was immediately centrifuged (in theatre) at 4°C for 10 minutes at 1 500g. The plasma was aliquoted into further polypropylene skirted screw top tubes and stored at -70°C.

**Preparation of the standard curve**

The endotoxin stock solution was vortexed for 10 minutes, and then diluted 1:10 (100µl of stock solution + 900µl water), before vortexing again for 1 minute. Endotoxin standards were made up according to Table 1 and vortexed for 1 minute.

**Assay procedure**

All reagents and laboratory utensils were sterile and endotoxin free. All analyses were calculated as mean values of either duplicate or triplicate samples. Controls were also used in this assay. A high (100 pg/ml) and a

low (10pg/ml) standard were made from donor plasma and EFW. These were ready for use after thawing and vortexing for one minute.

(1) Samples and standards were heated at 75°C for 10-15 minutes and left to cool for 15 minutes. This step is required to denature plasma inhibitors of endotoxin (Friberger P, 1985)(Cooperstock M et al., 1985).

(2) After each sample had been vortexed for 1 minute, the microtitre plate was loaded with standards and samples (50µl per well) and placed on a heating block to equilibrate to 37°C for 5 minutes.

(3) 50µl of LAL was added to each well. The timer was started on addition of LAL to the first well. The plate was incubated at 37°C for exactly 30 minutes.

(4) During the incubation, the chromogenic substrate was reconstituted with 6.6ml of EFW and a solution of one part substrate + one part buffer (sufficient for 100µl per well) was prepared and incubated for 10 minutes at 37°C.

(5) At the end of the 30 minute assay incubation, substrate was added to each well (100µl per well). The timer was restarted on addition of substrate to the first well.

(6) Incubation continued at 37°C for exactly 5 minutes after which the reaction was stopped with 20% Acetic acid (100µl per well).

**Spectrophotometry**

This was performed using the same equipment as for the cytokine studies (section 3.71). Samples were read at a wavelength of 405nm without wavelength correction and a linear standard curve was drawn using a linear/linear transformation.

Assay sensitivity: the detection limit, defined as the concentration corresponding to the mean $OD_{405}$ of the blank sample of the standard curve plus

three times the standard deviation, was 3.65pg/ml. On the basis of other studies of healthy volunteers, the upper limit of the normal range with this assay system was considered to be 4.5pg/ml (van Deventer SJH et al., 1988).

## 3.9 GLUTAMINE

### Preparation of plasma samples

Five ml of venous blood was aspirated directly from a central venous catheter into standard disposable pyrogen free sterile syringes and added to pyrogen free glass tubes containing EDTA (0.34M). After mixing, the blood was transferred to Eppendorf tubes and immediately centrifuged (in theatre) for 2 minutes at 1 100g. The plasma was aliquoted into further Eppendorf tubes and stored at -70°C.

Plasma was extracted in ice-cold perchloric acid (5%) and, after centrifugation, the supernatant was neutralised with ice-cold potassium hydroxide (3.5M, plus 7.7mM triethanolamine). The neutralised samples were centrifuged at 9000g for 90 seconds and the supernatant collected and stored at -20°C. All procedures were carried out in previously cooled Eppendorf tubes on ice and only two samples were extracted and neutralised at one time.

### Assay procedure

Glutamine concentration was measured in plasma extracts using the method of Windmueller & Spaeth (Windmueller HG et al., 1974), with some minor modifications. Asparaginase was dialysed for 24 hours against two changes of potassium dihydrogen phosphate buffer (80mM, pH 6.6) prior to use. This is an enzyme-linked spectrophotometric assay based on the disappearance of nicotinamide adenine dinucleotide hydride (NADH), which is followed at 340nm. Measurements were made using a

spectrophotometer (Gilford, Stasar III) or a fluorimeter (Perkin-Elmer). Standard solutions of glutamine and a blank (water) were included in each assay. The composition of the assay system is outlined below.

| Substance | Final concentration in 1ml cuvette |
|---|---|
| $KH_2PO_4$, pH 8.0 | 45 mM |
| NADH | 172 uM |
| 2-oxoglutarate | 3.6 mM |
| glycerol | 8 % |
| BSA | 0.05 % |
| glutamate dehydrogenase | 0.5 mg |
| asparaginase | 20 units |

Neutralized perchloric acid extracts of plasma were also analysed using enzymatic assays for concentrations of glutamate (Bernt E et al., 1974), alanine (Williamson DH, 1974) and branched chain amino acids (Livesey G et al., 1980). These three similar methods are also enzyme linked spectrophotometric assays. However they are based on the formation of NADH, rather than its disappearance, and the reaction is again followed at 340nm.

### 3.10 REVERSE HAEMOLYTIC PLAQUE ASSAY

The description and methodology of this assay is described in full in Chapter 7.

### 3.11 STATISTICAL ANALYSIS

Data was analysed by Mann-Whitney U testing and Spearman correlation. Criticism of the use of peaked curves in the analysis of serial biological measurements has recently been restated (Matthews JNS et al., 1990). This

prompted a comparison of some results (Chapter 4) with the method of summary measures which requires the calculation of the area under the response curve (Matthews JNS et al., 1990). Statistical methods used in the analysis of reverse haemolytic plaque assay results are described in Chapter 7.

# CHAPTER FOUR

# THE SYSTEMIC CYTOKINE RESPONSE FOLLOWING MAJOR SURGERY

# CHAPTER FOUR

## THE SYSTEMIC CYTOKINE RESPONSE FOLLOWING MAJOR SURGERY



### 4.1 INTRODUCTION

Surgery and trauma have been shown to induce a generalised state of immunodepression (Lennard TWJ et al., 1985), and this state has been shown to correlate with sepsis (Baker CC et al., 1980) and late death (Christou NV et al., 1986). Cytokines act as mediators of both the immune and the acute phase response. Each mediates a variety of often overlapping effects and their actions are frequently additive.

Tumour necrosis factor alpha, IL-1 and IL-6 are regarded as major mediators of the acute phase response in humans. While TNF and IL-1 are thought to be primarily responsible for the non-hepatic manifestations of the response such as fever, prostaglandinaemia, tachycardia and catabolism (Dinarello CA, 1989b), IL-6 has been shown *in vitro* to be primarily responsible for the hepatic component of the acute phase response, resulting in the synthesis of acute phase proteins (Castell JV et al., 1989)(Gauldie J et al., 1987). Nonetheless IL-1 and TNF also induce synthesis of acute phase proteins, in particular C-reactive protein (CRP) (Hasselgren P et al., 1988)(Perlmutter DH et al., 1986)(Baumann H et al., 1990). The transcription and production of IL-6 in fibroblasts, endothelial cells, keratinocytes and monocytes has been shown to be enhanced *in vitro* by IL-1 and TNF (Bauer AR et al., 1978)(Kohase M et al., 1986)(Tosatu G et al., 1990)(Shalaby MR et al., 1989). Therefore there is evidence that IL-1 and TNF may be partly responsible for the induction of IL-6, and all three of these cytokines then mediate the acute phase response.

These cytokines also have in common immunostimulatory capabilities. Interferon gamma acts primarily on monocytes and macrophages to enhance cell mediated cytotoxicity (Trinchieri G et al., 1985). The release of these cytokines may therefore represent an attempt by the body to counter the observed immunodeficiency of injury or sepsis. Indeed the current explosion of interest in immunotherapy, in both oncological and inflammatory conditions, centres around the evaluation of the administration of both cytokines and their inhibitors in various disease states.

The measurement of cytokines in the peripheral blood has proved difficult and most reports of circulating cytokines in humans have been of

sporadic detections involving single or daily measurements. There have been very few attempts to assay cytokines longitudinally over a predetermined series of time points during an acute illness or after a severe insult. Tumour necrosis factor has seldom been consistently detected except after endotoxin administration, which does not induce a detectable IL-1 response *in vivo* (Michie HR et al., 1988a). Recently Shenkin detected a consistent rise in the serum IL-6 of elective surgical patients but was unable to detect a consistent rise in IL-1 or TNF (Shenkin A et al., 1989). All these patients had an uncomplicated clinical course, and the difference, therefore, between a pathological and a physiological IL-6 response remains far from clear.

The aim of this study was to sample patients frequently before, during and after major surgery, and to explore the association between cytokine levels in their plasma and their clinical course and CRP response. The levels of IL-1, IL-6, TNF and IFNγ have been assessed in patients undergoing elective aortic aneurysm surgery. A study of patients undergoing this operation has several advantages. Besides being a relatively uniform population in terms of age, race and general health, they also undergo a reasonably comparable surgical insult. They are tumour free and have a relatively low risk of bacterial contamination and sepsis, thus allowing the impact of major surgical trauma on cytokine levels to be evaluated without the conflicting influence of bacterial contamination or malignancy on the immune system.

A smaller group of inguinal hernia patients were also assessed in order to determine whether the cytokine response was related to the magnitude of surgical insult.

## 4.2 PATIENTS AND METHODS

**4.2.1 Patients:** (Group 1) Twenty patients (17 men and three women), aged between 59 and 83 years, were studied. All underwent elective aortic aneurysm repair. Operative and post-operative details were recorded, including the durations of operation, aortic clamping and ventilation, as well as blood transfusion volume, post-operative temperatures, leucocyte count, APACHE II scores and duration of admission. APACHE II scoring was performed on the basis of the initial and worst physiological parameters during the first 24 hours post-operatively.

(Group 2) Five patients (four men and one woman), aged between 50 and 75 years, who all underwent elective inguinal hernia repair, were studied.

**4.2.2 Samples:** A detailed description of sample preparation is given in section 3.6. In brief, cold spun plasma was prepared from venous blood collected in pyrogen free tubes containing EDTA (0.34M) (Becton-Dickinson) and the proteinase inhibitor aprotinin (20μl/ml) (Sigma). Aliquots of plasma were stored at -70°C.

**4.2.3 Sample times:** Samples were taken the day before surgery and a preoperative sample was taken after induction of anaesthesia. Thereafter patients were sampled after incision at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6, 8, 12, 24, 48, 72, 96, 120 hours and selected times thereafter. Patients in Group 2 were sampled until their discharge at 24 hours.

**4.2.4 Cytokine assays:** A detailed description of these assays is given in section 3.7. Interleukin-6, IL-1 and IFNγ were measured using commercially available "sandwich" ELISA. Tumour necrosis factor was measured by ELISA using a murine monoclonal antibody as previously

described (Kwiatkowski D et al., 1990). In each assay a standard curve using recombinant cytokine was constructed and each sample was assayed in duplicate. The minimum sensitivities of the assays were: IL-1 and IFNγ 3pg/ml; IL-6 4pg/ml; TNF 10pg/ml. In addition, 20 random samples were assayed independently for IFNγ using a different ELISA at the National Institute for Biological Standards and Controls, Hertfordshire, England (minimum sensitivity 10pg/ml).

**4.2.5 C reactive protein assay:** This was measured using flow nephelometry (Array Protein System, Beckman Diagnostics) with a minimum detectable level of 6mg/l.

## 4.3 RESULTS

**4.3.1 Clinical:** Seventeen patients had an uncomplicated post-operative course. Three patients suffered profound complications. The first (patient 1), who had chronic obstructive airways disease, was thought to be progressing well after uncomplicated surgery until he developed hypotensive shock at 36 hours. In view of associated abdominal distension and no evidence of sepsis, a relook laparotomy was performed at 50 hours which was normal. Shortly thereafter pus was aspirated from the bronchial tree and antibiotic therapy initiated for septicaemia secondary to pneumonia. The patient made a slow recovery and was discharged 3 months after admission.

The second (patient 2) was thought to have done well after a routine operation and was discharged from the Intensive Care Unit after 22 hours. At 36 hours her renal function was noted to be grossly abnormal and she went on to develop intractable renal failure. One week later she infarcted her gallbladder and required an emergency cholecystectomy. Three weeks

later she underwent resection of her left colon for ischaemic colitis. She was discharged to a high dependency nursing home after 7 months.

The third patient (patient 3) had a routine operation and was not admitted to the Intensive Care Unit. At about 24 hours renal failure was noted and he required dialysis. He went on to develop a bleeding peptic ulcer, which required oversewing, and bowel infarction for which he underwent a sigmoid colectomy. He died on post-operative day 13 after developing adult respiratory distress syndrome and respiratory failure.

All five hernia patients underwent a routine operation and were discharged on the first post-operative day.

**4.3.2 Interleukin 6:** This cytokine was detectable in 5 patients from both groups before surgery. In Group 1 patients this basal level did not increase until a continuous rise began at 90-180 minutes after skin incision in all patients. Interleukin 6 peaked at four-48 hours (median=eight hours) after the commencement of the operation and had fallen rapidly by 48-72 hours in all patients who had an uncomplicated post-operative course. The three patients who suffered major complications each had a greater and more prolonged IL-6 response (Figure 4.1). In each case this difference was apparent during surgery or within eight hours of incision, which was well in advance of clinical suspicion of complications (Figure 4.2). Figure 4.3 is a plot of peak IL-6 levels in the complicated and uncomplicated patients. This cytokine was not detectable in stored blood prior to transfusion.

In Group 2, the IL-6 response showed similar kinetics to Group 1 and concentrations peaked at four-12 hours (median 8 hours) after commencement of operation. The peak concentrations obtained in the 5

patients were very significantly lower than those seen in Group 1 (6, 20, 21, 51, 37 pg/ml) ($p<0.001$), with the highest value seen in the only patient who underwent bilateral inguinal hernia repair.

Recent criticism of the use of peaked curves in the analysis of serial biological measurements prompted a comparison of these results with the method of summary measures which requires the calculation of the area under the response curve (Matthews JNS et al., 1990). Peak IL-6 levels were found to correlate closely with the IL-6 response as derived from the area under the IL-6 curve ($r=0.88$; $p<0.001$) (Figure 4.4).

**4.3.3 Interleukin 1** was not detected preoperatively in any patient (Figure 4.5). However between one and four hours, a short-lived rise in IL-1 was detected in 17 patients in Group 1. In each patient it preceded the IL-6 response by several hours and disappeared rapidly. An example of this sequential pattern, from a patient without subsequent complications, is given in Figure 4.6. There was no significant difference in the IL-1 response of complicated and uncomplicated patients. This cytokine was not detected in any Group 2 patients.

**4.3.4 Interferon gamma** was not detected by either ELISA in any samples either before, during or after surgery.

**4.3.5 Tumour necrosis factor** was detected in only one sample (42pg/ml) which was taken at 12 hours from one of the complicated patients in Group 1 (patient 3).

**4.3.6 C reactive protein:** In all patients plasma CRP was noted to increase between eight and 24 hours, peaking around 48 hours after incision

(Figure 4.7). Although the CRP values were slightly higher in the complicated patient group, the difference was not statistically significant nor was it apparent until 48 hours, by which time the complications were clinically evident.

Correlations were sought between various clinical data and the cytokine responses (Table 4.1). There was no correlation between the IL-1 and IL-6 responses, nor between either of these cytokines and the post-operative temperatures, WCC and the durations of operation, aortic clamping and ventilation. Weak correlation was seen between the IL-6 response and the APACHE scores, between IL-6 and the blood transfusion volume, and between the peak CRP and IL-1 responses.

Table 4.1: Correlation coefficients between peak IL-1 and IL-6 responses and various clinical parameters.

| | Correlation Coefficients (p values) | |
|---|---|---|
| | IL-1 | IL-6 |
| Peak IL-1 response | -- | 0.019 (0.94) |
| Duration of operation | 0.01 (0.96) | 0.079 (0.74) |
| Aortic clamp time | -0.39 (0.10) | 0.065 (0.79) |
| Duration assisted ventilation | 0.02 (0.47) | 0.41 (0.07) |
| Blood transfusion volume | 0.13 (0.58) | 0.49 (0.03) |
| Total fluid infusion volume | 0.07 (0.08) | 0.42 (0.07) |
| Postoperative temperature | 0.04 (0.89) | 0.04 (0.87) |
| Postoperative leucocyte count | 0.04 (0.67) | -0.19 (0.43) |
| APACHE II score | 0.36 (0.12) | 0.44 (0.05) |
| Peak CRP response | 0.60 (0.02) | 0.37 (0.18) |

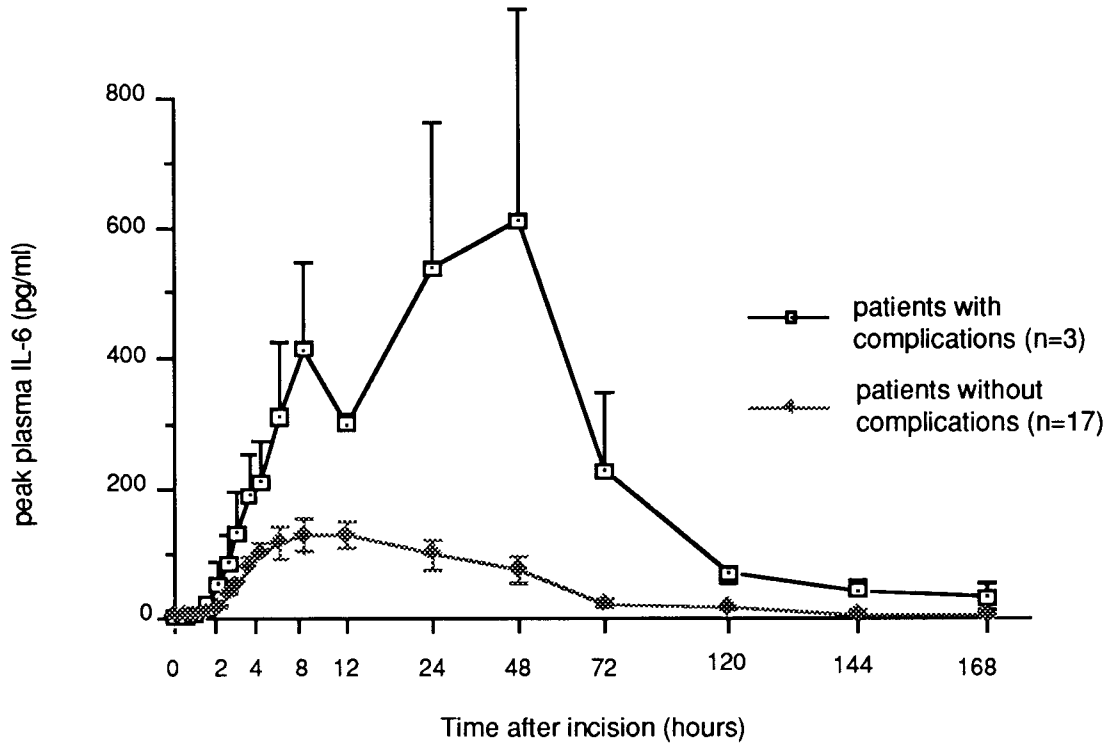



Figure 4.1: The different plasma IL-6 response (mean + SEM) in Group 1 patient subgroups (time 3.5-6hrs:$p<0.05$; time 8-48hrs: $p<0.01$; time 72-120hrs: $p<0.001$).

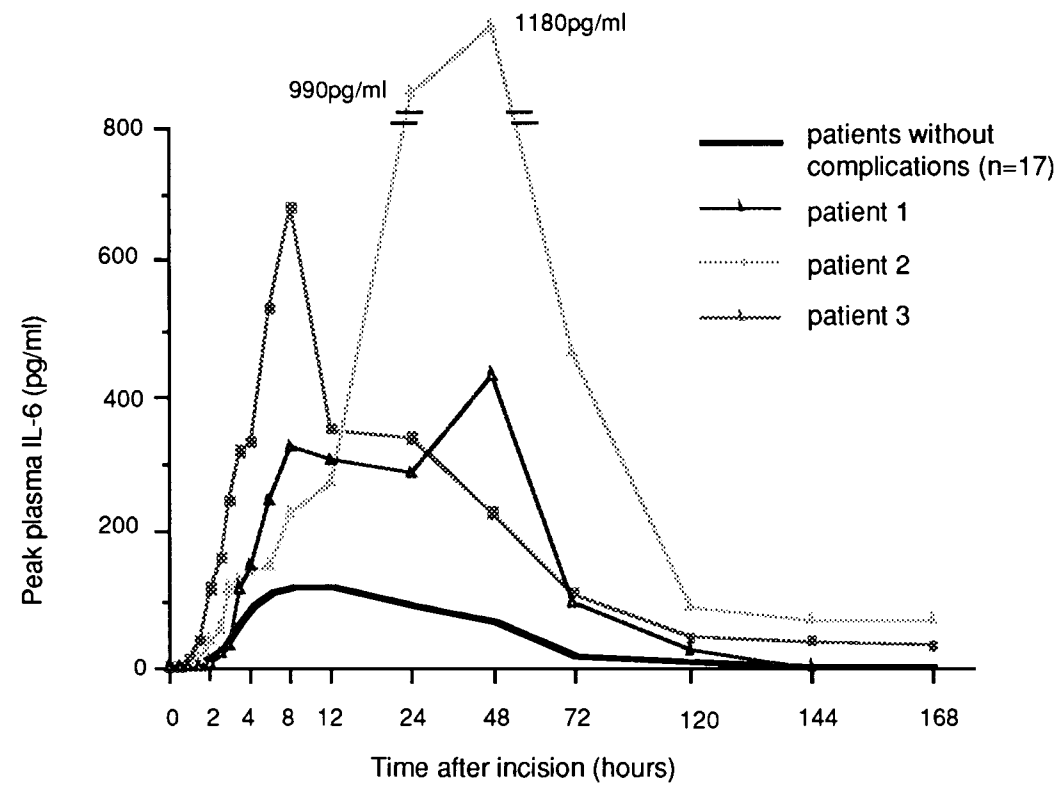



Figure 4.2: The individual plasma IL-6 response in three patients in .Group 1 with major post-operative complications.

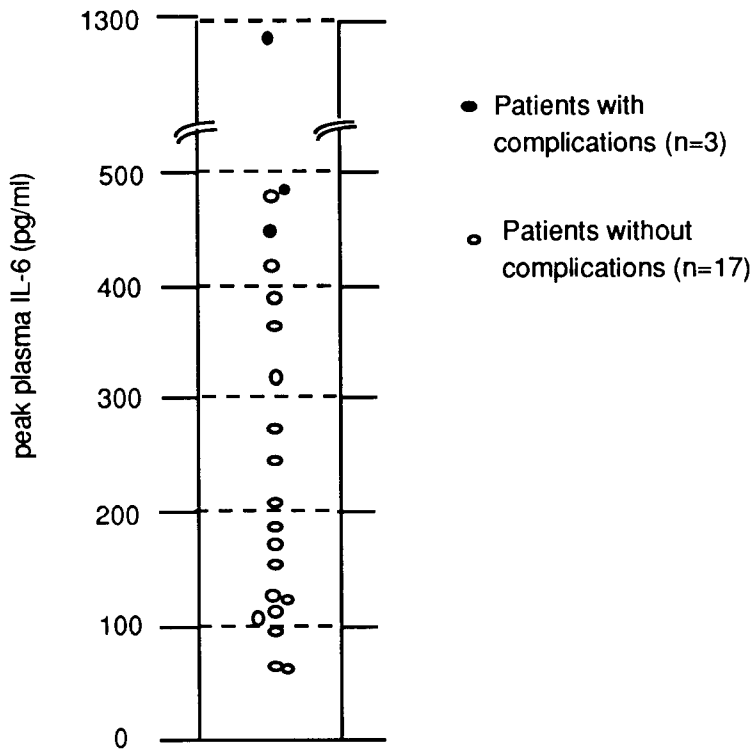



Figure 4.3: Scatter plot of peak IL-6 level in complicated and uncomplicated patients in Group 1.

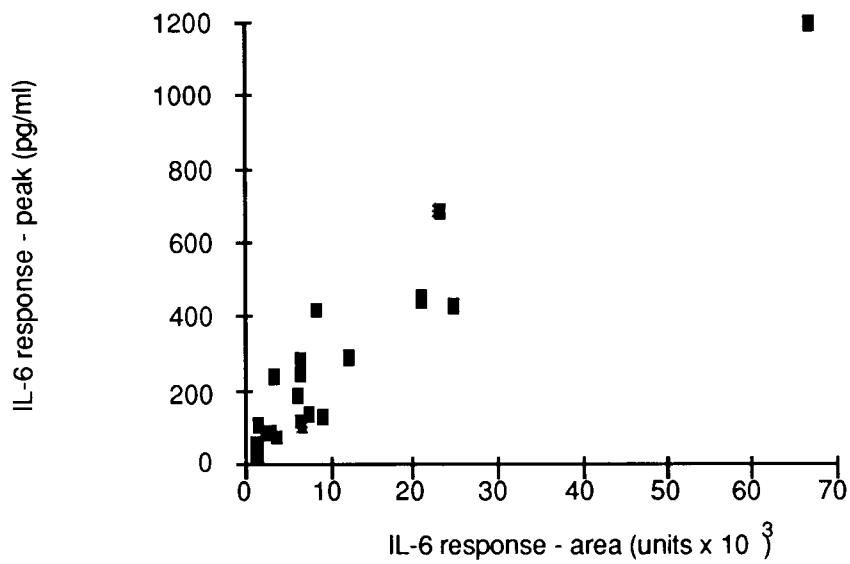



Figure 4.4: Correlation between the IL-6 response area and the peak IL-6 concentration ($r=0.88$; $p<0.001$).

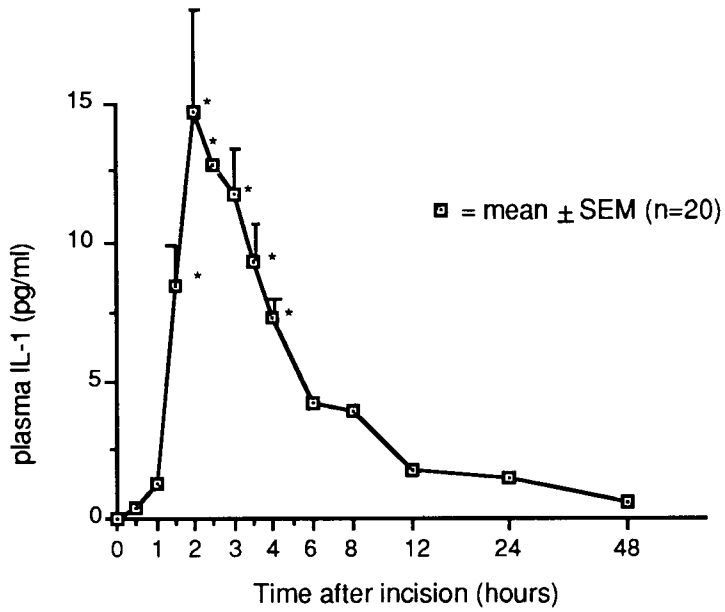



Figure 4.5: The plasma IL-1 response in Group 1 patients. * = significantly different from time 0.5 hrs ($p<0.001$).

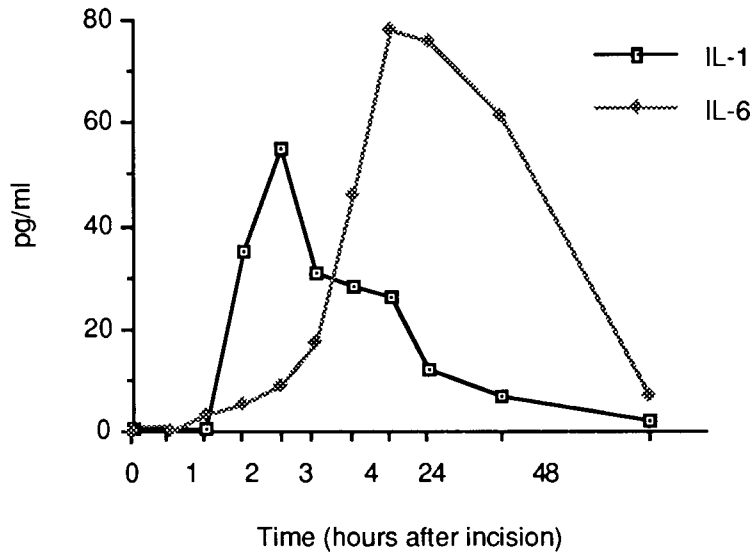



Figure 4.6: An example in one patient of the plasma IL-1 response preceding IL-6 after major surgery.

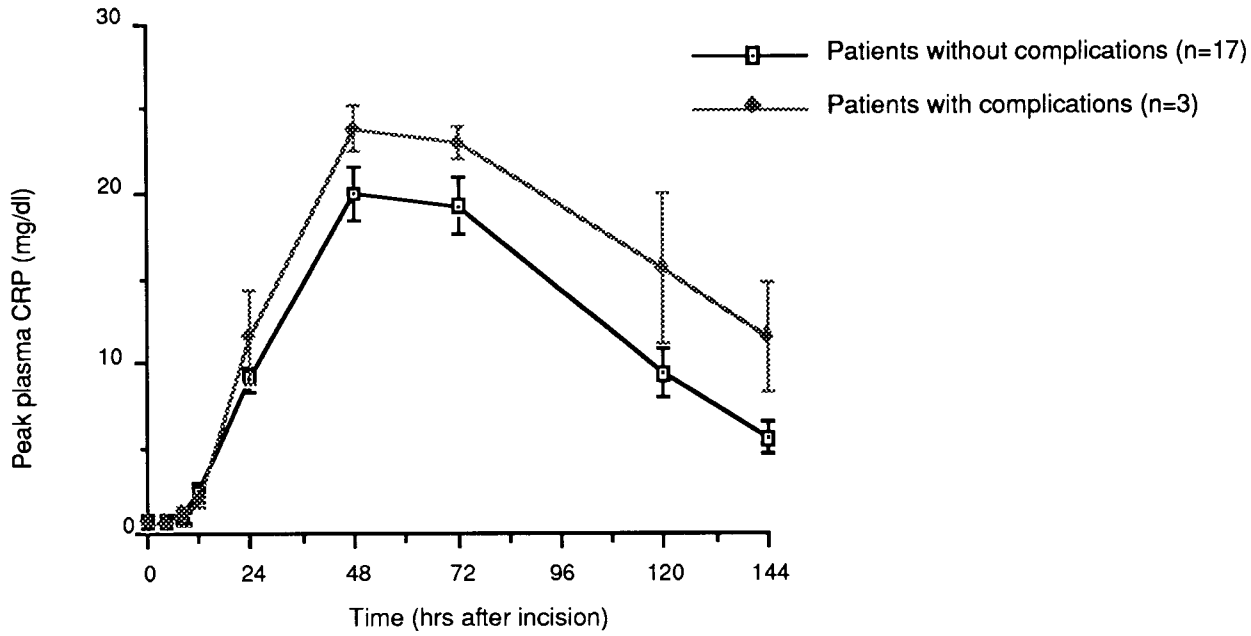



Figure 4.7: The plasma CRP response (mean + SEM) in Group 1 patients (n.s.).

## 4.4 DISCUSSION

An early and brief IL-1 response to major surgical injury has been shown. This always preceded the IL-6 response in any individual patient. These data provide *in vivo* evidence consistent with the *in vitro* observation that IL-1 induces IL-6 synthesis and release (Bauer AR et al., 1978)(Kohase M et al., 1986)(Tosatu G et al., 1990)(Shalaby MR et al., 1989). While the absence of any correlation between the IL-1 and IL-6 response in certain patients could be viewed as less consistent with this conclusion, it could be explained by the action of IL-1 being more local than IL-6, with a less consistent overflow into the peripheral circulation. An IL-1 receptor antagonist (IL-1ra) has recently been described (Hannum CH et al., 1990) and the lack of correlation between the IL-1 and IL-6 response could also be explained thus: differences in IL-1ra concentration cause similar IL-1 concentrations to induce a different IL-6 response in different patients. In

view of the recent development of an IL-1ra assay it is hoped to test this hypothesis in the near future.

Interleukin-1 has only occasionally been detected in the peripheral plasma, for example in four patients with meningococcal septicaemia (Baumann H et al., 1990). In most studies where IL-1 was not detected, the patients were only sampled daily and the period when detectable IL-1 was present in these patients could easily have been missed. In another study in which human volunteers were administered TNF and endotoxin, IL-1 was not detected in the circulation (Michie HR et al., 1988a)(Michie HR et al., 1988b), but no samples were taken between one and three hours, the time at which the results reported in this chapter suggest that IL-1 might have been present. Alternatively, the radioimmunoassay used in the latter study may have been less sensitive than the ELISA used in the present work. It is clear from these results that an IL-1 response will be detected only by frequent sampling of the intra-operative or equivalent period after a major insult, as represented by major aortic surgery.

The IL-6 response seen in the uncomplicated patients is similar to those published in two recent reports (Cruickshank AM et al., 1990)(Pullicino EA et al., 1990), but of interest are the patients who had a complicated post-operative course. Their IL-6 levels rose above the mean within a few hours of incision, well in advance of their subsequent complications. In each case there was no suspicion of poor progress until 24-48 hours had passed. Their IL-6 response not only preceded their clinical signs by 12-36 hours but was also significantly greater and persisted longer than the response seen in the uncomplicated group.

An exaggerated IL-6 response after major surgery was associated with the development of significant complications in this small series. Interleukin-6 is released by most tissues in the body and its plasma concentration may therefore provide a measure of tissue damage during injury. The smaller IL-6 response seen in the Group 2 hernia patients is consistent with this hypothesis. The surgeon is unable to assess tissue damage at the cellular level resulting from harmful influences such as ischaemia and pH change. This tissue damage may induce the observed early rise of IL-6, either directly or via IL-1 mediation. Such intra-operative changes may have a direct impact on the subsequent development of major complications and are reflected in the correlation between IL-6 and APACHE II scores. Thus the high levels of IL-6 seen may represent a relatively passive accessory cell response reflecting the degree of tissue damage or alternatively a more active elaboration of the cytokine in an attempt to ameliorate the adverse factors giving rise to later complications.

There have been many attempts to correlate isolated cytokine levels with patient prognosis, the most notable of these being the detection of TNF. Although the increases have not been consistent, circulating levels of TNF have been associated with poor clinical outcome in septic or burnt patients (Kwiatkowski D et al., 1990)(Girardin E et al., 1988)(Hack CE et al., 1989)(Debets JMH et al., 1989). Serum levels of TNF, IL-1 and IFNγ were shown by Girardin et al to correlate with the severity of meningococcaemia and they suggested that the level of TNF may have prognostic value in this disease. Debets et al in Holland,reported a study of critically ill septic patients which demonstrated that sepsis was accompanied by detectable circulating TNF in 25 percent of patients and for those patients mortality was twice that of comparable TNF negative patients. On the other hand, serum levels of TNF have also been shown to

have negligible impact on the prediction of outcome when assessed in conjunction with simple clinical and laboratory variables (Calandra T et al., 1990). Despite intensive sampling and the use of a sensitive immunoassay, TNF was not detected in this study nor in a similar study of trauma patients (Pullicino EA et al., 1990). Nonetheless TNF has been detected in plasma 90-120 minutes after endotoxin administration (Michie HR et al., 1988a) which corresponds to the period of IL-1 appearance in this study. It is possible therefore, that a very low concentration of TNF may be present which, along with IL-1, may be involved with IL-6 induction at this time. Alternatively endotoxin may induce different cytokine responses from surgical trauma.

Plasma IL-6 has been shown by others to correlate well with duration of surgery when either cholecystectomies alone or a selection of operations, including hip replacement, colorectal, vascular and minor surgery were studied (Shenkin A et al., 1989)(Cruickshank AM et al., 1990). However, in this study of a single major surgical procedure, namely replacement of an abdominal aortic aneurysm, no correlation with length of surgery was observed. It seems reasonable to assume that the duration of many operations influence the extent of surgical tissue injury and therefore perhaps IL-6 release. This assumption may not be as true of aneurysm surgery in which ischaemia and marked changes in blood pressure may be more important determinants of cell damage than duration of surgery. The blood transfusion requirement may also be a better reflection of surgical trauma than operative duration and there was a significant correlation between IL-6 and blood transfusion requirement in this study.

The CRP response confirms that described in previous studies (Colley CM et al., 1983)(Stahl WM, 1987). Moreover the 20-30 hour delay between the

appearance of the cytokines and the CRP response is consistent with the *in vitro* and *in vivo* evidence that IL-1 and IL-6 are mediators of acute phase proteins synthesis (Geiger T et al., 1988)(Gauldie J et al., 1990). The results of this study suggest that CRP is less sensitive than IL-6 as a marker of surgical complications. Moreover the comparatively slow CRP response times of about 48 hours mean this parameter has little predictive value in this setting. These CRP findings are in line with clinical experience, where its value in the early post-operative period has proved limited.

The source of IL-1 and IL-6 released in patients undergoing surgery remains to be established, although data presented in Chapter 7 implicates the bowel as the major organ producing IL-6. Preliminary studies appear to rule out cytokine induction by endotoxaemia in this setting (Chapter 7) and peripheral blood mononuclear cells do not increase IL-6 secretion in response to surgery (Chapter 8).

This study demonstrates that the IL-1 and IL-6 response increases with the severity of the surgical insult and suggests that IL-6 levels are maximal in patients who go on to develop major complications. Routine measurement of IL-6 after major surgery may have a valuable role in identifying patients in need of careful post-operative monitoring. Further studies are needed to determine whether the plasma IL-6 concentration is simply reflecting the degree of tissue insult or whether it is playing a more active role in either the induction of, or defence against, post-operative complications.

## 4.5 SUMMARY

The systemic cytokine response to major surgical trauma has been studied in 20 patients undergoing elective aortic surgery and five patients after inguinal hernia repair.

Tumour necrosis factor alpha and IFNγ were not detected in these patients. An early and short-lived IL-1 response to major surgery was detected only by intensively sampling the peri-operative period. The IL-1 peak preceded a more marked IL-6 response which peaked between four and 48 hours post-operatively. Interleukin-6 levels had fallen sharply by 48-72 hours in all patients who had an uneventful post-operative course. The IL-6 peaks were significantly lower after minor hernia surgery compared to major aortic surgery ($p<0.001$) and IL-1 was not detected in any samples.

Three aortic aneurysm patients developed major post-operative complications not anticipated during surgery. Their IL-6 levels were significantly higher than the other aneurysm patients, even within 6-8 hours of skin incision, and their levels remained elevated longer. These rises in plasma IL-6 levels preceded the clinical onset of major complications by 12-48 hours.

The systemic IL-1 and IL-6 response to surgical trauma increased with the severity of the surgical insult. An early, exaggerated IL-6 response was associated with the subsequent clinical development of major complications.

# CHAPTER FIVE

# THE SYSTEMIC CYTOKINE RESPONSE FOLLOWING MAJOR TRAUMA: A PILOT STUDY

# CHAPTER FIVE

# THE SYSTEMIC CYTOKINE RESPONSE FOLLOWING MAJOR TRAUMA: A PILOT STUDY



## 5.1 INTRODUCTION

The work reported in this chapter is the natural sequel to Chapters 4 and 5, which studied patients undergoing comparatively uniform surgical injury. This study was similar in structure, but the patients studied were all victims of road traffic accident trauma. Abdominal aortic aneurysm patients have a relatively low risk of bacterial contamination and sepsis which allowed assessment of the impact of a major surgical injury on cytokine levels, without the conflicting influence of such bacterial contamination. Major accidental trauma patients, on the other hand, have a higher risk of bacterial contamination and sepsis, although it is

obviously impossible to obtain baseline cytokine levels on these patients prior to their trauma.

## 5.2 PATIENTS AND METHODS

### 5.2.1 Patients

The victims of major trauma were all admitted via the Accident and Emergency Department of the John Radcliffe Hospital, between January 1991 and July 1991. It is department policy to transfer to the Regional Neurosurgical Unit at the Radcliffe Infirmary, those with major head injuries and relatively minor involvement of other systems. These patients were therefore excluded. Severely injured patients with minor head injuries, or those with both major head injuries and major injuries of other systems were included. This last group were managed on the intensive care unit of the John Radcliffe Hospital. All major burns patients are admitted directly to the Regional Burns Unit in Aylesbury and therefore thermally injured patients were not available for inclusion. Using these criteria, six patients were studied. At admission, patients were assigned an Injury Severity Score (ISS) using the Abbreviated Injury Scale (AIS) scoring system (Greenspan L et al., 1985).

### 5.2.2 Samples and sampling times

Patients were sampled as soon as possible after admission, and thereafter along a longitudinal time course as before: 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120 hours and selected times thereafter. Samples were taken from a peripheral or central vein and prepared for cytokine analysis as described in section 3.3.

### 5.2.3 Cytokine assays

Cytokines were assayed using the methods described in section 3.7, using commercially available "sandwich" ELISA for IL-1 and IL-6 (IL-6 by R&D Systems, from British Biotechnology Ltd, Oxford, England and IL-1 from Medgenix Diagnostics, High Wycombe, Bucks, England). Tumour necrosis factor was measured by ELISA using a murine monoclonal antibody. In each assay a standard curve using recombinant cytokine was constructed and each sample was assayed in duplicate. The minimum sensitivity of the assays were: IL-1 3pg/ml, IL-6 4pg/ml and TNF 10pg/ml.

## 5.3 RESULTS

**5.3.1 Clinical:** Six male patients were studied and their injuries are summarised in Table 5.1. All were road traffic accident victims. Five underwent emergency surgery, of which four were solely for orthopaedic indications and one required a thoracotomy to repair an aortic arch tear. There was one death, specifically the single patient who did not undergo surgery. Under normal circumstances this patient would have undergone laparotomy for a major retroperitoneal haemorrhage, but his neurological prognosis was assessed as very poor and it was decided to provide only supportive management. Although his haemodynamic condition stabilised and he stopped bleeding, life-support was withdrawn 24 hours after admission when he was confirmed brain dead.

**5.3.2 Interleukin-6:** Plasma levels of this cytokine varied greatly, with peak levels ranging from 180 to 9 000pg/ml (Figure 5.1).

Patient DS, who had relatively stable injuries requiring minimal initial resuscitation, had a small IL-6 response which was declining until he underwent major surgery to his thoracic aorta. This was followed by an IL-6 peak. A subsequent Klebsiella septicaemia was also accompanied by a

Table 5.1 Synopsis of patients' injuries, blood transfusion requirements in the first 24 hours, injury severity scores and outcome. Abbreviations: C=compound; F=fracture; ISS=injury severity score.

| Patient | Age | Injuries & blood requirements | ISS | Outcome |
|---|---|---|---|---|
| JG | 21 | Bilateral C F tibia & fibula; C F femur & humerus; avulsion brachial plexus; myocardial contusion; disrupted pelvis; pneumothorax; F ribs; head injury; 52 units blood. | 38 | alive |
| CB | 52 | C F tibia; myocardial contusion; Pneumocephalus; minor injuries; 13 units blood | 41 | alive |
| PH | 40 | F clavicle, humerus & lumbar vertabrae; severe head injury; major retroperitoneal haematoma; 6 units blood. | 38 | brain dead |
| DS | 24 | F femur; bilateral small pneumothoraces; transected aorta; 5 units blood; Klebsiella septicaemia on day four. | 38 | alive |
| BM | 49 | F pelvis and ribs; bilateral femurs & ankles; CF radius & ulna; head injury; 10 units blood. | 29 | alive |
| FH | 63 | Flail ribs; haemopneumothorax; F humerus, ankle & mandible; 4 units blood. | 24 | alive |

major rise in levels of IL-6. Surgery (bilateral femoral nailing) also affected the response in patient BM, but had little effect when it was less traumatic, as seen in Patient FH who underwent plating of his mandible and patient CB who had external fixators applied to his tibia.

Patient JG suffered by far the most extensive soft tissue damage. His initial level of 2 200pg/ml was prior to significant resuscitation but over the next 16 hours he received 50 units of blood. Despite this rapid and continuous replacement of his blood volume with stored blood (which contains no IL-6), which continued during prolonged orthopaedic surgery, IL-6 levels remained above 500pg/ml for more than 48 hours.

Patient PH, who died, had by far the greatest IL-6 response (peak level=9000pg/ml) despite apparently having less soft-tissue damage than patient JG. However, he did not undergo laparotomy which meant the extent of injury to the gut, kidneys and other organs resulting from the retroperitoneal haemorrhage could not be assessed.

**5.3.3 Interleukin-1:** While IL-1 again proved detectable with frequent sampling, the profile of this cytokine was neither consistent nor similar to that seen in Chapter 4 (Figure 5.2). A high IL-1 peak in response to surgery was seen in only two patients, DS and CB. An early, brief IL-1 response was seen in another two patients, PH and BM, while FH and JG had detectable IL-1 only at 12 hours. Four patients were incompletely sampled within the first two hours of injury when a brief IL-1 response may have been detectable.

**5.3.4 Tumour necrosis factor:** Within four hours of injury TNF was detected in all six patients (Figure 5.3). In five patients it was detected in the first sample obtained. There appeared to be a pattern of an early TNF peak followed by an absence or a decline in plasma levels. Every patient

had at least one sample in which the cytokine was not detectable above the minimum sensitivity of the assay, indicating that none of these patients were intrinsic producers of TNF as discussed in section 2.3.4. The TNF concentration rose above 100pg/ml (highest concentration=263pg/ml at 3 hours) in only one patient, PH, who was also the only patient to die. This patient also had by far the greatest IL-6 response (peak concentration=9000pg/ml at 6 hours). Tumour necrosis factor was not detected in association with the Klebsiella septicaemia in patient DS, although sampling was infrequent at this time and a brief response may have been missed.

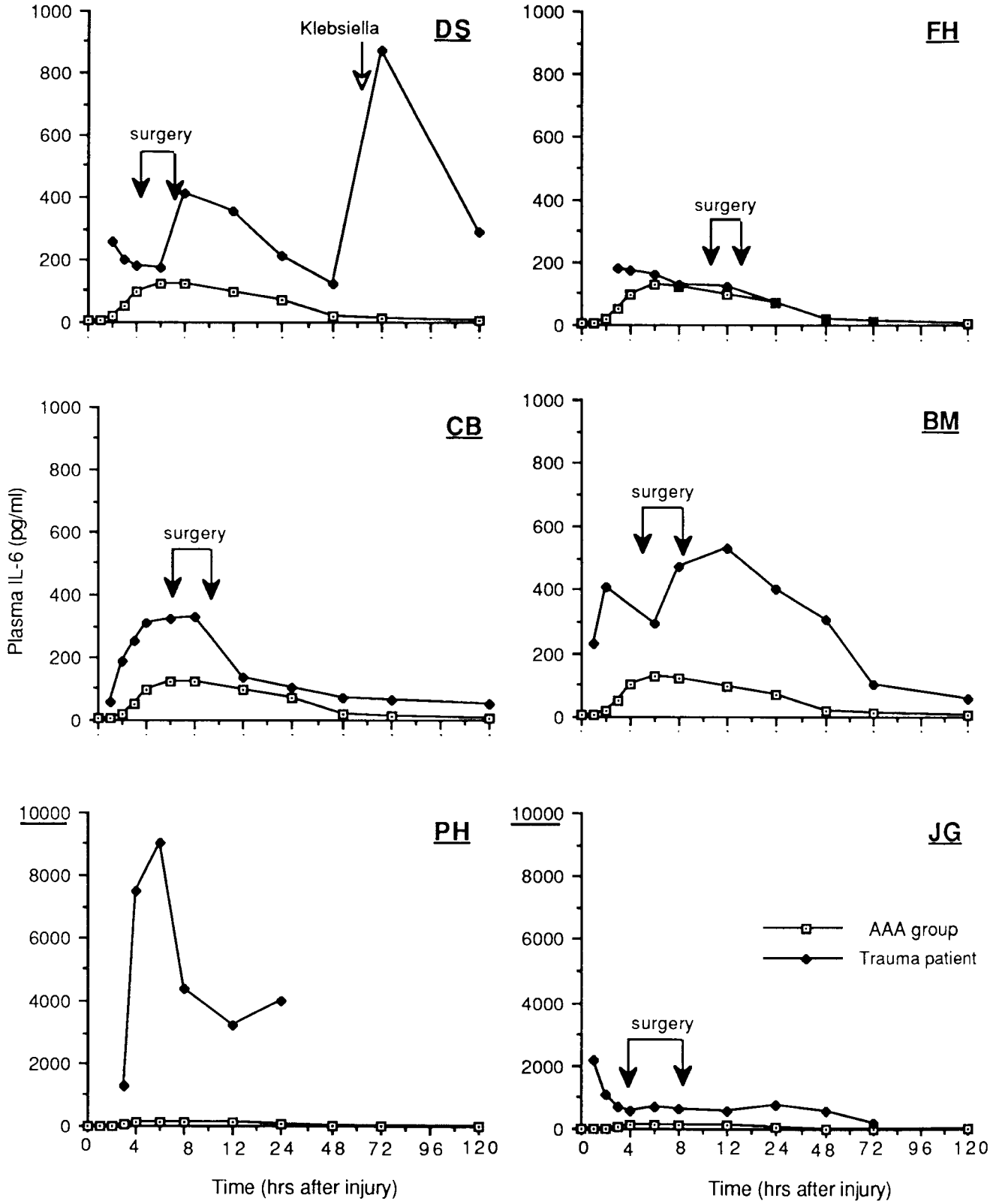



Figure 5.1 The plasma IL-6 response of six accidental trauma patients, compared with the IL-6 response of the 17 uncomplicated abdominal aortic aneurysm (AAA) patients in Chapter 4. Note that the time scale has been chosen to accentuate the sample times, and is neither linear nor logarithmic. The "y" axis scale extends to 10 000pg/ml in patients PH and JG. Times of surgery and diagnosis of Klebsiella sepsis are indicated.

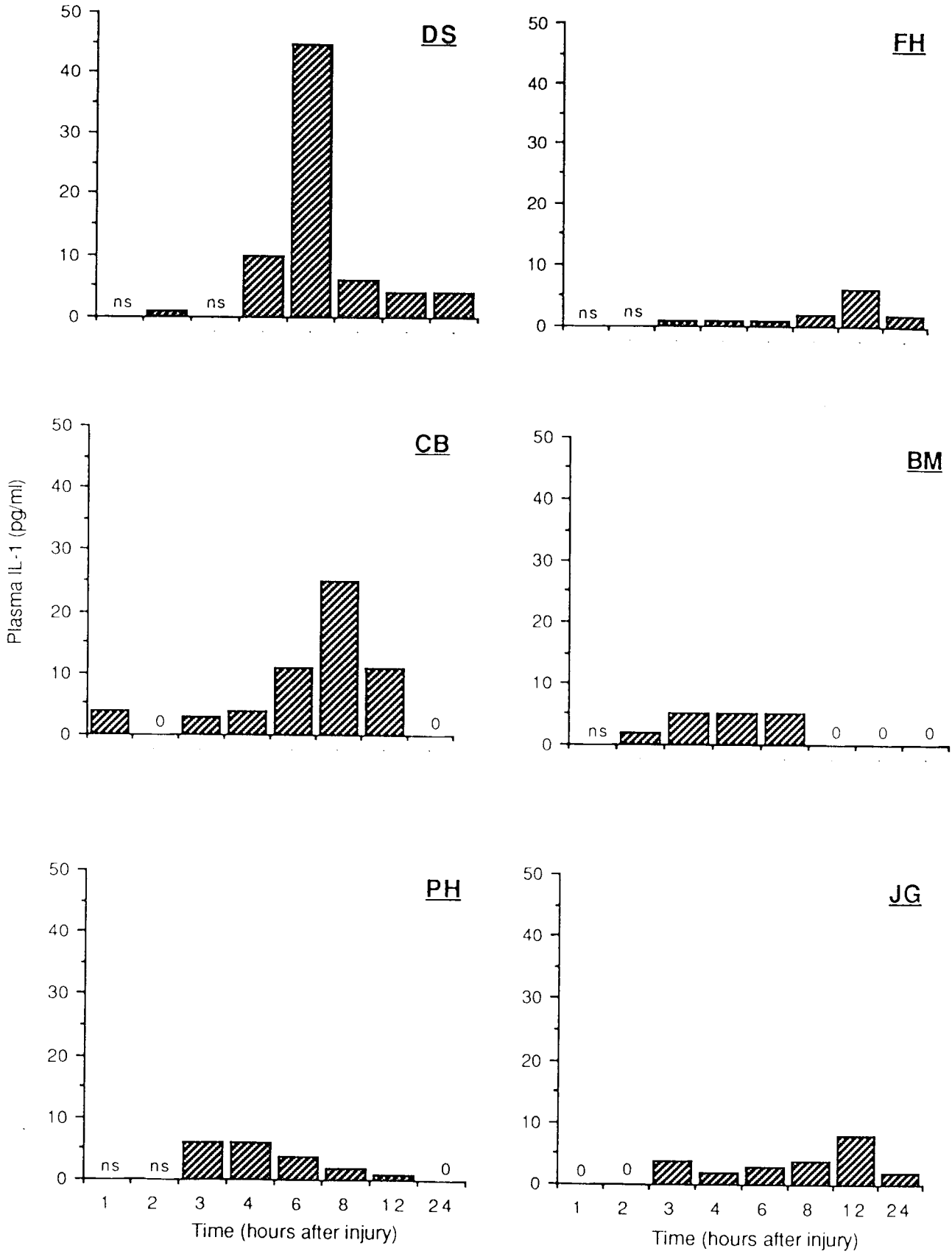



Figure 5.2 The plasma IL-1 response in six accidental trauma patients. Abbreviations: ns=no sample taken at this time point; 0=no IL-1 detected at this time point. Assay minimum sensitivity=3pg/ml.

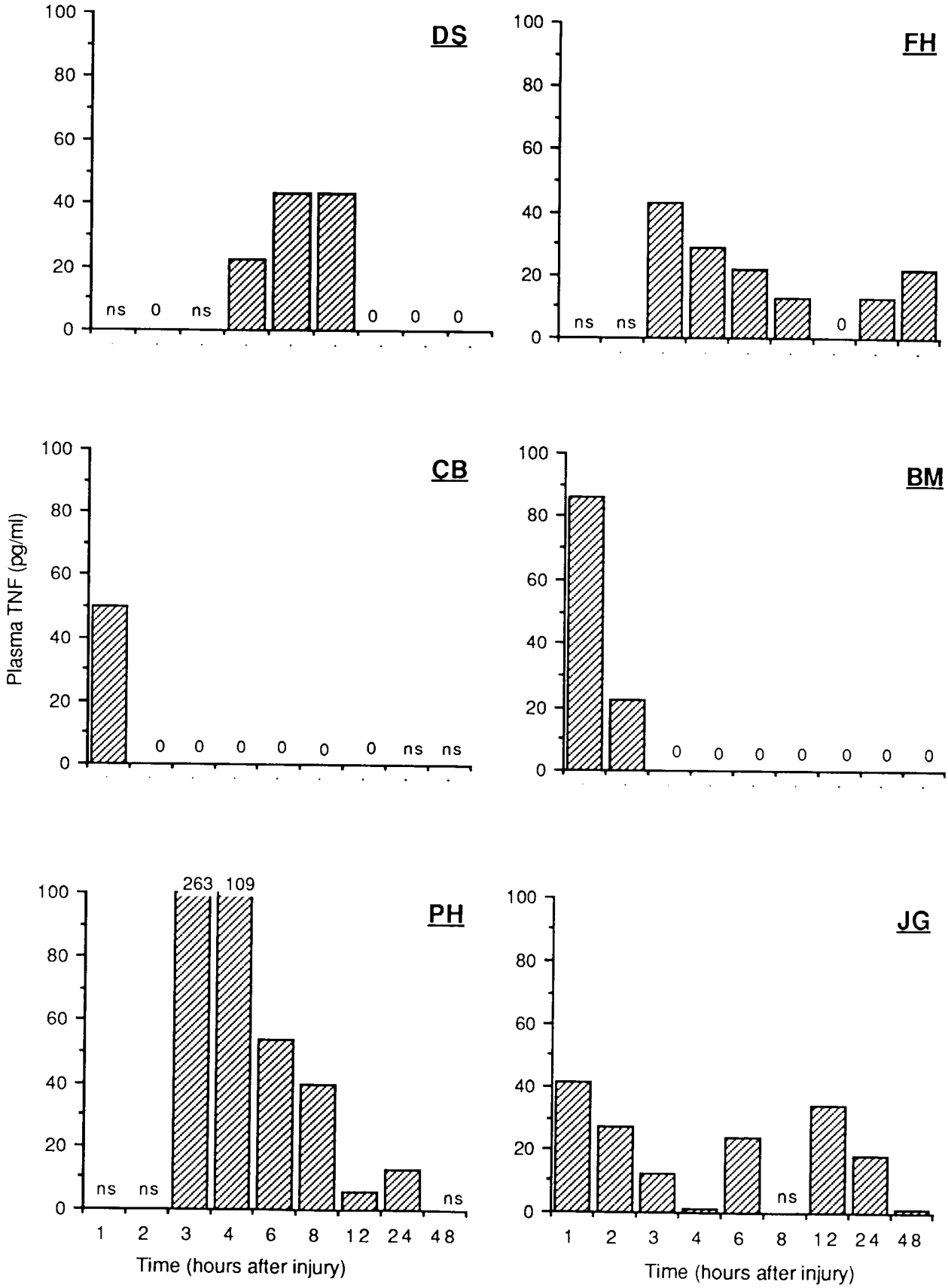



Figure 5.3 The plasma TNF response in six accidental trauma patients. Abbreviations: ns=no sample taken at this time point; 0=no TNF detected at this time point. Assay minimum sensitivity=10pg/ml.

## 5.4 DISCUSSION

This small pilot study has demonstrated wide variability in cytokine responses in major trauma patients, but has provided several useful conclusions.

It has emphasised the value of a comparatively uniform cohort of patients (as in Chapter 4) in a study of cytokine elaboration. An initial study of 20 major accident trauma patients would have been unlikely to yield as much useful information as the aneurysm study. To increase the likelihood of any patterns of cytokine production emerging in this heterogeneous group, it would be necessary to study a very large number of patients or, alternatively, focus on particular categories of injury such as burns or neurological damage. For this reason the pilot study was concluded after only six patients. Only one patient developed major sepsis and this was accompanied by a rise in IL-6. Again, more information on trauma patients developing sepsis would require large numbers.

This study has confirmed that tissue injury results in major IL-6 release within a few hours of injury. In addition, minor surgery did not to induce the significant IL-6 response seen in patients undergoing extensive orthopaedic and thoracic surgery. These results suggest that, in a larger study, a correlation may be found between IL-6 and severity of tissue damage. In contrast, the ISS attempts to score injuries according to expected outcome and does not take cognisance of severity of tissue damage, for example, one fractured femur is scored identically with bilateral compound tibial fractures plus bilateral fractured femurs. Therefore, as this small series suggests, plasma IL-6 would be unlikely to correlate with the ISS in a large series.

Several trauma patients had levels of IL-6 far in excess of the complicated patients in Chapter 4 and yet had an uncomplicated clinical course. This observation is consistent with the hypothesis that IL-6 levels simply reflect tissue damage and this cytokine does not mediate adverse inflammatory events. As mentioned in Chapter 2, evidence that IL-6 adversely influences cardiovascular stability or cellular integrity has not been found, so that the major effects of this protein appear to be beneficial to the host by enhancing immune function and acute phase protein synthesis (van Snick J, 1990). It may also prove to be clinically useful, serving as a circulating measure of cell damage.

Interleukin-1 again proved to be detectable provided patients were sampled regularly. Levels were usually low, probably reflecting "overflow" into the plasma from sites of its production. Late peaks (12 hours) were seen in two patients. The diverse nature of these injuries compared to those in the aneurysm patients, means that ongoing haemorrhage, hypotensive episodes and changes in tissue tension could all be responsible for these secondary peaks of cytokines.

The detection of TNF was an important finding, for it proved that this cytokine is detectable in the plasma of trauma patients using the ELISA described. It suggests that the trauma suffered by the aneurysm patients was simply insufficient to generate an overflow of TNF into plasma. Moreover, its absence in the plasma of the three complicated aneurysm patients argues against it having any predictive association with major morbidity, as seen with IL-6. Tumour necrosis factor generally appeared early in the clinical course before IL-6. Although this sequential appearance is not as consistent as that seen with IL-1 and IL-6 in the previous chapter, if one takes into consideration the diversity of the

injuries, then this finding could be said to support the *in vitro* evidence of IL-6 induction by TNF.

Data in Chapter 7 will show that the bowel is the major source of IL-6 in patients undergoing abdominal aortic surgery. Five patients in this study definitely did not have significant bowel injuries, yet produced very high levels of IL-6. This may provide indirect evidence for the elaboration of cytokines by a wide variety of tissues besides bowel, and this will be discussed further in Chapter 10. Alternatively splanchnic shutdown, in response to the injuries, could result in Il-6 production by the gastro-intestinal tract. However it is interesting to speculate over the source of the prodigious IL-6 response in patient PH who had severe head injuries, but appeared to have comparatively little soft-tissue damage. The possibility of undiagnosed intestinal ischaemia has been mentioned, but studies have also shown that brain tissues readily produce IL-6, and elevated levels of IL-6 have been noted in the cerebrospinal fluid of patients with a wide range of neurological diseases, including bacterial and viral meningitis and encephalitis (Frei K et al., 1989)(Helfgott DC et al., 1989). It could prove clinically useful to explore the possibility of a correlation between IL-6 production and the severity of brain damage. On the basis of this, a pilot study of plasma IL-6 levels in patients undergoing major brain surgery is planned.

There is only one report in the English language literature about plasma cytokines in non-thermal accidental trauma patients. Moore et al studied trauma patients requiring laparotomy and measured their portal and systemic cytokines (Moore FA et al., 1991). Therefore this paper is reviewed at length in Chapter 7. Researchers in this field have tended to concentrate on *in vitro* lymphocyte or macrophage studies (Browder W et

al., 1990)(Ertel W et al., 1990)(Faist E et al., 1988)(Rodrick Ml et al., 1986). Others have studied specific models of trauma such as elective surgery (discussed in Chapter 4) or burns. Nijsten et al followed 13 patients with 30 percent burns and correlated IL-6 with temperature and acute phase proteins. The IL-6 response, measured with a bioassay, was remarkably uniform (Nijsten MWN et al., 1991). Others attempted to show that the presence of TNF in plasma in critically ill burns patients correlated with sepsis and mortality (Marano MA et al., 1990).

This pilot study has suggested that a great deal of information would emanate from a large, well constructed study of cytokine elaboration and clinical parameters in trauma victims. In order to recruit sufficient numbers, a multicentre trial would almost certainly be required.

# CHAPTER SIX

# ENDOTOXIN AND CYTOKINE STUDIES ON THE PORTAL BLOOD OF MAJOR SURGERY PATIENTS

# CHAPTER SIX

# ENDOTOXIN AND CYTOKINE STUDIES ON THE PORTAL BLOOD OF MAJOR SURGERY PATIENTS



## 6.1 INTRODUCTION

Major surgical and other trauma induces the release of IL-6 into the circulating blood (Chapters 4, 5 and 6) and this cytokine is produced by innumerable cell types (Section 2.4.5). However, in the steady state IL-6 is not produced constitutively by normal cells which require a stimulus to begin production. Cytokine production is part of a cascade of mediator responses (Chapter 2) and the main stimuli, earlier in the cascade, for the production of IL-6 and other cytokines in the injured patient have not been demonstrated *in vivo*.

*In vitro* IL-6 expression has been shown to be induced by a variety of factors, including viral infection and endotoxin (Helfgott DC et al., 1987). In addition a variety of cytokines including IL-1, TNF, platelet derived growth factor, IL-3, GM-CSF and IFNγ (van Snick J, 1990) also induce IL-6 production. However not all cells respond similarly to all these factors: for example, IL-1 is probably the most potent inducer of IL-6 in fibroblasts but has little effect on bone marrow cells which, in contrast, respond very well to IL-3 or GM-CSF.

### 6.1.1 The biology of endotoxin

Lipopolysaccharides (LPS), whose toxic properties account for their other name, endotoxins, were first identified and purified in 1943 by Shear (Shear M et al., 1943) and are constituents of the outer membrane of Gram-negative bacteria (Ryan JL, 1985). They are now known to consist of a polysaccharide and a covalently bound lipid, designated lipid A, which is thought to mediate most of their biological effects. The biochemical structure of lipid A from different Enterobacteriaceae and Pseudomonaceae are similar. Endotoxins have been implicated as a causal factor in complement and coagulation activation, the generation of vasoactive kinins and the induction of cytokine mediators (Morrison DC et al., 1987).

The gut contains large amounts of Gram-negative bacteria, that continuously shed endotoxins. Under normal circumstances, the intestinal mucosa permits only the selective passage of nutrients into the circulation, while maintaining a protective barrier against the absorption of various intra-luminal particles, including bacteria and their toxins. The impermeability of the cellular membranes and the tight junctions between the mucosal cells play an essential role in this function. Low flow states

have been shown to cause the disruption of the gut mucosal barrier (Gurd FN, 1965). Hypoxia per se has been held to be the key factor causing the ischaemic mucosal lesion. However there has been recent evidence to suggest that reperfusion after an episode of ischaemia releases oxygen free radicals responsible for increasing the severity of the mucosal injury (Deitch EA et al., 1988). During abdominal aortic aneurysm surgery the aorta is cross-clamped and, even in the presence of a chronically occluded inferior mesenteric artery, there is at least 30 to 60 minutes of partial intestinal ischaemia.

The mechanism of endotoxin clearance in man is not well understood, but involves binding to high-density lipoproteins and naturally occurring anti-endotoxin antibodies as well as detoxification in the Kupffer cells of the liver (Saadia R et al., 1990). The portal vein is considered the most important absorption route, but lymphatic transport has also been identified in dogs and rats (Olofsson P et al., 1986).

There have been several studies of endotoxin levels in portal and peripheral blood and results have varied widely. Jacobs et al studied patients undergoing laparotomy and concluded that endotoxins were a normal constituent of portal blood, as 97 per cent of their portal blood samples yielded a positive LAL assay (Jacob AI et al., 1977). Prytz et al drew a similar conclusion after a study of laparotomy patients (Prytz H et al., 1978). A Danish group detected high levels in all their patients (up to 64pg/ml) in both peripheral and portal blood drawn during aortic cross-clamping (Andersen LW et al., 1988). In patients undergoing surgery for Crohns disease (Dr I Halliday, Belfast; personal communication) and in post-colonoscopy patients (Kelley CJ et al., 1985), significant endotoxaemia was measured.

Thus the classical hypothesis has always been that continuous endotoxin transmigration through the bowel mucosa, into the portal circulation, is a normal physiological event and systemic endotoxaemia is prevented by removal of these lipopolysaccharides by the Kupffer cells of the liver (Nolan JP, 1981). However some recent research has questioned this dogma. Brearley et al detected no endotoxin, or only very low levels (5-10pg/ml) in portal blood prior to cholecystectomy (Brearley S et al., 1985). van Deventer et al detected no endotoxin in peripheral and portal samples from cholecystectomy patients and only low levels (5-7pg/ml) in a small minority of peripheral and portal samples taken during aortic cross-clamping (van Deventer S et al., 1988a).

The role of endotoxin in liver disease itself remains controversial. Although the importance of endotoxin in the experimental animal in relation to liver disease is well established (Nolan JP et al., 1968)(Bhagwandeen et al., 1987), its place in clinical practice remains controversial. The development of the chromogenic LAL assay has not solved the controversies in liver disease any more than it has in other clinical settings. Recent publications have reported no less than a 1000-fold difference in endotoxin levels in cirrhotics using this technique (Triger DR. 1991). Although plasma endotoxin levels have recently been shown to be higher in alcoholic cirrhosis than in non-alcoholic cirrhosis, concentrations were significant in the latter (Fukui et al., 1991). In the same way, while high levels of circulating endotoxins are undoubtedly associated with terminal liver failure (Wilkinson et al., 1974), such an association does not necessarily imply causality. The failure of Fukui et al to demonstrate any correlation between peripheral endotoxin levels and

the clinical complications of cirrhosis adds doubt to the validity of the association.

Infusions of endotoxin in animals simulate the changes of clinical infection and injury (Hinshaw LB, 1982). These physiological changes are however not simply the direct effect of the endotoxin, but are orchestrated by a complex network of endogenous mediators amongst which IL-1, IL-6 and TNF are dominant. Hesse et al and Michie et al reported a rise in circulating TNF in endotoxin-challenged volunteers (Hesse DG et al., 1987)(Michie HR et al., 1988a), while the former group and others were able to produce a monophasic rise in circulating IL-6 in man (Fong Y et al., 1989b)(van Deventer SJH et al., 1990)(Spinas GA et al., 1991), peaking two to four hours after the endotoxin challenge and one to two hours after the TNF response. In addition, endotoxin has been shown to enhance IL-6 mRNA expression and IL-6 activity in human fibroblasts and monocytes in culture (Helfgott DC et al., 1987). Thus there is considerable evidence supporting endotoxin as a key mediator of IL-6 production in injured patients.

If endotoxin was indeed the prime inducer of the IL-6 response seen in the abdominal aortic aneurysm patients (Chapter 4), then it seemed likely that its origin would be the colon and its highest concentration would be found in the portal blood. The results of the endotoxin administration studies mentioned above noted a lag-time of two to four hours between endotoxaemia and detectable plasma IL-6. Interleukin-6 levels reported in the abdominal aortic aneurysm patients began their rapid rise about four to six hours after incision. With these kinetics in mind it seemed likely that portal and systemic endotoxin levels would be highest during the first two hours after incision. This also coincides with the timing of

intraoperative aortic cross-clamping when any hypoxia-induced mucosal permeability would logically be initiated.

The aim of the work reported in this chapter was to test a hypothesis that arose out of the findings in Chapter 4: Major injury and colonic ischaemia, which occur during abdominal aortic aneurysm surgery, result in early endotoxin release into the portal blood and this, in turn, induces the cytokine responses seen in these patients. Therefore this chapter reports a study of the endotoxin and cytokine levels in blood samples collected synchronously in both the systemic and portal circulation, at sequential times, in patients undergoing abdominal aortic aneurysm surgery.

### 6.1.2 Endotoxin assays

Twenty seven years ago Drs Frederik Bang and Jack Levin published the initial paper describing blood coagulation and the role of endotoxin in Limulus blood (Levin J et al., 1964). They also described the first *in vitro* assay for the detection of endotoxins in the blood - the Limulus amoebocyte lysate (LAL) gelation assay and used it in the clinical setting (Levin J et al., 1970). However acceptance of the test took some time and not until the 1980s did its use become widespread. Prior to this a number of other assays were used, which have all gradually lost popularity (Table 6.1).

Table 6.1: Other assays which correlate with the LAL test for endotoxin.

| | |
|---|---|
| Pyrogenicity | Chick embryo lethality |
| Dermal Schwartzman reaction | Mitogenicity |
| Tissue factor generation | Complement activation |

The most widely used of these was the rabbit pyrogen test, indeed endotoxin was equated with pyrogenicity. This is probably no longer appropriate as pyrogenicity is one of the safer and better tolerated effects of endotoxin.

*Limulus polyphemus*, the horseshoe crab (Figure 6.1), is the source of the LAL reagent used in the assay. It is not really a crab at all, but is in the phylum Arthropoda (subphylum Chelicerata) and therefore related more to spiders and scorpions than crabs. It can be considered a living fossil and, indeed, is very similar to Trilobitis, a common fossil with a worldwide distribution. This primitive yet successful creature has evolved very little in 300 million years.

The plasma of the crab is blue due to the presence of the oxygen carrying protein haemocyanin, which contains copper (Figure 6.2). Amoebocytes are the only type of circulating blood cell in limulus and are packed with cytoplasmic granules (Figure 6.3). The presence of the entire coagulation mechanism within these granules allows it to be isolated in a convenient package, free of plasma proteins.

The principles and method of the LAL assay are described section 3.8. The original LAL assay was based on the formation of a gel clot, but the use of chromogenic substrates in conjunction with the lysate has become increasingly popular. However, although they increase the sensitivity of the assay these substrates create their own difficulties, because different endotoxins have different relative activities when measured with a single chromogenic substrate and small differences in incubation time (minutes) may alter the results significantly (van Noordwijk J, 1985). These problems

have resulted in numerous assay modifications in various labs, including the development of various kinetic assays.

Figure 6.1: Dorsal view of Limulus polyphemus, the horseshoe crab.

Figure 6.2: The horseshoe crab is bled directly from a heart puncture and then returned to the sea alive. The plasma is blue due to the presence of haemocyanin.

Figure 6.3: Limulus amoebocytes. Three complete cells are shown. Normal amoebocytes are flat discs like mammalian platelets, but are the size of human monocytes. The nucleus occupies the the area devoid of cytoplasmic granules (seen in the two cells on the left).

## 6.2 PATIENTS AND METHODS

### 6.2.1 Patients

Eight patients undergoing elective abdominal aortic aneurysm surgery were studied. No patient had liver disease, inflammatory or ischaemic bowel disease. Preoperative bowel preparation was not performed, but all patients received a prophylactic dose of cefuroxime (1.5gram) at induction.

### 6.2.2 Sampling

Samples were obtained from both a central venous catheter (systemic sample) and the inferior mesenteric vein (portal sample) using a pyrogen-free needle and syringe. Sample collection times are given in Table 6.2. Systemic and portal samples were taken simultaneously and prepared for both cytokine and endotoxin analysis as described in sections 3.3 and 3.8. At least three aliquots of each sample were prepared.

Table 6.2: Sample times during abdominal aortic aneurysm surgery. Sample A was aspirated immediately after induction of anaesthesia, Sample B just prior to cross-clamping of the aorta; Sample C at the end of the proximal anastomosis; Sample D at the end of the distal anastomosis; Sample E just prior to wound closure and Sample F was taken 15 minutes after skin closure.

| | systemic blood | portal blood |
|---|---|---|
| A. Post induction | + | 0 |
| B. Pre cross-clamping | + | + |
| C. During cross clamping (1) | + | + |
| D. During cross clamping (2) | + | + |
| E. Post cross-clamping | + | + |
| F. 15 minutes post-operation | + | 0 |

### 6.2.3 Endotoxin assay

All samples were tested with Coatest Endotoxin (Kabi Diagnostica), a chromogenic LAL assay. A detailed description of methodology is given in section 3.8. All reagents and laboratory utensils were sterile and endotoxin free. All analyses were calculated as mean values of duplicate samples. At the completion of the assay, samples were read at a wavelength of 405nm without wavelength correction and a linear standard curve was drawn using a linear/linear transformation. The detection limit was 3.7pg/ml.

Note: Samples were also assayed in two independent laboratories which used the same LAL assay supplied by Kabi Diagnostica.

### 6.2.4 Cytokine assays

Cytokines were assayed using the methods described in section 3.7, using commercially available "sandwich" ELISA for IL-1 and IL-6 (IL-6 by R&D Systems, from British Biotechnology Ltd, Oxford, England and IL-1 from Medgenix Diagnostics, High Wycombe, Bucks, England). Tumour necrosis factor was measured by ELISA using a murine monoclonal antibody. In each assay a standard curve using recombinant cytokine was constructed and each sample was assayed in duplicate. The minimum sensitivities of the assays were: IL-1 3pg/ml, IL-6 4pg/ml and TNF 10pg/ml.

## 6.3 RESULTS

### 6.3.1 Cytokines

In all patients studied, there was a dramatic difference between systemic and portal IL-6 levels (Figure 6.4 and 6.5). The IL-6 concentration in the portal circulation was significantly higher and the portal-systemic ratio increased with time. The appearance of IL-6 in the portal blood preceded the systemic appearance in only one patient. The levels seen in the

systemic circulation were similar to those in the other group of abdominal aortic aneurysm patients (three patients are shared by both studies) reported in Chapter 4.

Tumour necrosis factor was not detected in any systemic or portal blood samples. Interleukin-1 levels in the portal blood did not differ significantly from those in the systemic circulation (Table 6.3), while the systemic levels were similar to those reported in Chapter 4.

Table 6.3: Mean interleukin-1 levels (pg/ml) in the portal and systemic circulation of eight patients undergoing abdominal aortic aneurysm surgery.

| | systemic IL-1 | portal IL-1 |
|---|---|---|
| A. Post induction | 0 | - |
| B. Pre cross-clamping | 0 | 0 |
| C. During cross clamping (1) | 4 | 3 |
| D. During cross clamping (2) | 8 | 8 |
| E. Post cross-clamping | 11 | 9 |
| F. 15 minutes post-operation | 14 | 13 |

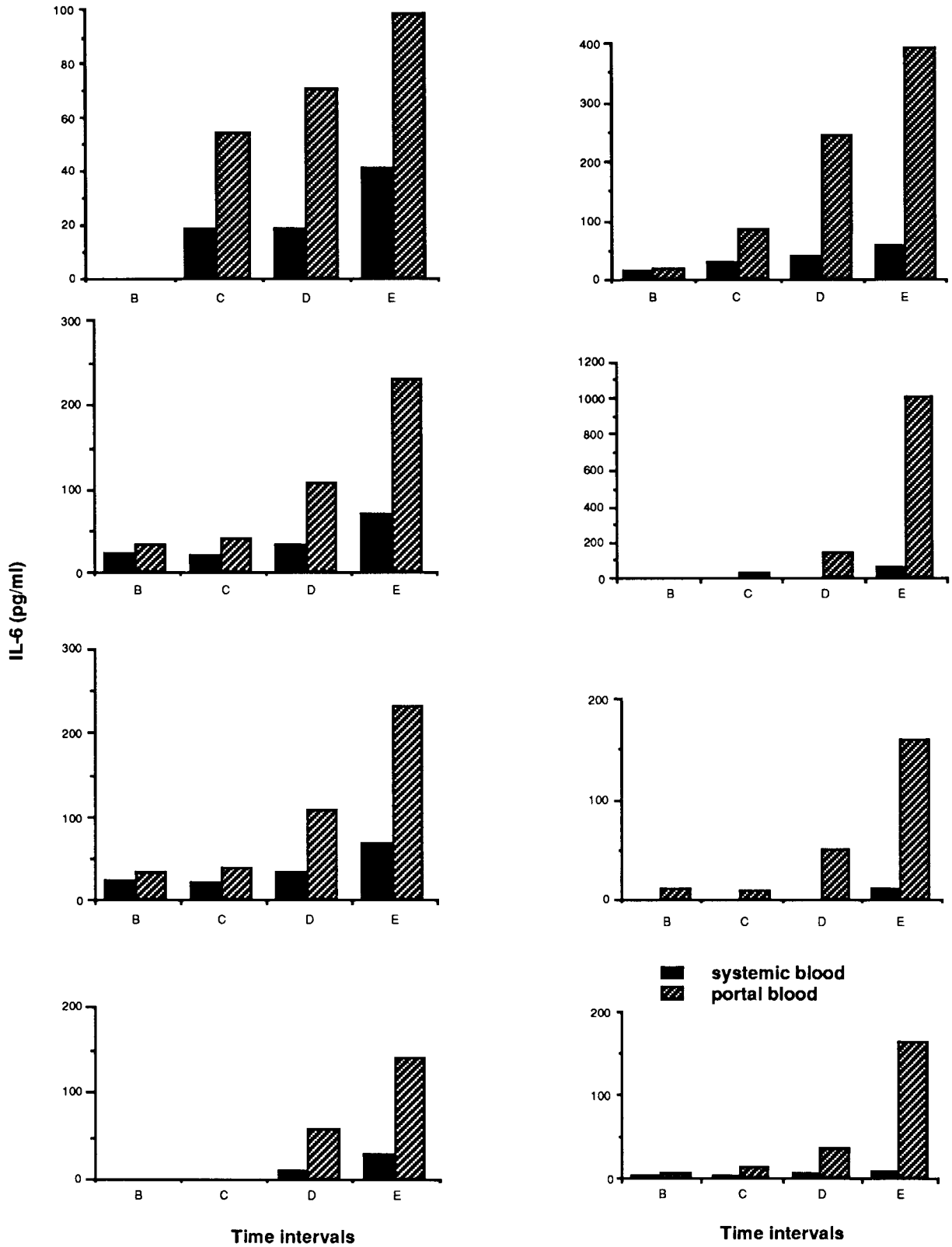



Figure 6.4: Portal and systemic IL-6 levels, in each of the eight patients studied, at time points B. Pre cross-clamping; C. During cross-clamping (1); D. During cross clamping (2); E. Post cross-clamping;

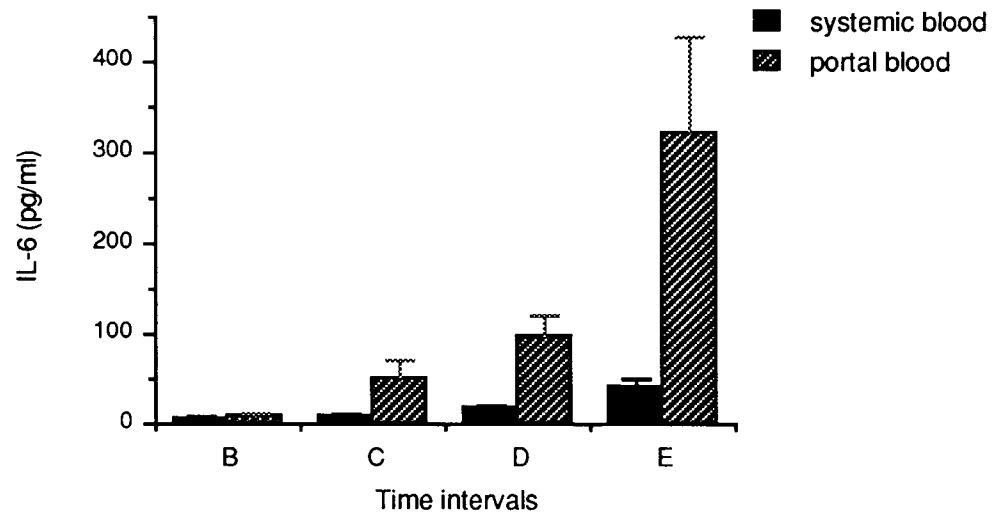



Figure 6.5 Mean portal and systemic IL-6 levels of all patients studied, at time points B. Pre cross-clamping; C. During cross-clamping (1); D. During cross clamping (2); E. Post cross-clamping. Error bars represent the standard errors of the mean. The significance of the portal-systemic difference at each time point is: B $p=0.22$, C $p<0.05$, D and E $p<0.001$.

### 6.3.2 Endotoxin

The results obtained were inconsistent and interassay variation was high. In order to investigate these inconsistencies further, coded and unopened sample aliquots were sent to two other laboratories (Institute of Clinical Science, Queens University, Belfast and Celltech Laboratories, Slough) for blind analysis and comparison with the Nuffield Department of Surgery findings. Both these laboratories were actively engaged in clinical endotoxin studies with recent publications on the subject in the international literature. Results from the independent laboratories are simply labelled (x) and (y) to preserve a degree of anonymity.

There had been three aliquots prepared at each time point for each of the eight patients. One complete set of sample aliquots was assayed in the Nuffield Department of Surgery. However technical difficulties meant that several of the second set of aliquots were required to obtain a valid

result. In spite of this, no result was obtained for two patients. The remaining aliquots from the second set were measured in laboratory (y). The third complete set of samples was reserved for testing by laboratory (x).

Thus at each sample point in Figure 6.6, there are eight results from laboratory (x), six from the Nuffield Department of Surgery and a variable number from laboratory (y). Uniformly high levels were found by laboratory (y). Results from both laboratory (x) and the Nuffield Department of Surgery were generally below the limit of detection, but there was a wide range observed.

**Laboratory (y)** range: 10-150pg/ml; median 13pg/ml.

**Laboratory (x)** range: 0-125pg/ml; median 0pg/ml.

**Nuffield Department of Surgery** range: 0-134pg/ml; median 0pg/ml.

Because of the high number of negative results in the two sets of data (o) and (x), their overall correlation was high, but this did not apply when only positive samples, by either one assay or the other, were scrutinised. Even when disregarding their picogram values, there was only a 33 percent overlap of samples found to be positive in one laboratory or the other.

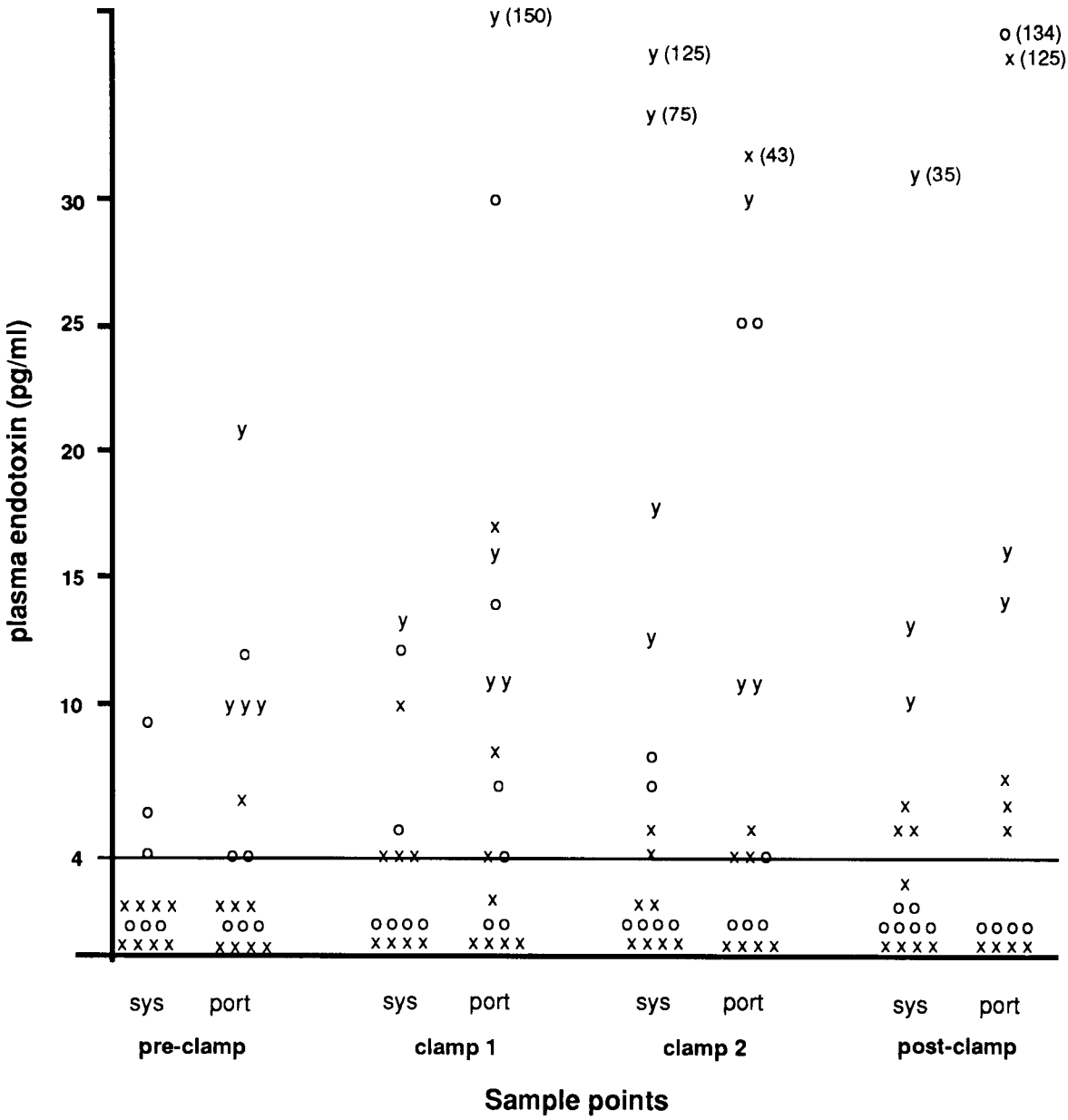



Figure 6.6 Endotoxin levels in systemic (sys) and portal (port) blood sampled during the operation at four time-points: before cross-clamping the aorta **(pre-clamp)**, at the end of the proximal anastomosis **(clamp 1)**, at the end of the distal anastomosis(es) **(clamp 2)**, and just prior to closure of the abdomen **(post-clamp).**

Aliquots of samples taken from eight patients were assayed in three different laboratories: Nuffield Department of Surgery (o), laboratory (x) and laboratory (y).

Minimum detectable concentration=4pg/ml.

## 6.4 DISCUSSION

This study is the first to report on portal and systemic cytokine analysis in patients undergoing major surgical injury. During the course of the study a report of portal and systemic cytokine analysis, in accidental trauma patients, was published by Moore et al. Their paper is relevant to this discussion and will be reviewed at length in this section.

While TNF was not detected and IL-1 levels were similar in both compartments, the portal IL-6 levels greatly exceeded systemic levels. This suggests that the colon is a major source of IL-6. In a comprehensive review of IL-6, van Snick suggested that this cytokine is tailored to function as an "SOS signal". His rationale for this was the absence of any evidence that IL-6 adversely influences cardiovascular stability or cellular integrity, so that the major effects of this protein appear to be beneficial to the host by enhancing immune function and acute phase protein synthesis. In addition, injury or infection are promptly followed by a massive induction of IL-6 and almost every cell is capable of making IL-6 (van Snick J, 1990). The colon is directly affected by aortic cross-clamping, as explained below, and therefore vulnerable to hypoxic injury. The release of large quantities of IL-6 by the colon, under the circumstances reported in this chapter, provides direct and substantial evidence for van Snick's suggestion.

Blood supply to the colon is primarily via the inferior mesenteric artery, with contributions from collaterals via the marginal artery and the haemorrhoidal arteries arising from the internal iliac arteries and their branches. In the majority of abdominal aortic aneurysm patients, the inferior mesenteric artery is occluded and collateral supply is dominant.

Aortic cross-clamping will affect not only a patent inferior mesenteric artery, but also iliac collaterals.

*In vivo*, the liver seems to be the major target organ for IL-6. Both the biological activity and radioactivity of $I^{125}$-labelled recombinant human IL-6, injected intravenously into rats, disappears very rapidly from the circulation. The kinetics of clearance are biphasic with a rapid initial elimination, corresponding to a half-life of about 3 minutes, and a second slower decrease corresponding to a half-life of about 55 minutes (Castell JV et al., 1989). This organ has a high first pass effect for protein and the efficient clearance of this cytokine from portal blood would account for the high portal-systemic ratio seen in the samples taken simultaneously from both circulatory compartments. Nonetheless it seems likely that the portal system is the major contributor to the systemic levels of IL-6 reported during injury.

On the other hand, IL-1 levels were similar and of low concentration in both portal and systemic blood. This cytokine is produced primarily by blood monocytes and tissue macrophages, although other cells may produce it in smaller quantities under certain circumstances (Dinarello CA, 1989b). Our findings are compatible with this knowledge.

Most of the endotoxin studies quoted in the introduction used exactly the same chromogenic endotoxin assay described in section 6.2.3, including the same chromogenic substrate, S-2423, from Kabi Diagnostica. This allows direct comparison of results (van Deventer SJH et al., 1988a)(van Deventer SJH et al., 1990)(Brearley S et al., 1985)(Andersen LW et al., 1988)(Kelley CJ et al., 1985) (Dr I Halliday, Belfast; personal communication).

The results obtained in the Nuffield Department of Surgery were inconsistent and difficult to interpret. Prior to using this assay, extensive enquiries were made within the scientific community in Oxford in search of a group with experience of the LAL test. Surprisingly no such expertise was found, moreover preliminary work with this assay proved that it was more difficult than the methodology suggested. This was confirmed during subsequent correspondence and meetings with, as well as demonstrations from other groups elsewhere in the country who were actively involved with the assay. As a result, a fresh series of samples was collected using "pyrogen-free" techniques, and the assay was carried out using the method of two other groups (Halliday et al, Belfast and McCathy et al, Glasgow - personal communications).

Despite these precautions, interassay variation was high. Nonetheless the overall picture was of low level endotoxaemia (less than 10pg/ml) in some patients, and an absence of endotoxaemia in others. This finding is consistent with the results reported by van Deventer and Brearley, but are different to those of Andersen, Kelly and Halliday, although the latter two groups were studying a different category of surgical patient (van Deventer SJH et al., 1990)(Brearley S et al., 1985)(Andersen LW et al., 1988)(Kelley CJ et al., 1985) (Dr I Halliday, Belfast; personal communication). All these groups were using the chromogenic LAL test.

However, attempts to validate these results by sending samples to two laboratories which had experience with the identical LAL assay provided contradictory data. The results from one group (x) were broadly similar to those obtained in the Nuffield Department of Surgery, in that they recorded low levels or an absence of endotoxin. However closer scrutiny of

the results for each sample revealed very poor correlation between the two sets of results. Moreover the results from the other group (y) were uniformly high, indeed higher than the levels they usually saw in the blood of patients with inflammatory bowel disease. The assay controls and blanks had all performed normally and therefore this group concluded that the results were accurate.

Thus, the inconsistency of the results makes it is impossible to draw any reliable conclusion from the endotoxin data reported in this study. Perhaps the only valid conclusion (and the most important) is that this assay remains extremely susceptible to experimental error, and results reported in the literature should be interpreted in this light. Nonetheless, the (unvalidated) impression obtained, is of, at most, low level endotoxaemia in the portal and systemic blood of abdominal aortic aneurysm patients.

The only other report of portal cytokines and endotoxin was published in May 1991. This report, by Moore et al in Colorado, of patients with "major torso trauma" is comparable in certain respects to the study reported in this chapter (Moore FA et al., 1991).

They inserted portal vein catheters into major injury patients who required laparotomy, and sampled portal and systemic blood synchronously at insertion and thereafter at 6, 12, 24 and 48 hours. They found no evidence of bacteraemia, endotoxaemia or elevated TNF in either vascular compartment. Levels of IL-6 were raised in both compartments but there was no difference observed between the two. Close scrutiny of their methodology reveals that their IL-6 assay was very similar to that used in this chapter and, indeed, the systemic IL-6 levels in both studies are

comparable. Curiously, these levels were considerably lower than those recorded in the trauma patients in Chapter 6.

They concluded: *"while animal studies (which implicate gut-derived bacteria or endotoxin as primary factors in the genesis of post-injury multiple organ failure) are consistent and compelling, the clinical evidence that bacterial translocation (and endotoxaemia) play a significant role in the the genesis of post-injury multiple organ failure is, at best, circumstantial."* The absence of endotoxaemia was a finding common to both their study and the work reported in this chapter. However, when this paper was discussed at the Fiftieth Annual Session of the American Association for the Surgery of Trauma, several delegates with an interest in endotoxin were reluctant to discuss the implications of this report and instead returned to the technical details of the chromogenic endotoxin assay, with at least one critic suggesting the results were negative because samples had been overdiluted and the incubation period was too short.

Moore et al did not observe a difference in their portal and systemic IL-6 levels. There are several possible reasons for this. They sampled the portal vein as opposed to the inferior mesenteric vein and hence their sample may have been diluted by superior mesenteric and splenic vein blood. It was for precisely this reason that the inferior mesenteric vein was chosen as the sample site in the aneurysm patients. A second reason relates to sample times. Although they did not detail the duration of the interval between injury and operation, it was apparent from the discussion that laparotomy rarely took place earlier than 4-6 hours after injury. Therefore their first sample was probably taken a substantial time after the initial rise in IL-6. In support of this, their highest IL-6 levels were seen in their first intraoperative sample, with a rapid decline thereafter. The porto-systemic

IL-6 differences recorded in this chapter were all observed within the first few hours of incision and it is possible that the two vascular compartments may have equilibrated by the time of their first sample.

The third consideration centres on the differences in injury. In abdominal aortic surgery, the insult of acute distal ischaemia resulting from aortic cross-clamping is unique and likely to have a direct and profound effect on distal tissues, including the gut. Moore et al do not give details of specific organ injuries, but no major bowel resection was performed. Thus the IL-6 "SOS signal" from the bowel may not be as significant in their patients. This point is discussed again in Chapter 10.

In summary, this paper provides further clinical evidence for an absence of detectable portal and systemic endotoxaemia after injury. Although the injury scores were high, the delay and infrequency of sampling probably prevented early TNF and IL-6 kinetics from being observed.

In 1985, at "The Fourth International Conference on the Detection of Bacterial Endotoxins with the LAL test" held at Woods Hole, Massachusetts, Dr Jack Levin, who pioneered the LAL test with Dr Frederik Bang, made these remarks in his conclusion:

*"I have alluded to the increasing use of the chromogenic substrates in conjunction with the Limulus test, but have not meant to imply unqualified approval...."*

He then spoke at some length of the pitfalls of this assay and finished:

*"My remarks are not meant to disparage this important development, but only to caution that much more information is needed before we assume the ability to calculate the concentration of an endotoxin based on this assay".*

(Levin J, 1985)

Our findings support his caution. Certainly, the technical difficulty of the assay is probably the single most important reason for the absence of widespread clinical studies which test van Deventer's finding of a predictive role for endotoxaemia in septic patients (van Deventer SJH et al., 1988b). Until an immunoassay for endotoxin is developed, the problems with this assay are likely to persist. The development of monoclonal "capture" antibodies for immunoassay use is likely to be problematical because of the variety of endotoxins that occur. However, anti-endotoxin monoclonal antibodies are already in therapeutic use (Ziegler EJ et al., 1991), and the small error which is likely to created by their use in immunoassays, may well prove to be outweighed by the advantages of a reliable and rugged immunoassay over the vagaries of the LAL test.

The absence of endotoxaemia in these patients does not argue against an important role for endotoxin in the induction of cytokines, because it is probable that the endotoxin concentration required to induce cytokine production is very low. In all the studies of endotoxin administration discussed in the introduction (Hinshaw LB, 1982)(Hesse DG et al., 1987)(Michie HR et al., 1988a)(Fong Y et al., 1989b)(van Deventer SJH et al., 1990)(Spinas GA et al., 1991), only van Deventer measured the level of endotoxaemia induced by this administration. This group noted a transient endotoxaemia peaking five minutes after administration (7 to 13pg/ml) and this low level was sufficient to induce TNF and IL-6 release approximately 90 and 120 minutes later.

Alternatively, our findings suggest the induction of IL-6 by endotoxin may be a local phenomenon, without the need for systemic or portal

endotoxaemia. The high IL-6 levels in the portal blood may reflect a local action of endotoxin, within the ischaemic bowel wall, where this bacterial product may induce IL-6 production without itself reaching detectable levels in the portal circulation.

# CHAPTER SEVEN

---

# THE EFFECT OF MAJOR SURGERY ON THE RELEASE OF CYTOKINES BY PERIPHERAL BLOOD MONONUCLEAR CELLS
# AN INVESTIGATION AT THE SINGLE CELL LEVEL USING THE REVERSE HAEMOLYTIC PLAQUE ASSAY

---

# CHAPTER SEVEN

# THE EFFECT OF MAJOR SURGERY ON THE RELEASE OF CYTOKINES BY PERIPHERAL BLOOD MONONUCLEAR CELLS
# AN INVESTIGATION AT THE SINGLE CELL LEVEL USING OF THE REVERSE HAEMOLYTIC PLAQUE ASSAY



## 7.1 INTRODUCTION

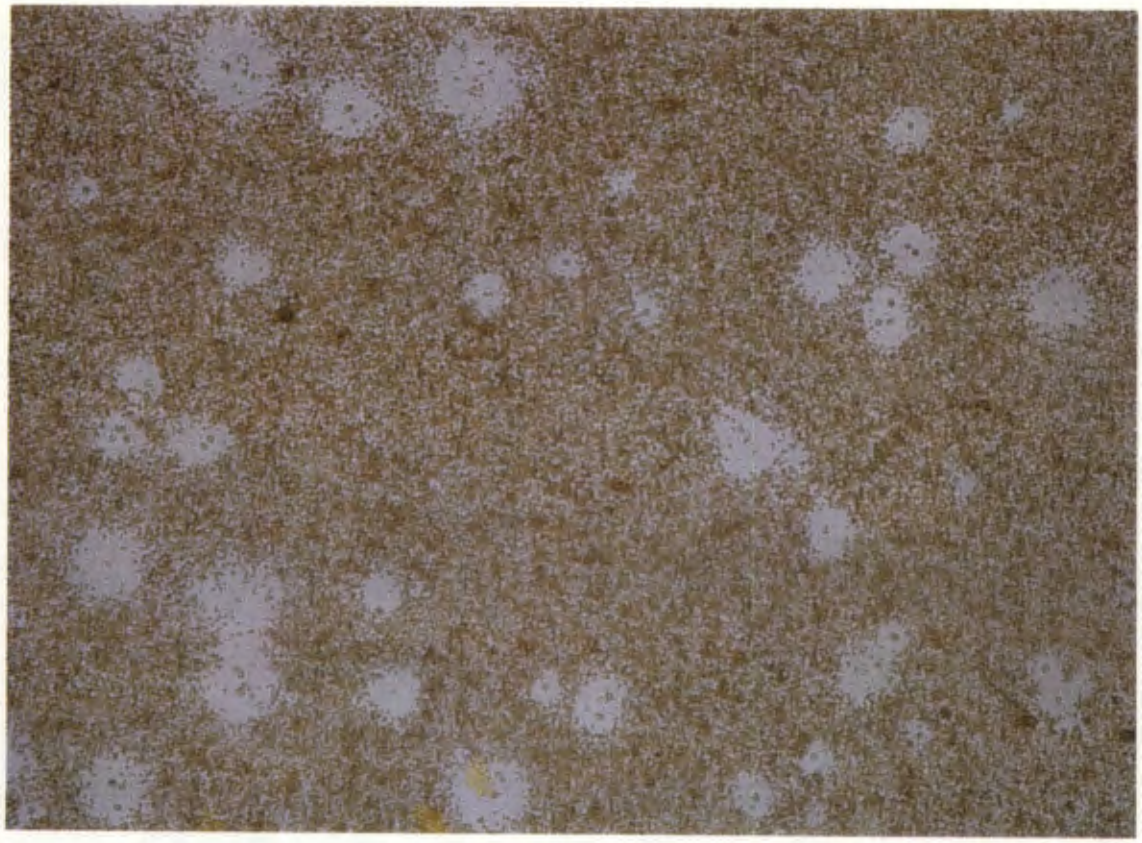
Generalised immunosuppression after major surgical injury is a well characterised phenomenon and is thought to contribute to the high incidence of associated septic complications.

*In vitro* studies have demonstrated a decrease in interferon gamma (IFNγ) production by peripheral blood mononuclear cells (PBMC) isolated from major injury patients (Faist E et al., 1988)(D Livingstone et al., 1988). However the bioassays and immunoassays used in these studies measured only the bulk release of cytokines by entire populations of cells in culture after lengthy mitogen stimulation. Not only are such studies unable to address the question of whether the spontaneous release of this cytokine may be affected by surgical trauma, but also the use of such 'bulk release' methods implies that all cells in a population secrete equal amounts of a given cytokine.

Scientists in the Nuffield Department of Pathology have recently applied a technique called the reverse haemolytic plaque assay (RHPA) (Neill JD et al., 1983) to detect and measure the secretion of a number of cytokines by individual human cells derived from the plasma and dispersed tumour biopsies of cancer patients (Lewis CE et al., 1990). In this immunoassay, secretory cells in a mixed cell population form plaques (zones of haemolysis around secretory cells) when incubated in a monolayer with protein A coated ovine erythrocytes in the presence of a specific antibody and complement (Figure 7.1).

Although TNF, IL-1 and IFNγ were not detected in the plasma of the aneurysm patients, there remained the possibility that these cells were secreting cytokines in concentrations that were too low to detect with ELISA, but which nevertheless could be important when these cells migrated to a site of inflammation. This chapter is a report of the use of this technique to study the release of cytokines by PBMC from patients undergoing abdominal aortic aneurysm repair.

(a)



(b)

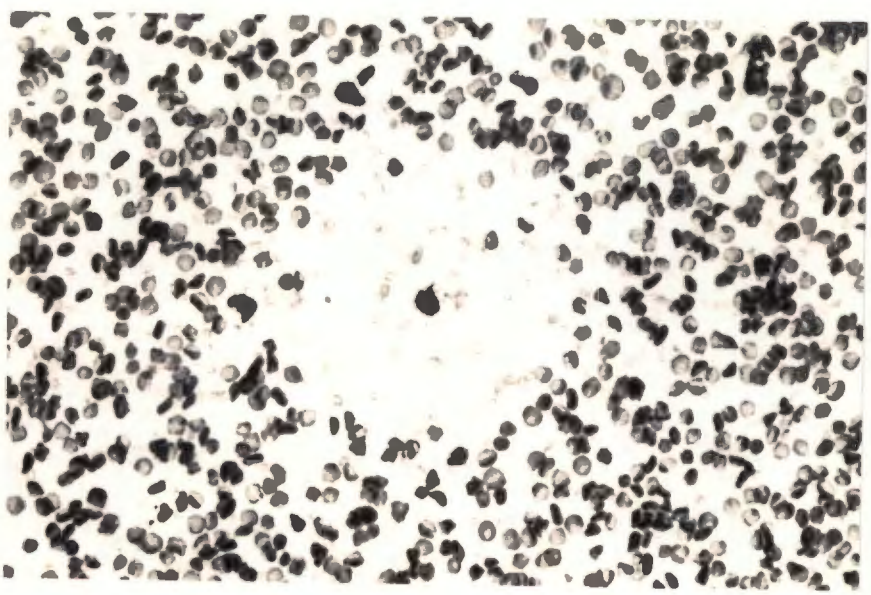


Figure 7.1a: Photomicrograph of IFNγ plaques (areas of erythrocyte lysis)

Figure 7.1b: An individual PBMC (arrowhead) at the centre of a plaque Magnification bar=20μm.

## 7.2 PATIENTS AND METHODS

### 7.2.1 Patients

Blood samples were taken from three patients undergoing abdominal aortic aneurysm repair. No patient developed significant post-operative complications. One patient received three units of blood between one and three hours after incision, the other two received only colloid and crystalloid fluids. All three patients were men aged between 60 and 75 years. Blood samples were also taken from a healthy male donor over the same period which were used to control for interassay variation at the four time-points studied for each patient.

### 7.2.2 Samples

Ten ml of venous blood was collected in standard disposable pyrogen-free sterile syringes and added to pyrogen free glass tubes containing EDTA (0.34M). Samples for the RHPA were taken prior to incision and again at nine hours, nine days and three weeks after incision.

### 7.2.3 Isolation of human PBMC

A modification of the technique described by Boyum was used (Boyum A, 1968).

(1) Venous blood anticoagulated with EDTA was diluted with an equal volume of phosphate buffered saline (PBS) and mixed.

(2) Aliquots (4ml) of the diluted blood were layered onto 3ml of Ficoll-Hypaque in 12ml polypropylene centrifuge tubes, and spun at 1700rpm (no brake) for 25 minutes.

(3) Following centrifugation, the upper plasma layer was discarded, leaving a PBMC rich layer at the interface which was transferred by pipette to a new tube and diluted with fresh calcium-and-magnesium-free-Hanks buffered salt solution (HBSS) (Flow Laboratories).

(4) Cells were resuspended, washed (1400rpm, 10 minutes) three times in fresh HBSS, and diluted to a final concentration of $5 \times 10^6$ mononuclear cells/ml in assay medium (see below).

### 7.2.4 Conjugation of Staphylococcal protein A to sheep red blood cells

(1) Four ml of sheep erythrocytes (oRBC) in Alsevers solution (Serotec) was diluted 1:1 with saline (0.9%) and subjected to a Ficoll-Hypaque density gradient treatment (see previous section) to remove the leukocytes.

(2) The erythrocyte pellet was harvested and pooled and washed three times by repeated suspension and centrifugation (1200rpm; 5 minutes) in saline.

(3) A 1ml pellet of oRBC was diluted with 5ml of saline to which 1ml of protein A (0.5mg/ml saline)(Sigma) and 5ml of Chromium chloride hexahydrate (BDH) (0.2mg/ml saline; stored in the dark at 4°C for at least 1 week before use) was added. The tube was then incubated at 30°C for 1 hour

(4) Protein A-oRBC (1ml) was then harvested by centrifugation at 1500rpm for 6 minutes and washed three times by repeated suspension and centrifugation steps in saline (first wash) and subsequent washes with Dulbeccos MEM (Gibco) {containing hepes, sodium pyruvate, 0.1% bovine serum albumin (Miles), penicillin (100U/ml) and streptomycin (100µg/ml)}. NB. if considerable lysis of erythrocytes occurred after the first centrifugation, or the pellet did not resuspend readily, the preparation was discarded and the procedure restarted using a different batch of oRBC.

(5) The Protein A-oRBC were resuspended in 50ml of Dulbeccos MEM {to create a 2% solution(v/v)} and kept at 4°C. for a maximum of 9 days. For best results, cells were left overnight before use.

### 7.2.5 Preparation of poly-l-Lysine coated Cunningham chambers

Chambers were freshly coated for each assay.

(1) Glass microscope slides (Denley) were washed in sterile $H_20$, immersed for 10 minutes in poly-l-Lysine (Sigma)(5mg/60ml $H_20$), then washed in sterile $H_20$ to remove excess poly-l-lysine and allowed to air dry.

(2) Cunningham chambers were constructed by applying two strips of double-sided adhesive tape across the slide, 2cm apart, and placing a coverslip on top, thereby creating a small chamber beneath the coverslip.

### 7.2.6 Protocol of the reverse haemolytic plaque assay

The RHPA was performed as previously described for the detection of luteinizing hormone secretion by rodent gonadotrophs (Neill JD et al., 1983). The protocol for the RHPA is shown in Figure 7.2.

(1) Freshly isolated human PBMC were mixed thoroughly with an equal volume of protein A-oRBC. Aliquots (100µl) of this cell suspension was applied to the entrance of each Cunningham chamber and allowed to settle for 30-45 minutes in an atmosphere of 5% $CO_2$/95% air, at 37°C.

(2) Excess unattached cells were removed by rinsing each chamber with warm assay medium {RPMI 1640 (100ml) containing streptomycin (100µg/ml), penicillin (100U/ml) and Pentex Bovine Albumin (0.1%) (Miles)}.to leave a virtually confluent monolayer of cells attached to the floor of the chamber. Chambers were then filled with a test solution of antibody at a dilution of 1:50 in assay medium {Anti-TNF, anti IL-6, anti IL-1, anti IFNγ: All antibodies were rabbit anti human polyclonal (Genzyme)}.

(3) The slides were incubated at 37°C for nine hours, after which the contents of the chambers were washed with medium to remove any

unbound antibody or stimulants and filled with guinea-pig complement at a dilution of 1:50 in assay medium to initiate plaque formation.

(4) After 25 minutes, cells were exposed to 3% (v/v) glutaraldehyde (Sigma) in phosphate buffer and the slides stored at 4°C for later analysis. Each antibody test condition was duplicated on 10 separate slides in each assay for both the patient and the control.

### 7.2.7 Quantitative analysis of plaque size and number and statistics

Studies involving the use of the RHPA to measure luteinizing hormone release from rodent gonadotrophs have coupled the plaque assay with a highly specific and sensitive radioimmunoassay for luteinizing hormone to demonstrate that the area of haemolysis (plaque) which forms around a secretory cell is directly proportional to the amount of product secreted. Thus, the size of haemolytic plaques provides a quantitative estimate of the amount of product secreted by single cells.

The area of haemolytic plaques was measured using a Wild M20 microscope to which a forward-projecting drawing device had been attached. This permitted the image of a computer (Apple Macintosh) screen to be superimposed on the image of each plaque in the Cunningham chamber. Plaque sizes were measured using a computer programme written by Dr J Lorenzen in this department which measured the area of each plaque image on the screen and stored the data in a file which could be accessed by the Statworks™ statistical analysis software program. The standard deviation of measurements was calculated to be ± 1.9μm. The first 10-20 plaques on replicate slides (until 100 plaques in total per treatment had been measured) were measured by this computer-assisted morphometry and the mean value expressed in $\mu m^3$.

Figure 7.2: Protocol for the RHPA (section 7.2.6). oRBC = sheep red blood cells.

The number of plaques produced in the Cunningham chambers was counted with a cell counter in each experiment, by scanning chambers with a microscope at a total magnification of 25X.

Data are presented as means $\pm$ SEM in Figure 7.3. Statistical analysis of the data was performed using the Mann Whitney U test.

**7.3 RESULTS** (Figure 7.3)

There was no significant difference in the amount of IFNγ released by individual PBMC derived from the healthy male donor over the various time points assayed for each patient. This uniformity of average plaque size for control patients indicates that inter-assay variation in the use of sequential RHPA was successfully controlled. In contrast, individual PBMC from all three surgical patients released significantly more IFNγ by nine hours after incision than that released prior to surgery ($p<0.001$). This change persisted up to nine days after surgery but was not present at 25 days, by which time IFNγ levels had returned to preoperative values. No significant difference in the proportion of PBMC secreting IFNγ was detected at the various time intervals. Plasma IFNγ was not detectable using either of the two ELISA techniques.

The release by PBMC of IL-1, TNF and IL-6 was not detected in any sample.

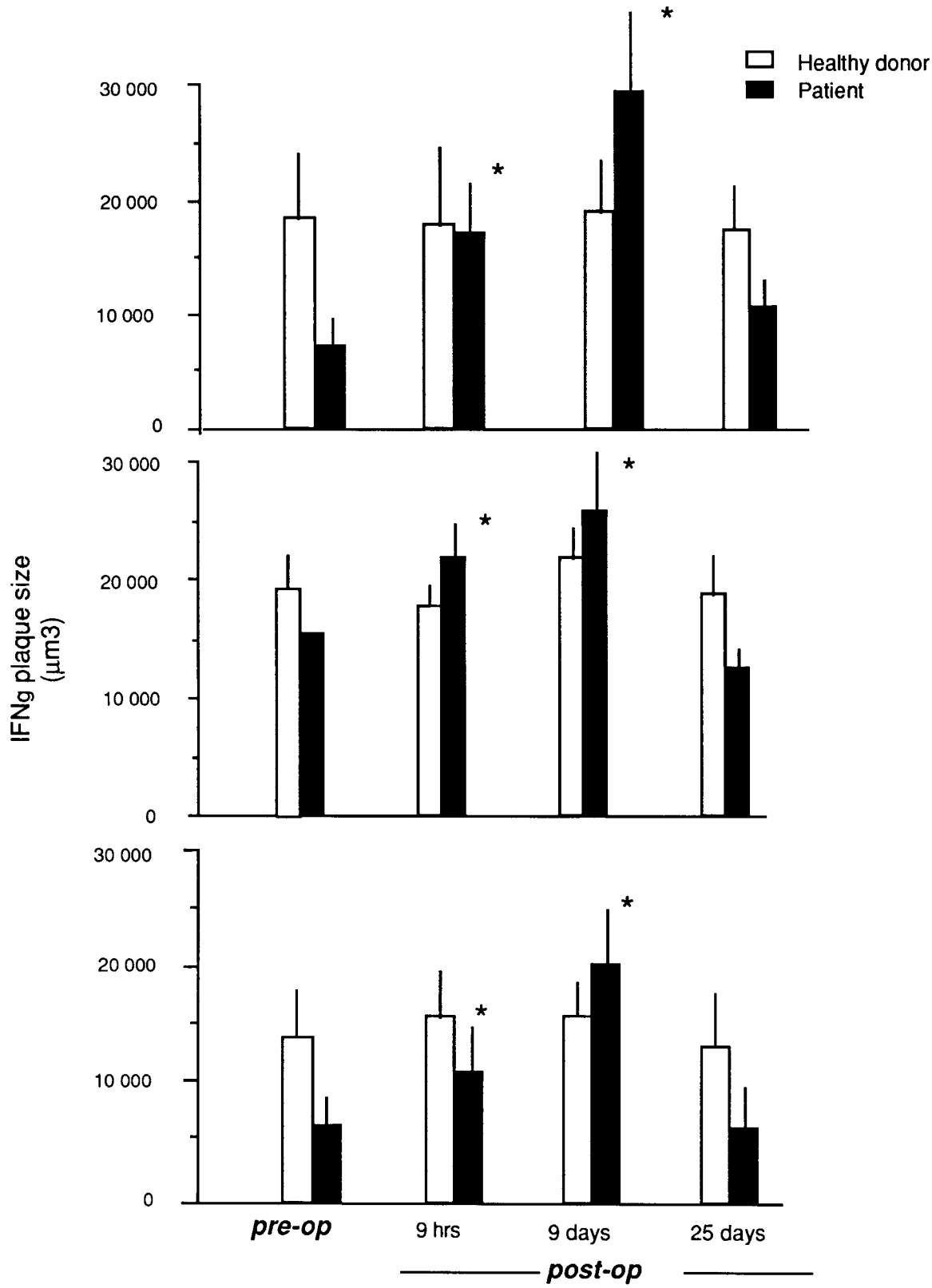



Figure 7.3: Mean (+ SEM) size of IFNγ plaques formed by PBMC isolated from sequential blood samples taken either from three patients undergoing major surgery or healthy control patients. * $p<0.001$ compared with pre-operative sample.

## 7.4 DISCUSSION

As a result of its increased sensitivity when compared with ELISA, the RHPA allows the measurement of the very low quantities of cytokine released by individual, unstimulated PBMC. While previous studies suggest that PBMC from patients undergoing major surgery have diminished responsiveness to lengthy mitogen stimulation in terms of *in vitro* IFNγ release (Faist E et al., 1988)(D Livingstone et al., 1988), use of the RHPA in this study has demonstrated the spontaneous release of IFNγ from PBMC to be augmented in the post-surgical patient. Although the number of patients studied was small, these results, involving four assays per patient, are remarkably consistent.

In this study, IFNγ release peaked at nine hours and was returning to preoperative levels by nine days. Kasahara et al have shown that increased transcription for IFNγ can be detected within six hours of stimulation with microbial antigens or IL-2 (Kasahara T et al., 1983), while release of this protein peaks at approximately 48-72 hours (Kelly CD et al., 1987) and may continue for seven to eight days (Green JA et al., 1969). In these three studies cells were given a single stimulus to produce IFNγ, compared to our model in which cells were derived from a patient recovering from major injury, where the postoperative host response was likely be providing an ongoing stimulus for IFNγ production. Given these differences in experimental model, the kinetics seen in our findings are not inconsistent with these *in vitro* reports.

This laboratory has recently used the RHPA, coupled to immunocytochemistry, to show that the majority of cells which spontaneously secrete IFNγ *in vitro* are $CD16^{+}$NK cells (Lewis C et al., 1991). This accords well with the recent finding by Young and Ortaldo that

NK cells are the predominant source of IFNγ *in vitro* (Young H et al., 1987). This implies that whilst the cytotoxicity of both NK and T cells in the peripheral blood of trauma patients may be suppressed (Faist E et al., 1988), another important aspect of their cellular activity, namely cytokine secretion, is augmented. Alternatively, these results could be due to an *in vitro* 'release phenomenon', in which the removal of these cells from their plasma environment results in their no longer being inhibited by possible post-surgical, blood-borne factors. Nonetheless, these results do demonstrate that PBMC from such patients are capable of enhanced IFNγ secretion, even in the absence of mitogen stimulation.

It was suprising that secretion of none of the other three cytokines was seen. Using this technique and the same antibodies, the secretion of these cytokines from cancer cells has been detected in this laboratory. Therefore technical shortcomings would seem an unlikely explanation. Tumour necrosis factor and IL-1 were also not detectable in plasma at any of the times assayed and it is therefore probable that these cytokines are simply not being secreted at these times. However IL-6 has been shown to be easily detectable in plasma at 9 hours. Therefore its secretion from PBMC might reasonably be expected at this time. It is known that a wide and heterogeneous range of cell types secrete IL-6 (Wolvekamp MCJ et al., 1990), but what is not known is the proportion of plasma IL-6 being secreted by any particular cell type at any given time after injury. For example, by nine hours IL-6 production by PBMC may have ceased, with plasma levels being derived from other cells. This explanation could be tested by undertaking more frequent early assays. However the nature of the RHPA means that a second worker would be required to perform the assay between 0 and 9 hours. This technical support was not available

during the study but it is hoped to extend this area of research in the future.

Studies in which the IFNγ has been administered to injured (thermal and non-thermal) animals have suggested an improvement in outcome after a bacterial challenge (Hershman MJ et al., 1988b)(M Malangoni et al., 1989), and preliminary results from a multicentre clinical trial have suggested some reduction in septic complications in certain subgroups of major trauma patients given IFNγ therapy (Polk HC, Proceedings of the 2nd International Congress on the Immune consequences of Trauma, Shock and Sepsis; Munich, March 1991, in press: Springer Verlag). The increased secretion of this cytokine by PBMC following major surgery could therefore be interpreted as a beneficial component of the host's immune response to injury, and a possible target for immunotherapy to augment the patient's own endogenous release of IFNγ.

## 7.5 SUMMARY

The release of IFNγ by peripheral blood mononuclear cells from patients undergoing abdominal aortic aneurysm repair was measured using a technique called the reverse haemolytic plaque assay. The plasma of these patients was also assayed for IFNγ using ELISA. Peripheral blood mononuclear cells from the surgical patients released significantly more IFNγ by nine hours after incision than that released prior to surgery ($p<0.001$). This change persisted up to nine days after surgery. Plasma IFNγ was not detectable using either of two different ELISA techniques. The increased secretion of IFNγ by PBMC following major surgery may be a beneficial component of the host's immune response to injury.

# CHAPTER EIGHT

---

# EFFECTS OF MAJOR AND MINOR SURGERY ON PLASMA GLUTAMINE LEVELS: A CORRELATION WITH INTERLEUKIN-6

---

# CHAPTER EIGHT

## EFFECTS OF MAJOR AND MINOR SURGERY ON PLASMA GLUTAMINE LEVELS: A CORRELATION WITH INTERLEUKIN-6



## 8.1 INTRODUCTION

The phenomenon of postoperative ileus has resulted in surgeons traditionally assuming that the gastrointestinal tract rests after major operations and injuries. In fact, this organ has been shown to play a key metabolic role after surgery and after other catabolic stresses. This role is largely related to the ability of the intestine to metabolise glutamine. Despite the lack of oral intake, the small intestine exhibits accelerated metabolic activity and the uptake of glutamine by the gastrointestinal tract far exceeds that of any other amino acid (Windmueller HG et al., 1974).

Glutamine has several unique properties that suggest it plays an important role in the acute phase response (reviewed in (Klimberg VS et al., 1990a)). It is the most abundant amino acid in blood and tissues (50-80 per cent of the free amino acid pool), and is avidly consumed by replicating cells such as fibroblasts, lymphocytes, tumour cells and intestinal epithelial cells. Glutamine uptake by the gastrointestinal tract is increased *in vivo* by 75 per cent after the stress of a standard laparotomy. This continues postoperatively despite a reduction in both portal blood flow and the arterial concentration gradient, which suggests that this change may be regulated by some circulating mediator. This accelerated consumption contributes to the low blood glutamine levels associated with catabolic states (Klimberg VS et al., 1990a). Skeletal muscle, the kidneys (Squires EJ et al., 1976), the lungs and cells of the immune system (Newsholme EA et al., 1988) are other tissues that have quantitative importance in the exchange of glutamine. Muscle glutamine release is accelerated after surgical stress, helping to maintain the circulating glutamine pool which fuels the enterocytes and renal ammoniagenesis.

Until recently, the purpose of these high rates of glutamine utilisation in rapidly dividing cells has been considered to be the provision of energy and/or the provision of both nitrogen and carbon for precursors for the synthesis of macromolecules, for example purine and pyrimidine nucleotides for DNA and RNA. However, these high rates of utilisation occur even in resting or quiescent human lymphocytes and mouse macrophages (Ardawi MSM, 1988), these rates are greatly in excess of requirements for DNA and RNA synthesis, and, furthermore, glutamine has been shown to have a relatively minor role in the generation of adenosine triphosphate (ATP)(Szondy Z et al., 1989).

The only explanation for these observations has come from Newsholme et al. They suggest that a high rate of glutamine utilisation has a more specific purpose, namely the "precise regulation of the rates of synthesis" of nucleotides and other compounds required in cell proliferation, or for other functions related to the role of these cells in the immune response (Newsholme EA et al., 1988). They point out that the rate of utilisation of glutamine by these cells depends on its external concentration, and therefore maintenance of physiological glutamine concentrations in blood would appear to be important for the normal functioning of these immune cells. Indeed, it has been shown that lowering the concentration of glutamine in culture medium, below that normally present in human plasma, decreases lymphocyte proliferation, antibody synthesis and macrophage phagocytosis despite the presence of other amino acids and other fuels, such as glucose (Parry-Billings M et al., 1990).

These considerations also apply to intestinal cells. Studies have shown that following irradiation there is a deterioration in intestinal cell structure in the rat and an increase in bacterial translocation, associated with a decrease in plasma glutamine. Feeding with glutamine leads to an increase in plasma glutamine and a restoration of gut structure and function (Klimberg VS et al., 1990b).

It is known that surgery increases the rate of utilisation of glutamine by the intestine (Souba WW et al., 1985) and it would be expected to increase the rate of utilisation by cells involved in the immune response to surgery. However, there have been no studies of the acute and sequential change in plasma glutamine levels in response to surgical operations of different severity and no studies following major surgery.

It has already been pointed out that the accelerated uptake of glutamine, despite a reduction in both portal blood flow and the arterial concentration gradient, suggests that this uptake may be regulated by some circulating mediator. Cytokines act as mediators of both the immune response and the response of other tissues in the body to injury. As previously discussed, IL-1, IL-6, TNF and IFNγ are considered to be important mediators of the integrated host response. Interleukin-6 is particularly important in amino acid metabolism in that it stimulates the production of acute phase proteins by the liver (Castell JV et al., 1989). The plasma level of this cytokine rises after surgery and excessively high postoperative IL-6 levels are associated with the development of major complications (Chapter 4).

In order to elucidate further the mechanisms of postoperative immunosuppression, this study examines the relationship between cytokine elaboration and glutamine metabolism. Consequently, the plasma levels of glutamine and other amino acids, IL-1, IL-6, TNF and IFNγ have been measured frequently in patients before, during and after elective abdominal aortic aneurysm surgery ("major surgery") or inguinal hernia repair ("minor surgery"). The advantages of studying this patient group, in terms of their uniformity of injury and relatively low risk of bacterial contamination, has already been stressed in Chapters 4, 5 and 6. The impact of major surgical trauma can be evaluated without the conflicting influence of bacterial contamination or malignancy on the immune and metabolic systems.

## 8.2 PATIENTS AND METHODS

**8.2.1 Major surgery:** Nine patients (7 men and 2 women), aged between 59 and 83 years, underwent elective aortic aneurysm repair. A preoperative

blood sample was taken after induction of anaesthesia. Thereafter patients were sampled after incision at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12, 24, 48, 72, 96, 120 and 168 hours.

**8.2.2 Minor surgery:** Five patients (4 men and 1 woman), aged between 50 and 75 years, underwent inguinal hernia repair. A preoperative blood sample was taken after induction of anaesthesia. Thereafter patients were sampled after incision at 0.5, 1, 4, 8 and 24 hours.
The patients from both these groups were also used as subjects in Chapter 4 and/or Chapter 6, and their cytokine results have therefore been analysed as part of the studies in those chapters.

**8.2.3 Samples:** A detailed description of sample preparation is given in section 3.9.

**8.2.4 Amino acid analysis** (section 3.9)**:** Neutralized perchloric acid extracts of plasma were analysed using enzymatic assays for concentrations of glutamine (Windmueller HG et al., 1974), glutamate (Bernt E et al., 1974), alanine (Williamson DH, 1974) and branched chain amino acids (Livesey G et al., 1980). Glutamine was present at physiological levels in infused blood.

**8.2.5 Cytokine assays:** IL-1, IL-6, TNF, and IFNγ were measured as before (section 3.7)

## 8.3 RESULTS

**8.3.1 Amino acids:** Following major surgery, the plasma concentration of glutamine was rapidly and markedly decreased in all patients: the concentration was decreased 2.5 hours after incision and remained so until the end of the study (168 hours after incision) (Table 8.1). Although the

extent of this decrease varied between subjects, it was by far the most significant change in amino acid concentration seen (as reflected in the p values in Table 8.1), and in four of the nine subjects the decrease was greater than 40 percent. The plasma concentrations of the other amino acids also changed after surgery, but in contrast to glutamine they returned to pre-surgery values by 168 hours after incision. The plasma concentration of alanine increased between 2 and 3.5 hours after incision and decreased between 48 and 120 hours after incision. The plasma concentration of glutamate decreased between 12-120 hours, and that of the branched chain amino acids was decreased between 12-24 hours after incision (Table 8.1). In contrast to the marked effect of major surgery on plasma glutamine levels, minor surgery was without effect on these levels (Table 8.2).

**8.3.2 Cytokines:** Following major surgery, the concentration of IL-6 was increased at 1.5 - 3 hours after skin incision in all patients. The concentration attained a maximum between 4 and 48 hours (median 8 hours) after the beginning of the operation and fell rapidly by 48-72 hours (Figure 8.1). The IL-6 response in patients undergoing hernia repair showed similar kinetics to that after major surgery, in that concentrations peaked at 4-12 hours (median 8 hours) after commencement of operation. However, the peak concentrations obtained were markedly lower than those following major surgery ($p<0.001$) (Figure 8.1)

Following major surgery, the concentration of IL-1 was increased in seven of the nine patients (mean$\pm$SEM = 17$\pm$5pg/ml) between 1 and 4 hours after incision (median 2 hours). This cytokine was not detected in any minor surgery patients while TNF and IFN$\gamma$ were not detected in any patients.

**8.3.3 The relationship between plasma glutamine and cytokine levels:** Following major surgery, there was no correlation between plasma levels of IL-1 and glutamine ($r= 0.01$, $p=0.97$), and since TNF and IFNγ were not detected, no correlation was observed between these cytokines and the plasma glutamine level. In marked contrast, there was a significant negative correlation between the plasma concentrations of IL-6 and glutamine (0-4 hours $r=-0.91$, 0-8 hours $r=-0.95$, 0-48 hours $r=-0.89$, $p<0.0001$ for all times) (Figure 8.1). However, following minor surgery there was no correlation (0-24 hours, $r= -0.64$, $p=0.17$).

Table 8.1. The effect of major surgery on plasma amino acid concentrations. Values are mean (SEM) and time is given in hours. BCAA denotes branched chain amino acids and nd denotes not determined. The significance of the differences from the preoperative values are denoted by [a] ($p<0.05$), [b] ($p<0.01$) and [c] ($p<0.001$).

| Time after incision | Number of patients | Plasma amino acid concentration (uM) | | | |
|---|---|---|---|---|---|
| | | Glutamine | Glutamate | Alanine | BCAA |
| 0 | 9 | 653(21) | 78(14) | 308(34) | 366(23) |
| 0.5 | 9 | 617(14) | 105(21) | 316(41) | 372(28) |
| 1 | 9 | 599(27) | 82(12) | 312(34) | 356(24) |
| 1.5 | 9 | 609(45) | 86(12) | 347(48) | 372(26) |
| 2 | 9 | 636(41) | 82(10) | 408(52)[a] | 398(26) |
| 2.5 | 9 | 589(39)[a] | 77(13) | 430(53)[a] | 405(38) |
| 3 | 9 | 573(42)[a] | 83(15) | 429(61)[a] | 415(37) |
| 3.5 | 9 | 554(46)[a] | 87(12) | 440(73)[a] | 429(42) |
| 4 | 9 | 521(40)[a] | 76(15) | 380(65) | 391(41) |
| 6 | 9 | 477(35)[b] | 76(12) | 365(58) | 369(36) |
| 8 | 9 | 478(26)[b] | 59(12) | 392(93) | 325(16) |
| 12 | 8 | 529(33)[b] | 44(12)[a] | 385(53) | 264(13)[b] |
| 24 | 9 | 542(30)[b] | 46(9) | 345(55) | 306(32)[a] |
| 48 | 9 | 455(26)[c] | 43(12)[a] | 204(29)[a] | 368(39) |
| 72 | 6 | 458(32)[b] | 25(6)[b] | 203(33)[a] | nd |
| 120 | 8 | 472(19)[c] | 39(13)[a] | 234(21)[a] | nd |
| 168 | 7 | 504(32)[b] | 54(15) | 278(24) | 415(38) |

Table 8.2. The effect of minor surgery on plasma glutamine concentrations. Values are mean (SEM) and time is given in hours. n=5 patients at all time points. The differences between control (0 hours) and all other times are not significant ($p>0.05$)

| Time after incision | Plasma glutamine concentration (uM) |
|---|---|
| 0 | 657(38) |
| 0.5 | 691(50) |
| 1 | 723(42) |
| 4 | 607(67) |
| 8 | 633(54) |
| 24 | 613(70) |

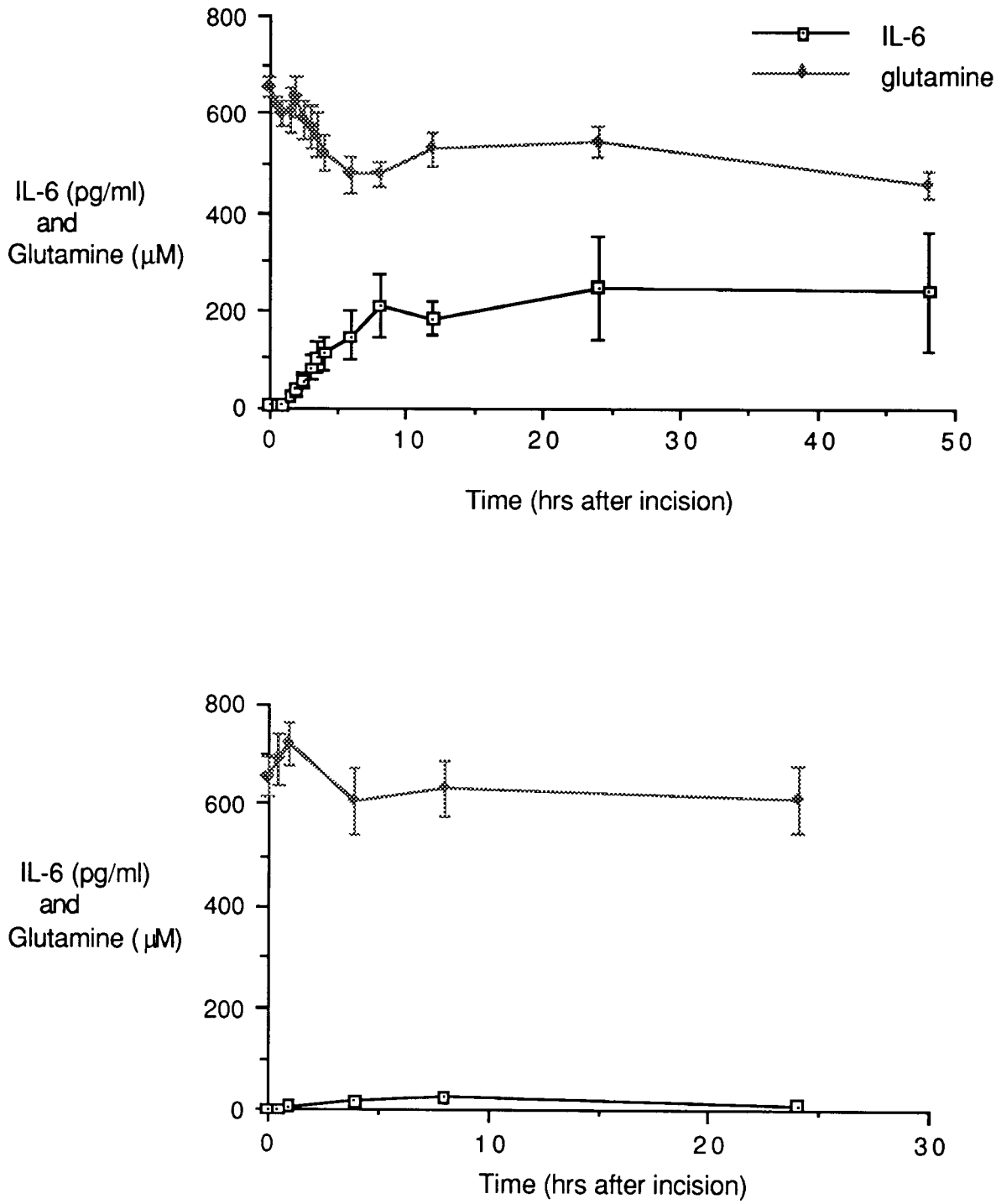



Figure 8.1. The relationship between the plasma concentrations of glutamine and interleukin-6 following major surgery in nine patients (upper graph) and minor surgery in five patients (lower graph). Values are means with standard error bars.

## 8.4 DISCUSSION

Previous studies have shown that surgery results in either no change (Hammarqvist F et al., 1990) or a small decrease (10-22 percent) (Vinnars E et al., 1975)(Stjernstrom H et al., 1986) in the plasma glutamine level at specific times post-surgery. However, neither a detailed time course of changes in concentrations nor the effect of major surgery upon plasma glutamine has been reported. Furthermore, in most studies the concentration of glutamine has been measured by automated amino acid analysis which may be unsatisfactory because glutamine is unstable in the acidic conditions used in this technique. The present study demonstrates that the plasma concentration of glutamine rapidly decreases after incision (2.5 hours) and remains markedly lower (30 percent) than preoperative values for at least 7 days following major surgery. Larger decreases (51-58 percent) in the plasma glutamine concentration have been reported following major burns (Parry-Billings M et al., 1990) and in patients suffering acute pancreatitis and multiple organ failure (Roth E et al., 1985). In contrast, in the present study minor surgery caused no change in the plasma glutamine level. The rate of glutamine release by muscle is increased following major surgery (Newsholme EA et al., 1988), therefore the rapid decline in plasma glutamine levels, reported in this chapter, is likely to result from increased rates of glutamine consumption by cells of the immune system and intestinal cells.

Given the proposed regulatory role of glutamine for cells of the immune system (Newsholme EA et al., 1988) and the experimental evidence which suggests that normal plasma levels of this amino acid are essential for a response of these cells to an immune challenge, it is suggested that this large and prolonged decrease in the plasma glutamine level may be one factor causing immunosuppression following major surgery. The

hypothesis that maintenance of an adequate supply of glutamine to the small intestine prevents malfunction of this organ and enhances survival following injury has recently been successfully tested (Klimberg VS et al., 1990b). Nutritional or pharmacological regimens that enhance the supply of glutamine to cells of the immune system after surgery may improve clinical outcome by maintenance or stimulation of the body's defence and repair mechanisms and require further evaluation in the clinical setting.

The correlation found in this work between changes in the plasma concentrations of glutamine and IL-6 is intriguing. Moreover the plasma levels of other cytokines (IL-1, TNF and INFγ) were not correlated to the plasma concentration of glutamine. It is possible that changes in the rate of glutamine utilisation and IL-6 production are controlled by the same factors (hormonal or biochemical), which change in response to the surgical insult. This hypothesis would be consistent with the synchronous timing of alterations in plasma IL-6 and glutamine concentrations after major surgery and the attenuated response of both parameters following minor surgery. Alternatively, IL-6 may be involved in the regulation of glutamine utilisation by intestinal cells, cells of the immune system and those involved in tissue repair. An increased rate of utilisation would enable these cells to respond more effectively to the immune challenge and the requirement for tissue repair following major surgery.

Tumour necrosis factor is another possible link between IL-6 and this amino acid. As discussed previously, this cytokine affects IL-6 production and is also known to induce glutamine release from muscle, independent of its effect on protein breakdown. Of itself, protein breakdown releases very little glutamine because the glutamine content of muscle protein is less than three percent. However there is a large free glutamine pool in

muscle, and the release of muscle glutamine by TNF is thought to occur via its effect on the transport mechanism of this free pool (Newsholme EA; personal communication).

Finally, the finding in Chapter 7 that the intestine, with its known high consumption of glutamine, is a major source of IL-6 but not of the other acute phase cytokines, adds substance to the suggestion that the correlation observed in this chapter reflects a significant physiological interaction between glutamine and IL-6.

The findings in both this chapter and Chapter 7 suggest that the measurement of plasma glutamine in the portal circulation may contribute to a better understanding of the utilisation of glutamine by the gut. However in addition to sampling the portal venous drainage the afferent arterial blood could also be sampled. In most surgical models, the relative inaccessibility of the portal vein has made it difficult to measure fuel requirements of the gastrointestinal tract. Samples from the arterial and hepatic venous blood have provided information about the splanchnic bed as a whole (Fong Y et al., 1990b) but are unable to partition the individual contributions of intestine and liver. Therefore abdominal aortic aneurysm patients provide a unique opportunity to study the immune and metabolic behaviour of the gastrointestinal tract.

## 8.5 SUMMARY

The plasma levels of glutamine and cytokines have been measured frequently in patients before, during and after elective aortic aneurysm surgery ("major surgery") or inguinal hernia repair ("minor surgery"). The plasma glutamine level declined rapidly following major surgery and

remained markedly below preoperative levels until at least 7 days post-surgery. This response of plasma glutamine concentration was significantly correlated with the elaboration of interleukin-6, but not with that of IL-1, TNF or IFNγ. In contrast, following minor surgery, the plasma glutamine concentration was unchanged and the elaboration of interleukin-6 was attenuated. It is concluded that the decrease in plasma glutamine following major surgery may contribute to immunosuppression following major surgery and the relationship between amino acid and cytokine metabolism is worthy of further study.

# CHAPTER NINE

# DISCUSSION

# CHAPTER NINE

## DISCUSSION

*"The measurement of any substance in the plasma reflects the net result of its synthesis and release into the circulating blood and degradation, binding or excretion of the substance with removal from the plasma. Thus sequential plasma levels reflect only the state of this dynamic balance."*

Aleksander Koj 1988

The aim of this thesis was to provide further insight into the sequence of events leading to the induction of the integrated host response to injury. There are many valid ways of investigating this subject. However, this work has concentrated primarily on studies of mediators and components in the plasma of injured patients.

Studies in the first experimental chapter (Chapters 4) examined cytokine and other responses in patients undergoing major surgery. The sequel to this, in Chapter 5, was a study of the cytokine response to major accidental trauma.

Using a clean, uniform model of major injury, namely abdominal aortic surgery patients, IL-1 was shown to be detectable in plasma after injury. This preceded a rise in IL-6 (Chapter 4). While no TNF was detected in

these patients, this cytokine was detected in the accidental trauma group within a few hours of injury, usually preceding the peak in IL-6 levels (Chapter 5). Together these two findings have provided unique*in vivo* clinical evidence in keeping with the widely accepted *in vitro* finding that IL-1 and TNF induces the production of IL-6.

It is likely that there are other mediators of IL-6 production in this trauma model, and the lack of correlation between plasma concentrations of these cytokines is not surprising. TNF and IL-1 probably exert their main effect locally, at the inflammatory site, where they are primarily produced.by monocyte/macrophages. They therefore "overflow" into the systemic circulation less freely than IL-6 whose major source is accessory cells. For these reasons the amount of TNF and IL-1 in the plasma is unlikely to reflect either their overall production or, therefore, their activity in the integrated host response at any particular time. Of more relevance, perhaps, is whether or not they are detectable at all in the plasma, regardless of concentration, because detection implies that overflow *has* occurred and hence a host response of some substance is in progress. For this reason, most studies describing a correlation between TNF and mortality have reported TNF in terms of its being either present or absent, and less in terms of the actual amounts detected (Debets JMH et al., 1989)(Marano MA et al., 1990). Tumour necrosis factor was not detected in any of the aneurysm patients who developed complications, but the highest levels detected in the trauma group were seen in the only patient who died. In contrast to reports linking TNF and mortality, others have shown that serum levels of TNF have a negligible impact on the prediction of outcome (Browder W et al., 1990)(Calandra T et al., 1990), and this research has not provided evidence to contradict this.

Endotoxin is usually placed at the beginning of the sequence of events leading to the host response. Encouraged by the finding of plasma IL-1 and TNF prior to IL-6 in the injured patients' time-course, it seemed logical to look for endotoxin in plasma samples early in the time course. Most research has implicated the gut as the source of endotoxin and bacteraemia in injured patients, therefore both the systemic and portal mesenteric blood were sampled (Chapter 6). Because of the special preparation required for endotoxin samples, sampling was begun afresh on a new series of patients and samples were also prepared for cytokine analysis. This work produced some interesting findings. The gut appears to be capable of massive IL-6 production and is almost certainly the primary source of this cytokine in aneurysm patients. The absence of TNF and IL-1 suggests that the same does not apply for these two cytokines. Endotoxin was not detected in either portal or systemic blood. Although the methodology of the LAL assay is open to question, this finding is in keeping with several recent studies casting doubt on the presence of detectable endotoxaemia in injured patients. Endotoxin may well be an important "trigger" in this setting, but not in quantities that make it easily measurable.

Interest in IL-6 and its role in the host response to injury is very recent, so that not even current text-books make particular mention of it. During the course of this research, evidence from other groups has emerged also stressing the value of IL-6 as a marker of injury severity. van Snick has suggested that it is tailored to function as an "SOS signal" because injury or infection are promptly followed by a massive induction of IL-6, and essentially every cell is capable of making this cytokine (van Snick J, 1990). Moreover its major effects appear to be beneficial to the host by enhancing immune function and acute phase protein synthesis. Our results, for both

the trauma and aneurysm patients, support van Snick's suggestion. However this observation begs another question: why is IL-6 produced in this way and which tissues are responsible for its production?

The work in Chapter 6 addresses this question. The portal blood contained very high levels of IL-6, implicating the bowel as the major source of IL-6 in the aneurysm patients. Aortic cross-clamping ensures that the colon undergoes considerable changes in tissue perfusion pressure, pH and oxygenation, all of which are likely to adversely alter cell homeostasis. If the cellular response to these stresses is to secrete IL-6, then the high portal levels would be explained. If this hypothesis is applied to the trauma patients in Chapter 5 then it would have to explain the very high levels of IL-6 seen in all these patients despite the absence of any obvious injury to the gut. However, they all had in common extensive injuries to other tissues (usually the soft tissues), no doubt resulting in considerable cell damage and alterations in cellular homeostasis within these tissues. The hypothesis would suggest that the high levels of IL-6 were released from these cells. The absence of high portal IL-6 levels in trauma patients, as reported in Moore's paper (discussed at length in Chapter 6), supports this hypothesis. It can also be applied to the aneurysm patients in Chapter 4, who suffered complications. These complications included acute renal failure and respiratory sepsis and were not related to bowel ischaemia. The hypothesis would propose that the high IL-6 levels seen in these patients reflects production by injured kidney or lung tissue, and it would require their portal IL-6 concentrations not to be any greater than those seen in uncomplicated patients' portal blood. Thus measurement of the portal IL-6 levels in these patients could have provided a means of indirectly testing the hypothesis. Unfortunately, portal sampling was not part of the protocol at that time.

Thus the proposed hypothesis states that IL-6 is released in response to cellular injury. There is no evidence in this thesis, or the literature, that this cytokine mediates any of the deleterious consequences of trauma which may result in the development of clinical complications. In fact all available evidence suggests that this cytokine attempts to ameliorate the consequences of injury through its immune and hepatic effects.

It seems clear from the literature, that production of cytokines *in vivo* may occur in a variety of tissues, for example gene transcription for TNF is increased in numerous sites after injection of endotoxin in rodents, including liver, kidney, lung and spleen. However what has not been clearly shown, is that the production of cytokines by these various tissues may be different, depending on the location of the injurious stimulus. Meningitis has provided an excellent model for testing this and, indeed, levels of TNF and IL-6 are decidedly higher in the cerebrospinal fluid than the systemic circulation. This concept of a discrete production of acute phase cytokines by different tissues, is supported by the results in Chapter 6 demonstrating large scale IL-6 production by "injured" colon, as well as the IL-6 and TNF production after soft tissue injury (Chapter 5). This same concept fits easily with the hypothesis proposed above.

Consideration can then be directed more specifically, from the level of production by different tissues to that of production by different cell types. Many cell types have been shown to be capable of acute phase cytokine synthesis. The monocyte is a well documented source of IL-1, TNF, IL-6 and IFNγ (NK cells) *in vitro*. The work in Chapter 8 set out to examine the production of cytokines by monocytes from a different and unique perspective, in the hope of learning more about this process.

Although TNF, IL-1 and IFNγ were not detected in the plasma of the aneurysm patients, there remained the possibility that these cells were secreting cytokines in concentrations that were too low to detect with ELISA, but which nevertheless could be important when these cells migrated to a site of inflammation. Results from cell culture studies (monocyte/macrophages isolated from trauma patients) showed reduced secretion of IL-1 and IFNγ (Faist E et al., 1988) and increased IL-6 secretion (Ertel W et al., 1990). These studies measure only the 'bulk release' of cytokines by entire populations of cells in culture after lengthy mitogen stimulation. Not only are such studies unable to address the question of whether the spontaneous release of this cytokine may be affected by surgical trauma, but also the use of such 'bulk release' methods implies that all cells in a population secrete equal amounts of a given cytokine. While no *in vitro* method provides a perfect model of cell behaviour in the body, it was hoped to address some of these shortcomings by the use of the reverse haemolytic plaque assay (RHPA) to study the release of cytokines by individual monocytes, without the influence of mitogens.

This *in vitro* technique demonstrated an increase in IFNγ production by NK cells at 9 hours after injury, which persisted for more than a week. Production of IL-6 was not found, suggesting that monocytes are not a particularly important source of the high plasma levels of IL-6 seen 9 hours after incision. The other two cytokines were not detectable by either ELISA or RHPA. When interpreting the results of any *in vitro* study of cell function, it has to be remembered that sampling cells after the injury means only those cells that have not already left the circulation or migrated to the site of inflammation will be studied.

As easily measurable products of cytokine mediation, acute phase proteins serve as useful indicators of cytokine activity. As in many studies, CRP has been measured in this thesis, but there have been no reports of a link between the amino acid glutamine and cytokine activity. This is surprising, because recent evidence has pointed to glutamine as an important component of the host response, making it seem plausible that this amino acid might indeed be associated with the activity of cytokine mediators of the host response. The observed correlation between plasma IL-6 and glutamine is remarkable and adds weight to the argument that the importance of this amino acid in immune function has been greatly underestimated.

There is an obvious sequel to this work namely the measurement of plasma glutamine in the portal circulation. However in addition to sampling the portal venous drainage we hope to sample the afferent arterial blood. In most surgical models, the relative inaccessibility of the portal vein has made it difficult to measure fuel requirements of the GI tract. Samples from the arterial and hepatic venous blood have provided information about the splanchnic bed as a whole but are unable to partition the individual contributions of intestine and liver. Our abdominal aortic aneurysm patients therefore provide us with a unique opportunity to study the immune and metabolic behaviour of the gastrointestinal tract.

Thus, the theme running through this thesis begins with injury and endotoxin as the initiators of the host response, which is mediated in turn by the acute phase cytokines. These are induced in different quantities and in different organs, tissues and cells, depending on the initial insult, but a sequential pattern of production seems apparent. Consideration has been

given to the role these cytokines might play in the pathogenesis of an inappropriate host response or their use as markers of this response.

The present data provide information which may be of value in the management of injured patients, namely the quantitation of the magnitude of injury and the prediction of its outcome. An important limitation in our current management is the lack of a reliable method of grading the severity of injured patients, with a view to predicting outcome. Various scoring systems have been devised including the ISS and APACHE methods reported in this thesis, as well as methods of sepsis scoring. Although these methods have proved moderately successful when used in the clinical evaluation of large series of patients for audit or research purposes, they have little predictive value for the individual patient. This may be due to their simply utilising numerous clinical and biochemical parameters which have only been shown to be of predictive value when multivariate analysis has been applied to large numbers of patients.

The present data suggests that the cytokine signal appears to antedate, by several hours, the maximum clinical response. Others have studied IL-6 levels in the urine of renal transplant patients and shown that high IL-6 levels precede clinical evidence of rejection by several days (van Oers MHJ et al., 1988)(Raasveld MHM et al, Proceedings of the 2nd Basic Sciences Symposium of the Transplant Society; Oxford, September 1991).

In principle, the use of cytokine assays, in particular the SOS signal of IL-6, would appear to be a potentially useful adjunct to the clinical assessment of critically ill patients. There is the added benefit of it being an isolated measurement, therefore able to be interpreted on its own without the

entry of complex data into a scoring data base. It is planned to test the value of this cytokine measurement in a large clinical study, which would be designed to include patients undergoing a wide variety of major surgery in all specialties. Each patient would be sampled only once, or perhaps twice, at five and ten hours after incision, and the results correlated with patient outcome.

## THE FUTURE

Cytokines are integral to normal homeostasis and to the response to injury, and their biologic activities and synergistic actions continue to be elucidated. In physiological quantities, these polypeptides are beneficial via activities such as myeloproliferation, immunostimulation and readjustments of metabolic processes to provide substrates important in an effective host response to injury. However, an excessive production of some cytokines may induce an inappropriate and detrimental host response. There is a precedent for this sort of biological behaviour, in the ability of a wholesale release of prostaglandins to induce deleterious effects on circulatory dynamics (Fink MP et al., 1985) and pulmonary vascular resistance and function (Demling RH et al., 1985). Therefore future effort will be focused increasingly on the enhancement of beneficial cytokine responses and, on the other hand, the development of antagonists to endotoxin and cytokines for use during an aberrant cytokine response. This will apply not only to the management of trauma and sepsis, but also to malignancy and immune disorders. Some success using IL-1 receptor antagonists, anti-TNF and anti-endotoxin have already been reported. The development of ever improving monoclonal antibodies and molecular biological techniques are two of the reasons behind this explosion of interest in immunotherapy. However the cost of this research is enormous and it must be hoped that constant appraisal and rigorous peer

review of this research, by the "critical research mass", will prevent bad and inappropriate research from obscuring the potential benefits of this exciting new field.

Thus, immuno-monitoring is in its infancy, while immunotherapy has moved on considerably from Coley's toxins. With the increased understanding of cytokines and other mediators elicited during the integrated host response, it is hoped that new treatments, perhaps representing modernised versions of Coley's toxins, will be able to arouse, enhance and modify the body's natural forces. These forces exist, the task ahead is to find ways to unleash them effectively.

# REFERENCES

## REFERENCES


Aarden LA, Brunner TK, Cerottini J-C, et al. (1979) The revised nomenclature for antigen non-specific T cell proliferation and helper factors. *J Immunol* **123:** 2928-2929.

Aarden LA, De Groot ER, Schaap OL, Lansdorp PM. (1987) Production of hybridoma growth factor by monocytes. *Eur J Immunol* **17:** 1411-1416.

Aggarwal B, Kohr W, Hass P, et al. (1985) Human tumor necrosis factor: production, purification, and characterization. *J Biol Chem* **260:** 2345-2364.

Alexander JW, Hegg M, Altemeier WA. (1968) Neutrophil function in selected surgical disorders. *Ann Surg* **168:** 447-458.

Alexander JW, Stinnett JD, Ogle CK, Ogle JD, Morris MJ. (1979) A comparison of immunologic profiles and their influence on bacteraemia in surgical patients with a high risk of infection. *Surgery* **86:** 94-104.

Andersen LW, Jensen TH, Jensen FM. (1988) Absorption of lipopolysaccharide from the intestine during aortic cross-clamping in humans. *Journal of Cardiothoracic Anaesthesia* **2:** 861-863.

Anon. (1974) Postoperative immunosuppression. *Lancet (editorial)* **ii:** 817-818.

Appelgren K, Bessey P, Wilmore D. Animal models in parenteral nutrition. In: Rombeau J, Caldwell M ed. Clinical Nutrition (volume 2). Philadelphia: WB Saunders Company, 1986.

Ardawi MSM. (1988) Glutamine and glucose metabolism in human peripheral lymphocytes. *Metabolism* **37:** 99-103.

Arend WP, Joslin FG, Massoni RJ. (1985) Effect of immune complexes on production by human monocytes of interleukin 1 or an interleukin 1 inhibitor. *J Immunol* **134:** 3868-3875.

Arend WP, Welgus HG, Thompson RC, Eisenberg SP. (1990a) Biological properties of recombinant human monocyte-derived interleukin 1 receptor antagonist. *J Clin Invest* **85:** 1694-1697.

Arend WP (1990b) Interleukin 1 receptor antagonist; a new member of the interleukin 1 family. *J Clin Invest* **88:** 1445-1451.

Arturson G, Fjellstrom KE. Complement-activated serum protein in clinical burns. In: Wallace AB, Wilkinson AW ed. Research in burns. Edinburgh: E and S Livingstone Ltd, 1966.

Arturson G, Hogman CF, Johannson SGO, Killander J. (1969) Changes in immunoglobulin levels in severely burnt patients. *Lancet* **i:** 546-548.

Athens JW, Haab OP, Raab SO, et al. (1961) Leukokinetic studies. IV. The total blood, circulating and marginal lymphocytic pools and the granulocyte turnover rate in normal subjects. *J Clin Invest* **40:** 989-995.

Atkins E. (1985) Fever: Historical perspectives and evolution of modern views. *Br J Rheumatol* **24(Suppl 1):** 1-5.

Auron PE, Warner SJC, Webb AC, et al. (1987) Studies on the molecular nature of human interleukin 1. *J Immunol* **138:** 1447-1456.

Ausobsky JR, Bean P, Proctor J, Pollock AV. (1982) Delayed hypersensitivty testing for the prediction of postoperative complications. *Br J Surg* **69:** 346-348.

Baker CC, Oppenheimer L, Stephens B, Lewis FR, Trunkey DD. (1980) Epidemiology of trauma patients. *Am J Surg* **140:** 144-150.

Balch HH. (1955) The effect of severe battle injury and of posttraumatic renal failure on resistance to infection. *Ann Surg* **142:** 145-163.

Bauer AR, McNeil C, Trentalman E, Swift SA, Mason JD. (1978) The depression of T lymphocytes after trauma. *Am J Surg* **136:** 674-680.

Baumann H, Gauldie J. (1990) Regulation of hepatic acute phase plasma protein genes by hepatocyte stimulating factors and other mediators of inflammation. *Mol Biol Med* **7:** 147-159.

Beeson PB. (1948) Temperature-elevating effect of a substance obtained from polymorphonuclear leucocytes. *J Clin Invest* **27:** 524.

Beisel W. (1975) Metabolic response to infection. *Ann Rev Med* **26:** 9- 20.

Belfrage S. (1963) Plasma protein pattern in the course of acute infectious disease. *Acta Med Scand* **369(Suppl):** 1-169.

Bennett B, Bloom BR. (1968) Reactions in vivo and in vitro produced by a soluble substance associated with delayed-type hypersensitivity. *Proc Natl Acad Sci USA* **59:** 756-762.

Bernheim HA, Block LH, Atkins E. (1979) Fever: Pathogenesis, pathophysiology and purpose. *Ann Int Med* **91:** 261-270.

Bernt E, Bergmeyer HU. L-glutamate, u-v assay with GDH and NAD. In: Bergmeyer HU ed. Methods of Enzymatic Analysis. London: Academic Press, 1974.

Bessey P, Watters J, Aoki T, Wilmore D. (1984) Combined hormonal infusion stimulates the metabolic response to injury. *Ann Surg* **200:** 264-281.

Beutler B, Greenwald D, Hulmes J, et al. (1985) Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. *Nature* **316:** 552-554.

Beutler B, Cerami A. (1987) Cachectin: more than a tumour necrosis factor. *N Eng J Med* **316:** 379-385.

Bhagwandeen BS, Apte M, Manwarring L, Dickeson J. (1987) Endotoxin induced hepatic necrosis in rats on an alcohol diet. *J Pathol* **151:** 47-53.

Bjornson AB, Altemeier WA, Bjornson HS. (1977) Changes in humoral components of host defense following burn trauma. *Ann Surg* **186:** 88-96.

Bjornson AB, Altemeier WA, Bjornson HS. (1978a) Host defense against opportunist microorganisms following trauma. I. Studies to determine the association between changes in humoral components of host defense and septicemia in burned patients. *Ann Surg* **188:** 93-101.

Bjornson AB, Altemeier WA, Bjornson HS. (1978b) Host defense against opportunist microorganisms following trauma. II. Changes in complement and immunoglobulins in patients with abdominal trauma and in septic patients without trauma. *Ann Surg* **188:** 102-108.

Bjornson AB, Altemeier WA, Bjornson HS. (1980) Complement, opsonins and the immune response to bacterial infection in burned patients. *Ann Surg* **191:** 323-329.

Blick M, Sherwin S, Rosenblum M. (1987) Phase I study of recombinant tumour necrosis factor in cancer patients. *Cancer Res* **47:** 2986-2989.

Bloom BR, Bennett B. (1966) Mechanism of a reaction in vitro asociated with delayed-type hypersensitivity. *Science* **153:** 80-82.

Bordet J. (1895) Les leukocytes et les proprietes actives du serum chez le vaccines. *Ann Inst Pasteur* **9:** 462.

Böyum A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g. *Scand J Clin Lab Invest* **21 (suppl 97):** 77-89.

Brearley S, Harris RI, Stone PCW, Keighley MRB. (1985) Endotoxin levels in portal and systemic blood. *Dig Surg* **2**: 70-72.

Browder W, Williams D, Pretus H, et al. (1990) Beneficial effects of enhanced macrophage function in the trauma patient. *Ann Surg* **211**: 605-613.

Calandra T, Baumgartner J-D, Grau GE, et al. (1990) Prognostic values of tumour necrosis factor/cachectin, interleukin-1, interferon alpha, and interferon gamma in the serum of patients with septic shock. *J Infect Dis* **161:** 982-987.

Cannon W. (1919) The course of events in secondary wound shock. *JAMA* **73:** 174-181.

Carswell E, Old L, Kassel R, Fiore N, Williamson B. (1975) An endotoxin induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA* **72:** 3666-3670.

Castell JV, Gomez-Lechon MJ, David M, et al. (1989) Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *Febs Lett* **242:** 237-239.

Chenu JC. Rapport au couseil de saute des armies sur les resultats due service medi medico-chururgical aux ambulances de Crimee et aux hospitaux militaires Francais en Turquie pendant la campagne d'orient en 1854-55-56. Paris: 1865.

Christou NV, Rode H, Larsen D, et al. (1984) The walk-in anergic patient: How best to assess the risk of sepsis following elective surgery. *Ann Surg* **199:** 438-444.

Christou NV, Morin JE. (1986) Host defence mechanisms in elective and emergency vascular surgery: prediciting sepsis-related mortality. *J Vasc Surg* **3:** 338-342.

Coley W. (1893) The treatment of malignant tumours by repeated inoculations of Erysipelas: with a report of ten original cases. *Am J Med Sci* **105:** 487-511.

Coley W. (1906) Late results of the treatment of inoperable sarcoma by the mixed toxins of Erysipelas and Bacillus prodigiosus. *Am Med J Sci* **131:** 375-429.

Colley CM, Fleck A, Goode AW, Muller BR, Myers MA. (1983) Early time course of the acute phase protein response in man. *J Clin Path* **36:** 203-207.

Constantian MB. (1978) Association of sepsis with an immunosuppressive polypeptide in the serum of burn patients. *Ann Surg* **188:** 209-215.

Cooper AJ, Irvine JM, Turnbull AR. (1974) Depression of immunological responses due to surgery. A model in the guinea-pig. *Immunology* **27:** 393.

Cooperstock M, Riegle L. (1985) Plasma limulus gelation assay in infants and children: correlation with gram negative bacterial infection and evidence for "intestinal endotoxaemia". *Prog Clin Biol Res* **189:** 329-45.

Crockson RA, Payne CJ, Ratcliff AP, Soothill JF. (1966) Time sequence of acute phase reactive proteins following surgical trauma. *Clin Chim Acta* **14:** 435-441.

Cruickshank AM, Fraser WD, Burns HJG, van Damme J, Shenkin A. (1990) Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. *Clin Sci* **79:** 161-165.

Cruse PJE, Foord R. (1980) The epidemiology of wound infection. *Surg Clin North Am* **60:** 27-40.

Cuthbertson DP. (1930) The disturbance of metabolism produced by bony and non-bony injury, with notes on certain abnormal conditions of bone. *Biochem J* **24:** 1244-1263.

Cuthbertson DP, Tompsett SL. (1935) Note on the effect of injury on the level of plasma proteins. *Br J Exp Pathol* **16:** 471-475.

Cuthbertson DP. (1942) Post shock metabolic responses. *Lancet* **i:** 433-437.

Dale HH. (1929) Chemical factors in control of the circulation. *Br Med J* **1:** 1093-1096.

Dale HH. (1933) Progress in autopharmacology. A survey of present knowledge of chemical regulation of certain functions by natural constituents of the tissues. *Bull Johns Hopkins Hosp* **53:** 329-347.

Davies CL, Newman RJ, Molyneux SG, Grahame-Smith DG. (1984) The relationship between plasma catecholamines and severity of injury in man. *J Trauma* **24:** 99-105.

Debets JMH, Kampmeijer R, van der Linden M, Buurman WA, van der Linden CJ. (1989) Plasma tumour necrosis factor and mortality in critically ill septic patients. *Crit Care Med* **17:** 489-494.

Deitch EA, Bridges W, Baker JL, et al. (1988) Hemorrhagic shock-induced translocation is reduced by xanthine oxidase inhibition or inactivation. *Surgery* **104:** 191-198.

Demling RH, Wong C, Jin LJ, et al. (1985) Early lung dysfunction after major burns: Role of edema and vasoactive mediators. *J Trauma* **25:** 959-966.

Di Luzio N, Al-Tuwaijri A, Williams D, Kitahama A, Browder W. Modulation of host susceptibility to endotoxin by reticuloendothelial system stimulation or depression. In: Agarwal M ed. Bacterial endotoxins and host response. Elsevier/North Holland Biomedical Press, 1980.

Dinarello CA. (1984) Interleukin-1. *Rev Infect Dis* **6:** 51-95.

Dinarello CA, Cannon JG, Wolff SM, et al. (1986) Tumour necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J Exp Med* **163:** 1433-1450.

Dinarello C. (1988) Biology of interleukin-1. *FASEB J* **2:** 108-115.

Dinarello CA, Savage N. (1989a) Interleukin-1 and its receptor. *Crit Rev Immunol* **9:** 1-20.

Dinarello CA. (1989b) Interleukin-1 and its biologically related cytokines. *Adv Immunol* **44:** 153-205.

Dumonde DC, Wolstencroft RA, Panayi GS, et al. (1969) "Lymphokines": non-antibody mediators of cellular immunity generated by lymphocyte activation. *Nature* **224:** 38-42.

Eiseman B, Beart R, Norton L. (1977) Multiple organ failure. *Surg Gynecol Obstet* **144:** 323-326.

Eisenberg SP, Evans RJ, Arend WP, Verderber E, Brewer MT, Hannum CH, Thompson RC. (1990) Primary structure and functional expression from complementary DNA of a human interleukin 1 receptor antagonist. *Nature* **343:** 341-346.

Ertel W, Faist E, Mewes A, et al. Terminal B cell maturation and immunoglobulin synthesis. In: Peiper HJ ed. Patients suffering major trauma. Berlin: Springer-Verlag, 1987.

Ertel W, Faist E, Nestle C, et al. (1990) Kinetics of interleukin-2 and interleukin-6 synthesis following major mechanical trauma. *J Surg Res* **48:** 622-628.

Faist E, Kupper TS, Baker CC, et al. (1986) Depression of cellular immunity after major injury. *Arch Surg* **121:** 1000-1005.

Faist E, Mewes A, Baker CC, et al. (1987) Prostaglandin $E_2$ ($PGE_2$) dependent suppression of interleukin alpha (IL-2) production in patients with major trauma. *J Trauma* **27:** 837-848.

Faist E, Mewes A, Strasser T, et al. (1988) Alteration of monocyte function following major injury. *Arch Surg* **123:** 287-292.

Faist E, Ertel W, Cohnert T, et al. (1990) Immunoprotective effects of cyclooxygenase inhibition in patients with major surgical trauma. *J Trauma* **30:** 8-18.

Fikrig SM, Karl SC, Suntharalingam K. (1977) Neutrophil chemotaxis in patients with burns. *Ann Surg* **186:** 746-748.

Fink MP, Homer LD, Fletcher JR. (1985) Diminished pressor response to exogenous norepinephrine and angiotensin II in septic, unanaesthetised rats: evidence for a prostaglandin-mediated effect. *J Surg Res* **38:** 335-342.

Fleck A. Metabolic response to injury. In: Ledingham I, Mackay C ed. Jamieson and Kay's textbook of surgical physiology. Edinburgh: Churchill Livingstone, 1978.

Fleck A, Colley CM, Myers MA. (1985) Liver export proteins and trauma. *Brit Med Bull* **41:** 265-273.

Flores EA, Bistrian BR, Pomposelli JJ, et al. (1989) Infusion of tumour necrosis factor/cachectin promotes muscle catabolism in the rat. *J Clin Invest* **83:** 1614-1622.

Fong Y, Tracey KJ, Moldawer LL, et al. (1989a) Antibodies to cachectin/TNF reduces interleukin-1 beta and interleukin-6 appearance during lethal bacteremia. *J Exp Med* **170:** 1627-1633.

Fong Y, Moldawer LL, Marano M, Wei He, et al. (1989b) Endotoxaemia elicits increased circulating $B_2$-IFN/IL-6 in man. *J Immunol* **142:** 2321-2324.

Fong Y, Moldawer LL, Shires GT, Lowry SF. (1990a) The biological characteristics of cytokines and their implications in surgical injury. *Surg Gynaecol Obstet* **170:** 366-378.

Fong Y, Marano MA, Moldawer LL, et al. (1990b) The acute splanchnic and peripheral tissue metabolic response to endotoxin in humans. *J Clin Invest* **85:** 1896-1904.

Frayn KN. (1986) Hormonal control of metabolism in trauma and sepsis. *Clin Endocrinol* **24:** 577-599.

Freeman R, Wheeler J, Robertson H, Paes ML, Laidler J. (1990) In-vitro production of TNF alpha in blood samples. *Lancet* i: 312-313.

Frei K, Malipiero UV, Leist TP, et al. (1989) On the cellular source and function of interleukin-6 produced in the central nervous system in viral diseases. *Eur J Immunol* **19:** 689-694.

Friberger P. The design of a reliable endotoxin test. In: ten Cate JW, Buller H, Sturk A, Levin J ed. Bacterial endotoxins: structure, biomedical significance, and detection with the Limulus Amoebocyte Lysate test. New York: Alan R Liss, Inc, 1985.

Fukui H, Brauner B, Bode JCh, Bode Ch. (1991) Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: Reevaluation with an improved chromogenic assay. *J Hepatol* **12:** 162-169.

Gann DS, Ward DG, Baertschi AJ, Carlson DE, Maran JW. (1977) Neural control of ACTH release in response to hemorrhage. *Ann NY Acad Sci* **297:** 447.

Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. (1987) Interferon-β2/B cell stimulatory factor 2 shares identity with monocyte-derived hepatocyte-stimulating factor (HSF) and regulates the major acute phase protein response in liver cells. *Proc Natl Acad Sci USA* **84:** 7251-7255.

Gauldie J, Northemann W, Fey GH. (1990) IL-6 functions as an exocrine hormone in inflammation. Hepatocytes undergoing acute phase responses require exogenous IL-6. *J Immunol* **144:** 3804-3808.

Gauperaa T, Giercksky K-E, Revhaug A, Rekvig OP. (1985) Fibronectin, complement and immunoglobulins in serum after surgery. *Br J Surg* **72:** 59-62.

Geiger T, Andus T, Klapproth J, et al. (1988) Induction of rat acute-phase proteins by interleukin-6 in vivo. *Eur J Immunol* **18:** 717-721.

Gelfand JA, Donelan M, Burke JF. (1983) Preferential activation and depletion of the alternate complement pathway by burn injury. *Ann Surg* **198:** 58-62.

Gershenwald JE, Fong Y, Fahey III TJ, Calvano SE, Chizzonite R, Kilian PL, Lowry SF, Moldawer LL. (1990) Interleukin 1 receptor blockade attenuates the host inflammatory response. *Proc Natl Acad Sci USA* **87:** 4966-4970.

Gery I, Gershon RK, Waksman BH. (1972a) Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. *J Exp Med* **136:** 128-142.

Gery I, Waksman BH. (1972b) Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s). *J Exp Med* **136:** 143-155.

Girardin E, Grau GE, Dayer JM, Roux-Lombard P, Lambert P-H. (1988) Tumour necrosis factor and interleukin-1 in the serum of children with severe infectious purpura. *N Engl J Med* **319:** 397-400.

Goldblum SE, Cohen DA, Jay M, McClain CJ. (1987) Interleukin-1 induced depression of iron and zinc: Role of granulocytes and lactoferrin. *Am J Physiol* **252:** E27-E32.

Goodall M, Alton H. (1965) Dopamine (3-hydroxytryptamine) replacement and metabolism in sympathetic nerve and adrenal medullary depletions after prolonged thermal injury. *Ann Surg* **162:** 893-900.

Goronzy J, Weyand C, Quan J, et al. (1989) Enhanced cell mediated protection against fatal Escherichia coli septicaemia induced by treatment with recombinant IL-2. *J Immunol* **142:** 1134-1138.

Green JA, Cooperband SR, Kibrick S. (1969) Immune specific induction of interferon production in cultures of human blood monocytes. *Science* **164:** 1415-1417.

Greenspan L, McLellan BA, Greig H. (1985) Abbreviated Injury Scale and Injury Severity Score: a scoring chart. *J Trauma* **25:** 60-64.

Grob P, Holch M, Fierz W, Glinz W, Geroulanos S. (1988) Immuno-deficiency after major trauma and selective surgery. *Pediatr Infect Dis J* **7:** S37-S42.

Gurd FN. (1965) Metabolic and functional changes in the intestinal mucosa during shock. *Am J Surg* **110:** 33-36.

Hack CE, De Groot ER, Felt-Bersma RJF, et al. (1989) Increased plasma levels of interleukin-6 in sepsis. *Blood* **74:** 1704-1710.

Hammarqvist F, Wernerman J, Ali R, Vinnars E. (1990) Effects of amino acid solutions enriched with either branched chain amino acids or ornithine-alpha-ketoglutarate on the post-operative intracellular amino acid concentration of skeletal muscle. *Br J Surg* **77:** 214-218.

Hannum CH, Wilcox CJ, Arend WP, et al. (1990) Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* **343:** 336-340.

Hansbrough JF, Bender EM, Zapata-Sirvent R, Anderson J. (1984) Altered helper and suppressor lymphocyte populations in surgical patients. *Am J Surg* **148:** 303-307.

Hansbrough JF, Zapata-Sirvent RL, Shackford SR, et al. (1986) Immuno modulating drugs increase resistance against sepsis in traumatized mice. *J Trauma* **26:** 625-629.

Hasselgren P, Pederson P, Sax HC, Warner BW, Fischer JE. (1988) Current concepts of protein turnover and amino acid transport in liver and skeletal muscle during sepsis. *Arch Surg* **123:** 992-999.

Heberman RB. (1984) Natural killer cells and their possible roles in host resistance against tumours. *Trans Proc* **16:** 476-478.

Heck EL, Brown L, Curreri PW, Baxter CR. (1975) Evaluation of leucocyte function in burned individuals by in vitro oxygen consumption. *J Trauma* **15:** 486-489.

Hedlund P. (1961) Clinical and experimental studies on C-reactive protein (acute phase protein). *Acta Med Scand* **169 (suppl 361):** 1-71.

Heideman M. (1979) Complement activation in vitro induced by endotoxin and injured tissue. *J Surg Res* **26:** 670-673.

Helfgott DC, May LT, Sthoeger Z, Tamm I, Sehgal PB. (1987) Bacterial lipopolysaccharide (endotoxin) enhances expression and secretion of $B_2$ interferon by human fibroblasts. *J Exp Med* **166:** 1300-1309.

Helfgott DC, Tatter SB, Santhanam U, et al. (1989) Multiple forms of IFN-beta/IL-6 in serum and other body fluids during acute bacterial infection. *J Immunol* **142:** 948-953.

Hershman MJ, Cheadle WG, George CD, et al. (1988a) The response of immunoglobulins to infection after thermal and non-thermal injury. *Am Surg* **54:** 408-411.

Hershman MJ, Polk HC, Pietsch JD, et al. (1988b) Modulation of infection by gamma interferon treatment following trauma. *Infect Immun* **56:** 2412-2416.

Hesse DG, Tracey KJ, Fong Y, et al. (1987) Cytokine appearance in human endotoxemia and primate bacteraemia. *Surg Gynecol Obstet* **166:** 147-153.

Hinshaw LB. Overview of endotoxic shock. In: Cowley RA, Trump BV ed. Pathophysiology of shock, anoxia and ischemia. Baltimore: Williams and Wilkins, 1982.

Hirano T, Yasukawa K, Harada H, et al. (1986) Complementary DNA for a novel human interleukin (BSF-2) that induces B lymphocytes to produce immunoglobulin. *Nature* **324:** 73-76.

Hoch-Ligeti C, Irvine K, Sprinkle EP. (1953) Investigation of serum protein patterns in patients undergoing operation. *Proc Soc Exp Biol Med* **84:** 707-710.

Howard RJ. (1979) Effect of burn injury, mechanical trauma, and operation on immune defenses. *Surg Clin North Am* **59:** 199-211.

Howes RM, Allen RC, Su CT, Hoopes JE. (1976) Altered polymorphonuclear leukocyte bioenergetics in patients with thermal injury. *Surg Forum* **27:** 558-560.

Hume DM, Egdahl RH. (1959) The importance of the brain in the endocrine response to injury. *Ann Surg* **150:** 697-712.

Hunter J. A treatise on the blood inflammation and gunshot wounds. London: 1794.

Jablons DM, Mule JJ, McIntosh JK, et al. (1989) IL-6/IFN-β-2 as a circulating hormone. Induction by cytokine administration in humans. *J Immunol* **142:** 1542-1547.

Jacob AI, Goldberg PK, Bloom N, Degenshein GA, Kozinn PJ. (1977) Endotoxin and bacteria in portal blood. *Gastroenterol* **72:** 1268-1270.

Jampel HD, Duff GW, Gershon RK, Atkins E, Duram SK. (1983) Fever and immunoregulation. III. Hyperthermia auguments the primary in vitro humoral immune response. *J Exp Med* **157:** 1229-1238.

Kahn CR. (1978) Insulin resistance, insulin insensitivity and insulin unresponsiveness. *Metabolism* **27:** 1893-1902.

Kampschmidt RF. Leucocytic endogenous mediator/endogenous pyrogen. In: Powanda MC, Canonico PG ed. Infection: The physiologic and metabolic responses of the host. Amsterdam, New York, Oxford: Elsevier, 1981.

Kampschmidt RF, Upchurch HF, Eddington CL, Pulliam LA. (1973) Multiple biological activities of a partially purified leucocytic endogenous mediator. *Ann J Physiol* **224:** 530-533.

Kasahara T, Hooks JJ, Dougherty SF, Oppenheim JJ. (1983) Interleukin 2 mediated immune interferon (IFN-gamma) production by human T cells and T cell subsets. *J Immunol* **130:** 1784-1789.

Kasakura S, Lowenstein L. (1970) Heterogeneity of blastogenic factors produced in vitro by antigenically stimulated and unstimulated leukocytes. *J Immunol* **105:** 1162-1167.

Kawakami M, Pekala P, Lane M, Cerami A. (1982) Lipoprotein lipase suppression in 3T3-Li cells by an endotoxin-induced mediator from exudate cells. *Proc Natl Acad Sci USA* **72:** 912-916.

Kehlet H, Brandt M, Hansen A, Alberti K. (1979) Effect of epidural analgesia on metabolic profiles during and after surgery. *Br J Surg* **66:** 543-546.

Kelly CD, Welte K, Murray HW. (1987) Antigen induced interferon gamma production: Differential dependence on interleukin-2 and its receptor. *J Immunol* **139:** 2325-2328.

Kelley CJ, Ingoldby CJH, Blenkarn JI, Wood CB. (1985) Colonoscopy related endotoxaemia. *Surg Gynaecol Obstets* **161:** 332-334.

King L. (1957) Dr. Koch's postulates. *J Hist Med* **7:** 350-361.

Klimberg VS, Souba WW. (1990a) The importance of intestinal glutamine metabolism in maintaining a healthy gastrointestinal tract and supporting the body's response to injury and illness. *Surg Ann* **22:** 61-76.

Klimberg VS, Salloum RM, Kasper M, et al. (1990b) Oral glutamine accelerates healing of the small intestine and improves outcome after whole abdominal radiation. *Arch Surg* **125:** 1040-1045.

Kluger MJ, Rothenburg BA. Fever, trace metals and disease. In: Lipton JM ed. Fever. New York: Raven Press, 1979.

Kohase M, Henriksen DeStefano D, May LT. (1986) Induction of beta 2-interferon by tumour necrosis factor: a homeostatic mechanism in control of cell proliferation. *Cell* **45:** 659-666.

Koj A. Metabolic studies of acute-phase proteins. In: Marini G ed. Patho-physiology of plasma protein metabolism. London: Macmillan, 1983.

Koj A. Definition and classification of the acute-phase proteins. In: Gordon AH, Koj A ed. The acute-phase response to injury and infection. Amsterdam, New York, London: Elsevier, 1985.

Koj A, Magielska-Zero D, Bereta J, et al. (1988) The cascade of inflammatory cytokines regulating synthesis of acute phase proteins. *Tokai J Exp Clin Med* **13:** 255-264.

Kushner I. (1982) The phenomenon of the acute phase response. *Ann NY Acad Sci* **389:** 39-48.

Kwiatkowski D, Hill AV, Sambou I, et al. (1990) TNF concentration in fatal cerebral, non-fatal cerebral and uncomplicated Plasmodium falciparum malaria. *Lancet* **ii:** 1201-1204.

Laurell CB. (1966) Quantitative estimation of proteins by electrophoresis in agarose gels containing antibodies. *Ann Biochem* **15:** 45-52.

Lee DM, Zentella A, Pekala PH, Cerami A. (1987) Effect of endotoxin induced monokines on glucose metabolism in the muscle cell line L-6. *Proc Natl Acad Sci USA* **84:** 2590-2594.

Lennard TWJ, Shenton BK, Borzotta A, et al. (1985) The influence of surgical operations on components of the human immune system. *Br J Surg* **72:** 771-776.

Leon C, Rodrigo MJ, Tomasa A, et al. (1982) Complement activation in septic shock due to gram negative and gram positive bacteria. *Crit Care Med* **10:** 308-310.

Levin J. The Limulus Amebocyte Lysate Test: Perspectives and problems. In: Watson SW, Levin J, Novitsky TJ ed. The Fourth International Conference on the Detection of Bacterial Endotoxins with the LAL test. Woods Hole, Massachusetts: Alan R Liss, Inc, 1985.

Levin J, Bang F. (1964) The role of endotoxin in the extracellular coagulation of Limulus blood. *Bulls Johns Hopkins Hosp* **115:** 265-274.

Levin J, Poore TE, Zauber NP, Oser RS. (1970) Detection of endotoxin in the blood of patients with sepsis due to Gram-negative bacteria. *New Eng J Med* **283:** 1313-1316.

Lewis CE, McCarthy SP, Richards PS, et al. (1990) Measurement of cytokine release by human cells: a quantitative analysis at the single cell level using the reverse haemolytic plaque assay. *J Immunol Methods* **127:** 51-59.

Lewis CE, Ramshaw A, Lorenzen J, McGee J. (1991) Basic fibroblast growth factor and interleukins 4 and 6 stimulate the release of IFN gamma by individual NK cells. *Cell Immunol* **132:** 158-167.

Lister J. (1870) On the effects of the antiseptic system of treatment upon the salubrity of a surgical hospital. *Lancet* **i:** 4.

Livesey G, Lund P. (1980) Enzymic determinations of branched chain amino acids and 2-oxoacids in rat tissues. *Biochem J* **188:** 705-713.

Livingstone D, Appel S, Wellhausen S, et al. (1988) Depressed interferon gamma production and monocyte HLA-DR expression after severe injury. *Arch Surg* **123:** 1309-1312.

Maclean LD, Meakins JL, Taguchi K, et al. (1975) Host resistance in sepsis and trauma. *Ann Surg* **182:** 207-217.

Macleod CM, Avery OT. (1941a) The occurrence during acute infections of a protein not normally present in the blood. II. Isolation and properties of the reactive protein. *J Exp Med* **73:** 183-190.

Macleod CM, Avery OT. (1941b) The occurrence during acute infections of a protein not normally present in the blood. III. Immumological properties of the C-reactive protein and its differentiation from normal blood proteins. *J Exp Med* **73:** 191-200.

Majno G. The healing hand: man and wound in the ancient world. Cambridge, Massachusetts: Commonwealth Fund Book, Harvard University Press, 1975.

Malangoni M, Livingstone D, Sonnenfeld G, Polk H. (1989) Interferon gamma and tumour necrosis factor alpha: use in gram-negative infection after shock. *Arch Surg* **125:** 444-446.

Mancini G, Carbonara AO, Heremans JF. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochem* **2:** 235-254.

Marano MA, Fong Y, Moldawer LL, et al. (1990) Serum cachectin/tumour necrosis factor in critically ill patients with burns correlates with infection and mortality. *Surg Gynae Obstets* **170:** 32-38.

March C, Mosley B, Larsen A, et al. (1985) Cloning sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* **315:** 641-647.

Mathison JC, Wolfson E, Ulevitch RJ. (1988) Participation of tumour necrosis factor in the mediation of gram negative bacterial lipo-polysaccharide-induced injury in rabbits. *J Clin Invest* **81:** 1925-1932.

Matthews JNS, Altman DG, Campbell MJ, Royston P. (1990) Analysis of serial measurements in medical research. *Br Med J* **300:** 230-235.

McCabe WP, Rebuck JW, Kelly AP, Ditmars DM. (1973) Leukocytic response as a monitor of immunodepression in burn patients. *Arch Surg* **106:** 155-159.

McIrvine AJ, O'Mahony JB, Saporoschetz I, Mannick JA. (1982) Depressed immune responses in burn patients: use of monoclonal antibodies and functional assays to define the role of suppressor cells. *Ann Surg* **196:** 297-304.

Menkin V. (1943) Chemical basis of injury in inflammation. *Arch Pathol* **36:** 269-288.

Mertz W. (1981) The essential trace elements. *Science* **213:** 1332-1338.

Merz B. (1986) Trials test new biologic, old therapeutic concept. *JAMA* **256:** 1249.

Metchnikoff E. Immunity in Infective Diseases. Cambridge, Massachusetts: Harvard University Press, 1905.

Michalek S, Moore R, McGhee J, Rosenstreich D, Mergenhagen S. (1980) The primary role of the reticular cells in the mediation of host responses to bacterial endotoxin. *J Infect Dis* **141:** 55-63.

Michie HR, Manogue KR, Spriggs DR, et al. (1988a) Detection of circulating tumour necrosis factor after endotoxin administration. *N Engl J Med* **318:** 1481-1486.

Michie HR, Spriggs DR, Manogue KR, et al. (1988b) Tumour necrosis factor and endotoxin induce similar metabolic responses in human beings. *Surgery* **104:** 280-286.

Miller CL. (1981) Secondary immunodeficiency in burns and after surgical trauma. *Clin Immunol Allergy* **1:** 641-667.

Miller SE, Miller CL, Trunkey DD. (1982) The immune consequences of trauma. *Surg Clin North Am* **62:** 167-181.

Minchin-Clarke HG, Freeman T, Pryse-Phillips W. (1971) Serum protein changes after injury. *Clin Sci* **40:** 337-344.

Moldawer LL, Andersson C, Gelin J, et al. (1987) Regulation of food intake and hepatic protein metabolism by recombinant-derived monokines. *Am J Physiol* **254:** 450-456.

Moore F. (1980) Energy and the maintenance of body cell mass. *J Parent Ent Nutrition* **4:** 228-259.

Moore FA, Moore EE, Poggetti R, et al. (1991) Gut bacterial translocation via the portal vein: A clinical perspective with major torso trauma. *J Trauma* **31:** 631-638.

Morgan DA, Ruscetti FW, Gallo R. (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science* **193:** 1007-1010.

Morrison DC, Ryan JC. (1987) Endotoxin and disease mechanisms. *Ann Rev Med* **38:** 417-432.

Neill JD, Frawley LS. (1983) Detection of hormone release from individual cells in mixed populations using a reverse haemolytic plaque assay. *Endocrinology* **112:** 1135-1140.

Newsholme EA, Newsholme P, Curi R, et al. (1988) A role for muscle in the immune system and its importance in surgery, trauma, sepsis and burns. *Nutrition* **4:** 261-268.

Newton R, Uhl J, Covington M, Back O. (1988) The distribution and clearance of radiolabelled human interleukin-1β in mice. *Lymphokine Res* **7:** 207-216.

Nijsten MWN, de Groot ER, ten Duis HJ, et al. (1987) Serum levels of interleukin 6 and acute phase responses. *Lancet (letter)* **ii:** 921.

Nijsten MWN, Hack CE, Helle M, et al. (1991) Interleukin-6 and its relation to the humoral immune response and clinical parameters in burned patients. *Surgery* **109:** 761-767.

Nohr CW, Christou NV, Rode H, et al. (1984) In vivo and in vitro humoral immunity in surgical patients. *Ann Surg* **200:** 373-380.

Nolan JP. (1981) Endotoxin, reticuloendothelial function, and liver injury. *Hepatology* **1:** 458-465.

Nolan JP, Ali MV. (1968) Endotoxin and the liver I. Toxicity in rats with choline deficient livers. *Proc Soc Exp Biol Med* **129:** 29-31.

O'Flaherty JT. (1982) Lipid mediators of inflammation and allergy. *Lab Invest* **47:** 314.

O'Mahoney JB, Palder SB, Wood JJ. (1984) Depression of cellular immunity after multiple trauma in the absence of sepsis. *J Trauma* **24:** 869-875.

O'Mahoney JB, Wood JJ, Rodrick ML, Mannick JA. (1985) Changes in T lymphocyte subsets following injury. *Ann Surg* **202:** 580-586.

Ohlsson K, Bjork P, Bergenveldt M, Hageman R, Thompson RC. (1990) Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature* **348:** 550-552.

Okusawa S, Gelfand JA, Ikejima T, Connolly RA, Dinarello CA. (1988) Interleukin-1 induces a shock-like state in rabbits. Synergism with tumor necrosis factor and the effects of cyclooxygenase inhibition. *J Clin Invest* **81:** 1162-1172.

Olofsson P, Nylander G, Olsson P. (1986) Endotoxin: Routes of transport in experimental peritonitis. *Am J Surg* **151:** 443-446.

Oomura Y. (1988) Chemical and neuronal control of feeding motivation. *Physiol Behav* **44:** 555-560.

Ottow RT, Bruining HA, Jeekel J. (1984) Clinical judgement versus delayed hypersensitivity skin testing for the prediction of postoperative sepsis and mortality. *Surg Gynecol Obstet* **159:** 475-477.

Packard FR. The life and times of Ambroise Pare. New York: Hoeber, 1927.

Park SK, Brody JI, Wallace HA, et al. (1971) Immunosuppressive effect of surgery. *Lancet* **i:** 53-55.

Parry-Billings M, Evans J, Calder PC, Newsholme EA. (1990) Immuno-suppression following major burns: could glutamine play a role? *Lancet* **i:** 523-525.

Pepys MB. (1981) Serum C-reactive protein, serum amyloid P-component and serum amyloid A protein in autoimmune disease. *Clinics in Immunology* **1:** 77-101.

Perlmutter DH, Dinarello CA, Punsal PI, Colten HR. (1986) Cachectin/tumor necrosis factor regulates hepatic acute phase gene expression. *J Clin Invest* **78:** 1349-1353.

Pollock RE, Babcock GF, Romsdahl MM, Nishioka K. (1984) Surgical stress-mediated suppression of murine natural killer cell cytotoxicity. *Cancer Res* **44:** 3888-3891.

Poupart P, Vandenabeele P, Cayphas S, Van-Snick J, et al. (1987) B cell growth modulating and differentiating activity of recombinant

human 26-kd protein (BSF-2, HulFN-beta 2, HPGF). *EMBO J* **6:** 1219-1224.

Powanda M. (1977) Changes in body balances of nitrogen and other key nutrients: description and underlying mechanisms. *Am J Clin Nutr* **30:** 1254-1268.

Powanda MC, Moyer ED. (1981) Plasma proteins and wound healing. *Surg Gynaecol Obs* **153:** 749-755.

Prytz H, Holst-Christensen, Korner B, Liehr H. (1978) Portal venous and systemic endotoxaemia in patients without liver disease and systemic endotoxaemia in patients with cirrhosis. *Scand J Gastroenterol* **11:** 857-863.

Pullicino EA, Carli F, Poole S, et al. (1990) The relationship between the circulating concentrations of interleukin 6 (IL-6), tumour necrosis factor (TNF) and the acute phase response to elective surgery and accidental injury. *Lymphokine Res* **9:** 231-238.

Riddle PR, Berenbaum MC. (1967) Postoperative depression of the lymphocyte response to phytohaemagglutinin. *Lancet* **i:** 746-748.

Robin M, Intrator L, Rapin M. (1975) Complement activation in septic shock. *N Eng J Med* **293:** 304.

Rodrick Ml, Wood JJ, O'Mahoney JB, et al. (1986) Mechanism of immunosuppression associated with severe nonthermal traumatic injuries in man: Production of interleukin 1 and 2. *J Clin Immunol* **6:** 310-318.

Rosenberg SA, Packard BS, Aebersold PM, et al. (1988) Use of tumour-infiltrating lymphocytes and interleukin-2 in the immunotherapy of patients with metastatic melanoma. *N Eng J Med* **319:** 1676-1680.

Roth E, Zoch G, Schulz F, et al. (1985) Amino acid concentrations in plasma and skeletal muscle of patients with acute hemorrhagic necrotizing pancreatitis. *Clin Chem* **31:** 1305-1309.

Ryan JL. Microbial factors in pathogenesis: Lipopolysaccharides. In: Root RK, Sande MA ed. Septic shock. New York: Churchill Livingstone, 1985.

Saadia R, Schein M, MacFarlane C, Boffard KD. (1990) Gut barrier function and the surgeon. *Br J Surg* **77:** 487-492.

Saba TM. (1975) Reticuloendothelial systemic host defence after surgery and traumatic shock. *Circ Shock* **2:** 91-107.

Sakai H, Daniels JC, Beathard GA, et al. (1974) Mixed lymphocyte culture reaction in patients with acute thermal burns. *J Trauma* **14:** 53-57.

Schmidt J, Olivier CN, Lepe-Zuniga JL, et al. (1984) Silica-stimulated monocytes release fibroblast proliferation factors identical to interleukin-1. *J Clin Invest* **73:** 1462-1472.

Seeger W, Stohr G, Wolf HRD, Neuhof H. (1985) Alteration of surfactant function due to protein leakage. *J Appl Physiol* **58:** 326-339.

Sehgal PB, Zilberstein A, Ruggieri RM, et al. (1987) Human chromosome 7 carries the $\beta_2$ interferon gene. *Proc Natl Acad Sci USA* **84:** 3633-3637.

Shalaby MR, Aggarwal BB, Rinderknecht E, et al. (1985) Activation of human polymorphonuclear neutrophil functions by interferon and tumour necrosis factor. *J Immunol* **135:** 2069-2073.

Shalaby MR, Waage A, Aarden L, Espevik T. (1989) Endotoxin, tumour necrosis factor-alpha and interleukin-1 induce interleukin-6 production in vivo. *Clin Immunol Immunopathol* **53:** 488-498.

Shaw GB. The Doctor's dilemma. London: Penguin, 1906.

Shear M, Turner F. (1943) Chemical treatment of tumors. V. Isolation of the hemorrhage-producing fraction from Serratia marcescens (Bacillus prodigiosus) culture filtrate. *J Natl Cancer Inst* **4:** 81-97.

Shenkin A, Fraser WD, Series J, et al. (1989) The serum interleukin 6 response to elective surgery. *Lymph Res* **8:** 123-127.

Shoham S, Davenne D, Cady AB, et al. (1987) Recombinant tumour necrosis factor and interleukin-1 enhance slow wave sleep. *Am J Physiol* **253:** R142-R149.

Slade MS, Simmons RL, Yunis E, Greenberg LJ. (1975) Immunodepression after major surgery in normal patients. *Surgery* **78:** 363-372.

Smith KA. (1988) Interleukin-2: Inception, impact and implications. *Science* **240:** 1169-1176.

Smith R, Kirstein M, Fiers W, Baglioni C. (1986) Species specificity of human and murine tumor necrosis factor. *J Biol Chem* **261:** 14871-14874.

Souba WW, Smith RJ, Wilmore DW. (1985) Glutamine metabolism by the intestinal tract. *J Parent Ent Nutr* **9:** 608-617.

Spinas GA, Bloesch D, Kaufmann M-T, Keller U, Dayer J-M. (1990) Induction of plasma inhibitors of interleukin 1 and TNFα activity by endotoxin administration to normal human volunteer. *Am J Physiol* **259:** R993-R997.

Spinas GA, Bloesch D, Keller U, Zimmerli W, Cammisuli S. (1991) Pretreatment with ibuprofen augments circulating tumour necrosis factor alpha, interleukin-6 and elastase during acute endotoxaemia. *J Infect Dis* **163:** 89-95.

Squires EJ, Hall DE, Brosnan JT. (1976) Arteriovenous differences for amino acids and lactate across kidney of normal and acidotic rats. *Biochem J* **160:** 125-128.

Stahl WM. (1987) Acute phase protein response to tissue injury. *Crit Care Med* **15:** 545-550.

Stjernstrom H, Lund J, Wiklund L, et al. (1986) The influence of abdominal surgical trauma on the exchange of blood borne amino acids in the human leg. *Clin Nutr* **5:** 123-131.

Sunderman FW Jr. (1964) Studies of the serum proteins. VI. Recent advances in clinical interpretation of electrophoretic fractionations. *Am J Clin Pathol* **42:** 1-21.

Szondy Z, Newsholme EA. (1989) The effect of glutamine concentration on the activity of carbamoyl-phosphate synthase II and on the incorporation of tritiated thymidine into DNA in rat mesenteric lymphocytes stimulated by phytohaemaglutinin. *Biochem J* **261:** 979-983.

Takada S, Ueda Y, Murakawa Y, et al. (1983) Functional heterogeneities among concanavalin A activated OKT4 and OKT8 cells by using autologous erythrocyte rosette technique. *J Clin Invest* **72:** 2060-2071.

Talcott J, Finberg R, Mayer R, Goldman L. (1988) The medical course of cancer patients with fever and neutropenia. *Arch Intern Med* **148:** 2561-2568.

Thomas Y, Rogozinski L, Irigoyen CH, et al. (1982) Functional analysis of human T cell subsets defined by monoclonal antibodies: V. Supressor cells within the activated OKT4 population belong to a distinct subset. *J Immunol* **128:** 1386-1390.

Tillet WS, Francis T Jr. (1930) Serological reactions in pneumonia with a non-protein somatic fraction of pneumococcus. *J Exp Med* **52:** 561-571.

Tosatu G, Jones KD. (1990) Inteleukin-1 induces interleukin-6 production in peripheral blood monocytes. *Blood* **75:** 1305-1310.

Tracey K, Beutler B, Lowry S, et al. (1986) Shock and tissue injury induced by recombinant human cachectin. *Science* **234:** 470-474.

Triger DR. (1991) Endotoxaemia in liver disease - time for re-appraisal? *J Hepatol* **12:** 136-138.

Trinchieri G, Perussia B. (1985) Immune Interferon: a pleiotrophic lymphokine with multiple effects. *Immunol Today* **6:** 131-136.

Ulich TR, Castillo JD, Keys M, et al. (1987) Kinetics and mechanism of recombinant human interleukin-1 alpha and tumour necrosis factor alpha induced changes in circulating neutrophils and lymphocytes. *J Immunol* **139:** 3406-3415.

Unanue ER, Rosenthal ES. Macrophage regulation of immunity. New York: Academic Press, 1980.

Van Damme J, Cayphas S, Van Snick J, Conings R, et al. (1987) Purification and characterisation of human fibroblast-derived hybridoma growth factor identical to T-cell-derived B-cell stimulatory factor-2 (interleukin-6). *Eur J Biochem* **168:** 543-550.

van Deventer S, ten Cate J, Tytgat G. (1988a) Intestinal endotoxaemia-clinical significance. *Gastroenterology* **94:** 825-831.

van Deventer SJH, Buller HR, ten Cate JW, Sturk A, Pauw W. (1988b) Endotoxaemia: An early predictor of septicaemia in febrile patients. *Lancet* **i:** 605-609.

van Deventer SJH, Buller HR, ten Cate JW, et al. (1990) Experimental endotoxaemia in humans: analysis of cytokine release and coagulation, fibrinolytic and complement pathways. *Blood* **76:** 2520-2526.

van Noordwijk J. Parallel line assays of endotoxins with the LAL chromogenic substrate method. In: ten Cate JW BH Sturk A, Levin J ed. Bacterial endotoxins. Structure, biomedical significance and detection with the Limulus amebocyte lysate test. New York: Alan R Liss, 1985.

van Oers MHJ, van der Heyden AA, Aarden LA. (1988) Interleukin-6 in the serum and urine of renal transplant patients. *Clin Exp Immunol* **71:** 314-319.

van Snick J. (1990) Interleukin-6: an overview. *Ann Rev Immunol* **8:** 253-278.

Vinnars E, Bergstrom J, Furst P. (1975) Influence of postoperative state on the intracellular free amino acids in human muscle tissue. *Ann Surg* **182:** 665-671.

Vogel S, Hansen C, Rosenstreich D. (1979a) Characterisation of a congenitally LPS-resistant athymic mouse strain. *J Immunol* **122:** 619-622.

Vogel S, Rosenstreich D. (1979b) Defective Fc receptor-mediated phagocytosis in C3H/HeJ macrophages; 1) Correction by lymphokine induced stimulation. *J Immunol* **123:** 2842-2850.

Waage A, Halstensen A, Espevik T. (1987) Association between tumour necrosis factor in the serum and fatal outcome in patients with meningococcal disease. *Lancet* **i:** 335-337.

Walter JS, Meyers P, Kreuger JM. (1989) Microinjection of interleukin-1 into brain: separation of sleep and fever responses. *Physiol Behav* **45:** 169-176.

Wannemacher RW Jr, Pekarek RS, Thompson WL, et al. (1975) A protein from polymorphonuclear leucocytes (LEM) which affects the rate of hepatic amino acid transport and synthesis of acute phase globulins. *Endocrinol* **96:** 651-661.

Warden GD. Leukocyte chemotaxis in thermally injured patients. In: Ninnemann JL ed. The immune consequences of thermal injury. Baltimore: Williams and Wilkins, 1981.

Warren RS, Donner DB, Starnes HF, Brennan MF. (1987) Modulation of endogenous hormone action by recombinant human tumour necrosis factor. *Proc Natl Acad Sci USA* **84:** 8619-8622.

Weissenbach J, Chernajovsky Y, Zeevi M, et al. (1980) Two interferon mRNAs in human fibroblasts: in vitro translation and *Escherichia coli* cloning studies. *Proc Natl Acad Sci USA* **77:** 7152-7156.

Whicher JT. (1985) Interleukin-1 and the acute phase proteins. *Br J Rheumatol* **24(suppl 1)):** 21-24.

White JC, Smithwick RH, Simeone FA. The Autonomic Nervous System (3rd ed.) New York: Macmillan, 1952.

Wiedermann CJ. (1989) Interleukin 1 interaction with neuroregulatory systems: Selective enhancement by recombinant human and mouse interleukin 1 of in vitro opioid peptide receptor binding in rat brain. *J Neurosci Res* **22:** 172-180.

Wilkinson SP, Arroyo V, Gazzard BG, Moodie H, Williams R. (1974) Relationship of renal impairment and haemorrhagic diathesis to endotoxaemia in fulminant hepatic failure. *Lancet* **i:** 521-525.

Williamson DH. L-alanine determination with alanine dehydrogenase. In: Bergmeyer HU ed. Methods of Enzymatic Analysis. London: Academic Press, 1974.

Wilmore DW, Long JM, Mason AD Jr, Skreen RW, Pruitt BA Jr. (1974) Catecholamines: Mediators of the hypermetabolic response to thermal injury. *Ann Surg* **180:** 653-669.

Wilmore D, Mason A, Johnson D, Pruitt B. (1975) Alterations in hypothalamic function following thermal injury. *J Trauma* **15:** 697-703.

Windmueller HG, Spaeth AE. (1974) Uptake and metabolism of plasma glutamine by the small intestine. *J Biol Chem* **294:** 5070-5079.

Wolff S. Biological effects of bacterial endotoxins in man. In: Wolff S, Kass E ed. Bacterial lipopolysaccharides. Chicago: The University of Chicago Press, 1973.

Wolvekamp MCJ, Marquet RL. (1990) Interleukin-6: historical background, genetics and biological significance. *Immunol Lett* **24:** 1-10.

Wood WB Jr. (1958) The role of endogenous pyrogen in the genesis of fever. *Lancet* **ii:** 53-57.

Yocum RS, Doerner AA. (1957) A clinical evaluation of the C-reactive test. *AMA Arch Int Med* **99:** 74-81.

Young H, Ortaldo J. (1987) One signal requirement for interferon-gamma production by human large granular lymphocytes. *J Immunol* **139:** 724-727.

Ziegler EJ, Fisher CJ, Sprung CL, et al. (1991) Treatment of Gram-negative bacteremia and septic shock with human monoclonal antibody against endotoxin. *N Eng J Med* **324:** 429-436.