STUDIES ON INTERLEUKIN-6: ITS ROLE IN THE ACUTE PHASE RESPONSE AND ITS CLINICAL VALUE AS A MARKER OF TISSUE DAMAGE

Anne McDonald Cruickshank

MB ChB (Glasgow)

MRCPath

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Institute of Biochemistry
Royal Infirmary
Glasgow

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SUMMARY

In vitro evidence suggests that interleukin-6 (IL-6) is a major mediator of the acute phase response. The aims of this work were threefold: firstly, to validate a bioassay for the measurement of IL-6 in serum; secondly, to characterise the role of IL-6 in the acute phase response and determine its potential as a marker of tissue damage using elective surgical patients as models of controlled trauma; and thirdly, to assess its value in four clinical situations : post-operative complications; suspected myocardial infarction; pancreatitis; and rheumatoid arthritis.

The validation of a hybridoma growth stimulation assay using the mouse 7TD1 cell line with colorimetric evaluation of cell numbers is described. This assay was used to measure serum IL-6 concentrations at timed intervals post-operation in 39 patients from 6 broad surgical categories. Serum IL-6 rose within 2 to 4 hours of incision in all surgical groups. The magnitude of the IL-6 response was strongly associated with duration of surgery, and was associated significantly with C-reactive protein (CRP) response, increase in axillary temperature and reduction in zinc/albumin ratio.

Surgical patients who subsequently developed complications had higher IL-6 concentrations 24 hours post-operation than those who did not.

Serum IL-6 was also measured in 15 patients with suspected myocardial infarction, in 23 patients with acute pancreatitis, and in 33 patients with rheumatoid arthritis. In patients with suspected myocardial infarction, admission serum IL-6 concentration discriminated completely between those with angina and those who had suffered myocardial infarction, and was strongly associated with left ventricular ejection fraction on the third hospital day. Patients who had severe pancreatitis had higher serum IL-6 levels on admission than those with mild

disease. Serum IL-6 concentrations in rheumatoid arthritis were significantly associated with clinical disease activity.

These results are consistent with the hypothesis that IL-6 is a major mediator of the systemic inflammatory response, and indicate that it behaves as an early, quantitative marker of tissue damage. Its measurement is likely to be of value in clinical practice, particularly in acute situations, but larger numbers of patients require to be studied to confirm these findings.

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DECLARATION OF WORK PUBLISHED AND PRESENTED

The following papers relating to work presented in this thesis have been published:

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Fearon KCH, McMillan DC, Preston T, Cruickshank AM, Winstanley P, Shenkin A. Reduced rate of hepatic protein synthesis and acute phase protein response, and elevated circulating interleukin-6 and tumour necrosis factor in advanced malignancy. Ann Surg 1991; 213: 26-31.

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Hamid SK, Scott NG, Sutcliffe NP, Tighe SQM, Anderson JR, Cruickshank AM, Kehlet H. The effect of continuous coeliac plexus blockade plus intermittent wound infiltration with local anaesthesia on pain relief, pulmonary function and the stress response following upper abdominal surgery: a double blind randomised study. Acta Anaesthesiol Scand 1992; 36: 534-9.

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STATEMENT OF AUTHOR'S CONTRIBUTION AND COLLABORATION

The concept of this project was developed by myself in conjunction with Professor Shenkin towards the end of 1987.

The analytical work and recruitment of patients necessary for the first part of the project, namely the study of the role of IL-6 in the acute phase response and its potential as an inflammatory marker, were carried out by myself. The second part of the project, namely the assessment of the value of IL-6 in different clinical situations, necessitated collaboration with clinical colleagues. I wished to study patients with both acute and chronic conditions. Mr Dugal Heath was in the process of recruiting patients with acute pancreatitis and we agreed that I should include these patients in this project. Likewise, I approached Dr Keith Aldroyd who was studying patients admitted with chest pain, and these patients were also included. I also approached Dr Rajan Madhok who collected samples and data on clinical disease activity from patients with rheumatoid arthritis.

The validation of the IL-6 bioassay described in this thesis was performed by myself between February 1988 and January 1989. The analysis of all patient samples for IL-6 and IL-1 was performed by myself with the assistance of Mrs Isobel Cameron. Collection of timed blood samples and clinical information from elective surgical patients was performed mainly by myself (Dr W D Fraser collected samples from 8 of the 39 patients studied).

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The illustrations presented in this thesis (excluding plate 1 and figure 1) were produced by myself using Apple MacIntosh CricketGraph software.

LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AMP	adenosine monophosphate
APACHE	acute physiology and chronic health evaluation
CI	confidence interval
CK	creatine kinase
CKMB	creatine kinase isoenzyme MB
CO_2	carbon dioxide
CRP	C-reactive protein
CSF	cerebrospinal fluid
CT	computerised tomography
CV	coefficient of variation
DNA	deoxyribonucleic acid
DVT	deep venous thrombosis
E coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ESR	erythrocyte sedimentation rate
HCl	hydrochloric acid
HGF	hybridoma growth factor
HIV	human immunodeficiency virus
IL-1	interleukin-1
IL-6	interleukin-6
IU	international enzyme unit
log	logarithm
LPS	lipopolysaccharide
LVEF	left ventricular ejection fraction
MI	myocardial infarction

MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NIBSC	National Institute of Biological Standards and Controls
NPV	negative predictive value
op	operation
paO_2	arterial partial pressure of oxygen
PBS	phosphate buffered saline
PMN	polymorphonuclear
PPV	positive predictive value
PTE	pulmonary thromboembolism
QC	quality control
r	Pearson product moment correlation coefficient
SDS	sodium dodecyl sulphate
TAP	trypsinogen activation peptides
TNFα	tumour necrosis factor alpha

INTRODUCTION

Interleukin-6 (IL-6) is one of the more recently described members of the interleukin family. The interleukins are cytokines (i.e. cellular messenger proteins) which are involved in the modulation of host defence mechanisms such as inflammation, the immune response and the metabolic response to injury. IL-6 has been found to have a wide spectrum of biological activities, and this diversity created considerable confusion over nomenclature (which was based mainly on functional properties) in the 1980s.

Historical Review of IL-6

Several groups involved in the identification of apparently unrelated cytokines eventually concluded that these cytokines were all one and the same protein molecule. As a result this protein factor was known by a number of different names based on identified characteristics.

Three main strands of investigation occurring in parallel were involved:

1. INTERFERON BETA-2 / 26 KILODALTON PROTEIN

IL-6 was discovered in 1980 as a product co-induced in human fibroblasts which had been stimulated for interferon-beta (IFNβ) expression (1). Weissenbach et al isolated two messenger RNA species, one coding for interferon-beta and the second coding for a protein with a molecular weight of 23 to 26 kilodaltons. This protein was named interferon beta-2 (IFNβ2) and was described independently by other investigators (2). However, Content et al named the protein "26 K factor" or "26 kD protein" because they concluded that it had no antiviral activity (3). The same workers also reported that its transcription was regulated differently from that of interferon beta (4), and that this protein was induced by a factor belonging to the interleukin-1 family (5), suggesting that it played a role as an intermediary or

effector molecule in inflammatory or immunoregulatory processes. Subsequent sequencing of the protein in 1986 confirmed the lack of structural homology with interferon beta (6). However, the physiological function of interferon beta-2/26 kilodalton protein remained obscure.

2. B CELL DIFFERENTIATION FACTOR / B CELL STIMULATING FACTOR-2

Meantime, Yoshizaki et al had isolated and purified a B cell differentiation factor (initially called BCDF) from a human B cell line (7). This factor was shown to induce the final maturation of B cells into immunoglobulin-secreting cells (8) and was renamed B cell stimulating factor-2 (BSF-2). It was cloned from a leukaemia virus transformed human T cell line and the primary sequence of B cell stimulating factor-2 was deduced from the complementary DNA (9). By 1987 it was apparent that B cell stimulating factor-2 was identical to interferon beta-2 (10).

3. INTERLEUKIN HYBRIDOMA PLASMACYTOMA-1 / PLASMACYTOMA GROWTH FACTOR / HYBRIDOMA / PLASMACYTOMA GROWTH FACTOR

The third line of research involved the study of growth factors for plasmacytomas and B cell hybridomas. In 1986, a hybridoma growth factor was purified from the supernatant of a murine helper T cell line (interleukin hybridoma plasmacytoma-1 (IL-HP1)) (11) and from murine macrophage supernatant (plasmacytoma growth factor (PCT-GF)) (12). This led Van Damme et al to purify a human hybridoma/plasmacytoma growth factor (HPGF) produced by an osteosarcoma cell line treated with interleukin-1 (13). Surprisingly, the amino terminal of this factor was unlike that of the murine factor but identical to that of the interferon beta-2 / B cell stimulating factor-2 molecule (14). This identity was confirmed by independent cloning and sequence analysis (15), and by demonstrating that

recombinant 26 kilodalton protein had hybridoma plasmacytoma growth factor activity (16).

The realisation that these apparently unrelated cytokines were one and the same molecule led to the proposal that it be designated 'interleukin-6' to avoid further confusion over nomenclature (16,17). Thus the molecule first isolated in 1980 was finally named interleukin-6 in 1987.

By 1988, three further biological actions had been attributed to IL-6:

1. In 1983 Ritchie and Fuller had described a monocyte-derived polypeptide involved in the regulation of hepatic acute phase protein synthesis, which they had called hepatocyte stimulating factor (HSF) (18). This factor seemed to be responsible for controlling the full hepatic acute phase protein response whereas interleukin-1 (IL-1) and tumour necrosis factor alpha (TNFα), cytokines previously thought to have been the principal inducers of this response, had been shown to produce only a restricted acute phase response in vitro (19). In 1987, Gauldie et al reported that hepatocyte stimulating factor was immunologically and functionally identical to IL-6 (20). In other words, IL-6 was a hormone which interacted with liver cells to induce the synthesis of a spectrum of plasma proteins characteristic of the hepatic acute phase reaction.

2. Other workers demonstrated that IL-6 was able to stimulate haematopoiesis (21,22).

3. IL-6 was shown to be active in the differentiation of cytotoxic T lymphocytes (cytotoxic T cell differentiation factor (CDF)) (23).

The nomenclature relating to IL-6 is summarised below:

IFNβ2
26 K
BCDF
BSF-2
IL-HP1
PCT -GF
HPGF
HSF
CDF

= INTERLEUKIN-6

IL-6 Gene and Protein Structure

The human IL-6 gene has been localised to chromosome 7 p 21 (24,25), and consists of five exons and four introns. Its organisation is strikingly similar to that of the granulocyte-colony stimulating factor gene (26) suggesting that these genes are evolutionarily derived from a common ancestor gene.

The complementary DNA for human IL-6 predicts that the protein consists of 184 amino acids with two potential N-glycation sites and four cysteine residues (9). It is formed by the cleavage of a 28 amino acid signal peptide from a 212 amino acid precursor protein with a molecular mass of 26 kilodaltons (thus the synonym 26 kilodalton protein) (9), and then N and O glycated before secretion (27). IL-6 thus exists in at least 5 different forms (molecular weight ranging from 21-28 kd) resulting from different glycations (28). These differences in post-translational modification may explain how such a multipotent cytokine can produce tissue specific effects, and may cause differences in its plasma half-life.

Regulation of IL-6 Production

IL-6 has been shown to be produced by an apparently endless variety of cells including monocytes (29), macrophages (30), lymphocytes (11), endothelial cells (31), fibroblasts (13), keratinocytes (32), mast cells (33), astrocytes (34), anterior pituitary cells (35) and numerous tumour cell lines (36-41). Usually these cells do not produce IL-6 constitutively but require appropriate stimulation, e.g. viral infection, lipopolysaccharide (LPS) or cytokine administration (table 1). Different cell lines require different stimulants for optimal IL-6 production. For example, monocytes/macrophages are preferentially stimulated by lipopolysaccharide whereas fibroblasts respond better to the cytokines IL-1 and TNFα, suggesting that these cytokines are key factors in IL-6 production. Lipopolysaccharide, TNFα and IL-1 are all potent inducers of IL-6 production by endothelial cells (table 1). It has been shown that glucocorticoids negatively regulate IL-6 gene expression in various cells (50). In spite of the large variety of cells known to secrete IL-6, it is probable that endothelial cells, fibroblasts and monocytes/macrophages are the principal producers of IL-6 during inflammation. These cells have a high capacity for IL-6 production and are distributed widely throughout the body.

The kinetics of IL-6 messenger RNA expression vary among the different cell types. Maximal messenger RNA expression occurs 3 hours after stimulation of monocytes (28) but over 24 hours after stimulation of T cells (51). These differences in kinetics probably reflect differing functions of the IL-6 molecule at different stages during the immune / inflammatory response. Both diacylglycerols, which activate the protein kinase pathway (52), and agents which increase intracellular cyclic AMP (53) enhance the accumulation of IL-6 messenger RNA. Thus it appears that at least two mechanisms exist for IL-6 gene expression.

In addition to much in vitro work, many in vivo studies have been performed to study the factors involved in IL-6 production. The first of these was the

Cells	Stimulants	Effect	
Monocytes/Macrophages	LPS	+++	(18, 28, 30)
	HIV	+++	(42)
	IL-1	++	(28)
	TNFα	-	(28)
Fibroblasts	IL-1	+++	(13, 16)
	Viruses	+++	(43, 44)
	E coli	+++	(44)
	TNF α	++	(13, 45, 46)
	LPS	+	(44)
Endothelial Cells	IL-1	+++	(47, 48)
	TNF α	+++	(47, 48)
	LPS	+++	(47, 48)
Keratinocytes	IL-1	+	(41)
Sinoviocytes	IL-1	+++	(49)
	TNFα	++	(49)

Key: $^{+++}$ = very strong stimulation of IL-6 production; $^{++}$ = strong stimulation; $^{+}$ = weak stimulation; - = no stimulation

Table 1: Regulation of IL-6 production in vitro.

demonstration by Coulie et al that injection of lipopolysaccharide into mice produced detectable IL-6 levels in plasma (30). High plasma IL-6 concentrations have been reported in rats subjected to psychological stress (54), and adrenalin administration to rats has been found to evoke high plasma concentrations of IL-6 (55). This effect was blocked by propanolol. Injection of recombinant IL-1 (56,57) and TNFα (57) into mice caused production of substantial amounts of IL-6 within 30 mins, while administration of endotoxin (E coli derived lipopolysaccharide) (57) and zymosan (58) resulted in peak circulating IL-6 levels occurring approximately 2 hours post-administration. The effects of endotoxin appear to be mediated at least in part by TNFα, since injection of anti-TNFα antibody caused a reduction in the IL-6 response to endotoxin (57). Further support for the premise that TNFα is crucial in the initiation or amplification of IL-6 release was provided by the finding that passive immunisation of baboons against TNFα prior to the administration of lethal doses of E coli resulted in an attenuated plasma IL-6 response (59).

Recent in vivo work in humans supports the hypothesis that IL-1 and TNFα are important stimulators of IL-6 production. These reports are discussed later in this chapter in the section 'Clinical Studies of IL-6' (page 39).

Matsuda et al reported that alpha-2 macroglobulin acts as a carrier protein for IL-6 in plasma (60). IL-6 bound to alpha-2 macroglobulin retains its biological activity and becomes resistant to proteases, whereas free IL-6 is easily degraded. By binding to alpha-2 macroglobulin, locally produced IL-6 is made available systemically.

Receptor and Signal Transduction

IL-6 receptor complementary DNA was found to encode a typical membrane protein consisting of 468 amino acids including a signal peptide of approximately 19 amino acids (61). The extracellular portion of the molecule contains

approximately 340 amino acids with a 90 residue domain resembling that of the immunoglobulin family. The transmembrane portion of the molecule consists of approximately 28 residues and is followed by a cytoplasmic segment containing approximately 82 amino acids. This last portion of the molecule appears to play no role in the transduction of the IL-6 signal (62), for which the mechanisms as yet remain unclear. However, it appears that cell-type specific responses to IL-6 are not due to cell-type specific forms of the receptor but to other unknown elements of the signal transduction cascade. Taga et al described how binding of IL-6 to its receptor triggered the association of the receptor with a non-ligand binding membrane glycoprotein (62). Fey et al have identified an 'IL-6-response-element' in the rat alpha-2 macroglobulin gene (a prototype rat liver acute phase gene) which serves as a binding site for nuclear factors, as yet uncharacterised, that facilitate hormone induced transcription (63).

IL-6 receptors have been found in hepatocytes, fibroblasts, epithelial cells, haematopoietic lines and cells of neural origin (64-66). Particularly high numbers have been found on human multiple myeloma cells (64). Two classes of binding sites have been described: high affinity sites with a dissociation constant of approximately 10 picomolar; and low affinity sites with a dissociation constant of approximately 1 nanomolar (61).

Biological Functions of IL-6

It will be apparent that IL-6 is a multipotent cytokine, and most of its functions will be considered here only briefly. Its role in the acute phase response is of particular relevance to this thesis and will be considered in more detail at the end of this section.

IMMUNE SYSTEM

B Cell

As mentioned previously, IL-6 used to be called B cell stimulatory factor 2 because it was found to induce the final maturation of B cells into antibody producing cells (8) and so stimulate immunoglobulin production in vitro (9,67). IL-6 also stimulated in vivo antibody production in mice (68). These and other studies demonstrate that IL-6 is an essential factor for B cell differentiation and antibody production, and may act synergistically with IL-1 (69). IL-6 is thought not to be involved in the proliferation of activated B cells (67). The effect of IL-6 on tumours of B cell origin is discussed in the sub-section 'Hybridoma/Plasmacytoma Cells' (page 31).

T Cell

Resting T cells express IL-6 receptors in contrast to resting B cells which do not (only activated B cells express IL-6 receptors) (64). This supports the premise that while IL-6 acts only on the final maturation stage of activated B cells, it is effective on resting T cells. IL-6 was found to promote the growth of phytohaemagglutinin stimulated thymocytes and peripheral T cells (70-72), an effect only partly inhibited by anti-interleukin-2 antibody which suggests that IL-6 may regulate T cell function independent of interleukin-2 to some degree (73). Moreover, the thymocyte co-stimulatory activity of macrophage derived conditioned medium was largely abrogated by anti-IL-6 antibody (74) (previously, IL-1 was thought to exert this activity). IL-6 stimulates interleukin-2 production by murine T cells (75) and enhances the interleukin-2 responsiveness of mitogen stimulated T cells and thymocytes (76). Since interleukin-2 is pivotal in T cell activation and proliferation (77), these results strongly support the premise that IL-6 is an essential accessory signal which promotes T cell activation and proliferation. It may act in synergy with IL-1 (76) and possibly TNFα (78). IL-6 has been found to

induce not only T cell proliferation but also differentiation of cytotoxic T cells from human thymocytes and splenic T cells in the presence of interleukin-2 (23,79).

MESANGIAL CELLS

Recent in vitro work suggests that IL-6 is an autocrine growth factor for mesangial cells. These cells express IL-6 messenger RNA and secrete IL-6 which has growth factor activity (80).

HAEMATOPOIESIS

The positive effect of IL-6 on haematopoiesis was first described by Ikebuchi et al who found that interleukin-3 and IL-6 acted synergistically to support the formation of multilineage blast cell colonies in murine spleen cell cultures (21). IL-6 appears to act on multipotent progenitors but not on more mature progenitors (81). The haematopoietic activity of IL-6 may prove valuable in the field of bone marrow transplantation. Prior incubation of murine bone marrow cells with IL-6 dramatically increased the survival rate when the marrow cells were transplanted to lethally irradiated mice (82). Moreover, perfusion of IL-6 in 5-fluorouracil treated mice was found to accelerate the recovery in the numbers of haematopoietic stem cells (83). Other in vivo studies in mice (84) and monkeys (85) have shown that injection of human recombinant IL-6 results in dose dependent increases in platelet count. This effect on thrombopoeisis is partly explained by the finding that the N terminal sequence of thrombopoetin is identical to that of human IL-6 (86).

HYBRIDOMA/PLASMACYTOMA CELLS

The growth stimulation of these cells by IL-6 has been discussed on page 23 in the section 'Historical Review of IL-6'. IL-6 has been implicated in the pathogenesis of multiple myeloma and plasma cell dyscrasias. Multiple myeloma cells have been shown both to produce and contain IL-6 (87,88), and to express the IL-6

receptor (64,87) - findings which support the existence of an autocrine positive feedback loop. IL-6 appears to act as a growth factor for myeloma cells (89,90), and in vitro myeloma cell responsiveness to IL-6 is directly related to disease severity (89). Moreover, Brandt et al demonstrated that transplantation of the coding sequences of the IL-6 gene into the haematopoietic cells of mice produced a syndrome (Castleman's Disease) characterised by anaemia, hypergammaglobulinaemia, splenomegaly and lymphadenopathy associated with extensive plasma cell infiltration of spleen, lung, liver and lymph nodes (91).

NEUROENDOCRINE

Stimulation of astrocytoma cells results in expression of IL-6 messenger RNA (34), suggesting that IL-6 may affect nerve cell growth or function. Indeed IL-6 has been shown to induce the differentiation of chromaffin cells into neural cells and to maintain the viability of these cells (92). In vitro work has shown that hypothalamic (93) and pituitary cells (94) can produce IL-6, and in vivo work has demonstrated that IL-6 may be involved in the regulation of the hypothalamic-pituitary-adrenal axis. Intravenous administration of IL-6 to rats results in dose-dependent increases in plasma adrenocorticotrophic hormone (ACTH) levels 30 minutes post-injection (95), an effect thought to be mediated via IL-6 stimulation of hypothalamic corticotrophin releasing factor since ACTH secretion was blocked by neutralising antibodies to corticotrophin releasing factor. Thus it appears that IL-6 may induce ACTH secretion during the acute phase response. IL-6 has also been shown to act synergistically with ACTH to stimulate the release of corticosterone from adrenal gland cells in vitro (96). Glucocorticoid has a synergistic effect on IL-6 induced acute phase protein synthesis (97) and also inhibits IL-6 production in various cells (50). Consequently, IL-6 induced secretion of ACTH may have a positive feedback effect on acute phase protein synthesis but a negative feedback effect on other aspects of the inflammatory/immune response. Although the above findings suggest that IL-6

acts on the hypothalamus, other in vitro work using anterior pituitary cells raises the possibility that IL-6 is involved in the paracrine modulation of pituitary hormone secretion. Anterior pituitary cells can themselves produce IL-6 while IL-6 has been shown to stimulate pituitary production of growth hormone (98), prolactin (98,99), luteinising hormone (98,99) and follicular stimulating hormone (99).

ACUTE PHASE RESPONSE

The acute phase response is the name given to the collection of systemic changes which occur in response to injury. This systemic inflammatory reaction comprises fever, leukocytosis, net protein catabolism, alterations in plasma steroid and metal concentrations and changes in the circulating concentrations of various proteins (the acute phase proteins), brought about by alterations in hepatic protein synthesis (100).

In the past IL-1 and TNFα have been implicated in causing these systemic inflammatory effects directly (101,102). However, the principal inflammatory actions of these cytokines such as increased vascular permeability (103), chemotaxis (104), leukocyte vascular adherence (105,106), coagulation (107) and bacterial killing (108) occur at a local level. Moreover, IL-6 is active in the thymocyte assay which was previously in widespread use to measure IL-1 (109). Consequently, the involvement of IL-1 and TNFα in most of the systemic changes which comprise the acute phase response may well occur either via, or in conjunction with, other cytokine mediators such as IL-6, the production of which may be stimulated locally by IL-1 and TNFα. The recent report that anti-IL-6 antibodies protected mice against lethal E coli infection suggests that IL-6 is pivotal in inducing the pathophysiological changes which occur in septic shock (110).

The evidence concerning the effect of IL-6 on plasma steroid concentrations and on haematopoesis has already been discussed. To date, there is no evidence that IL-6

is involved in muscle proteolysis. Both recombinant IL-1ß and TNFα have been shown not to induce proteolysis or prostaglandin E_2 production when incubated with isolated rat muscles (111) . Of particular interest to this work are the reported actions of IL-6 with regard to acute phase protein synthesis, fever and plasma metal concentrations. These actions are discussed below in more detail.

Acute Phase Proteins

The principal acute phase proteins in man are shown in table 2. After trauma, plasma levels of these proteins increase by a variable degree. Although our understanding of the biological significance of these changes is limited, it is generally assumed that these proteins perform a protective role by limiting tissue damage and promoting repair and healing (100). For example, C-reactive protein (CRP) is an effective precipitor and agglutinin of soluble and particulate ligands, and may be involved in activation of the classical complement pathway (115).

As described earlier, in vitro work showed that IL-6 was identical to hepatocyte stimulating factor , the principal inducer of acute phase protein synthesis (20). At the inception of this project, this was the only evidence to suggest that IL-6 was the major stimulator of hepatic acute phase protein synthesis. The importance of IL-6 as an inducer of the acute phase protein response has since been confirmed by the reports that it induced rat acute phase proteins in vivo in a dose-dependent manner(116), and that it stimulated the full spectrum of acute phase proteins in adult human hepatocytes (table 2). In contrast IL-1 and TNFα, previously thought to be important inducers of acute phase protein synthesis, failed to induce either serum amyloid A or CRP synthesis (112). IL-6 exerts its control on acute phase protein synthesis at least in part at the transcriptional level (117), and appears to function as an exocrine hormone rather than by an autocrine mechanism (118).

The theory that hepatocyte stimulation is one of the most important physiological roles of IL-6 is supported by the finding that about 30% of labelled recombinant

Protein	Magnitude of Increase in Serum Concentration	Stimulatory Effect of IL-6 on In Vitro Production
C-reactive protein	>1000%	+++ (112)
Serum amyloid A	>1000%	+++ (112)
α-1 Proteinase inhibitor (α-1 Antitrypsin)	100-1000%	+ (112)
α–1 Acid glycoprotein	100-1000%	+ (112)
α–1 Anti-chymotrypsin	100-1000%	++ (112)
Fibrinogen	100-1000%	++ (112)
Haptoglobin	100-1000%	++ (112)
C-1-Inactivator	<100%	+ (113)
Caeruloplasmin	<100%	++* (114)

*produced by human hepatoma cells (Hep 3B)

Table 2: The principal acute phase proteins in man, and the regulation by IL-6 of their production by adult human hepatocytes.

human IL-6 injected intravenously into rats was taken up by the liver by 20 minutes post-injection (119), although after 1 hour it disappeared from the liver and accumulated in the skin (120). (This observation indicates that the skin may be involved in IL-6 catabolism).

Fever

Fever is defined as a controlled elevation of body temperature which results from a shift in the hypothalamic set point (121). It is thought that tissue damage/infection stimulates cells such as macrophages to produce a substance(s), the so-called "endogenous pyrogen" (EP), which induces prostaglandin E_2 synthesis in the anterior hypothalamus resulting in a raised hypothalamic set point (121).

"Endogenous pyrogen" was the name given to a partially purified product derived from activated leukocytes which elicited fever when injected into animals, and subsequently IL-1 was thought to be equivalent to endogenous pyrogen (121). Certainly, numerous studies have since demonstrated that human recombinant IL-1α and IL-1β elicit fever when administered either intravenously or via the cerebroventricles to rats and rabbits (122-127). However, it has since become apparent that other cytokines are capable of eliciting fever and so may qualify as 'endogenous pyrogen'. TNFα produces increases in body temperature when administered to animals (127-129), and TNFα (130), interleukin-2 (131-133) and interferons alpha (131) and beta (132,133) have caused fever when administered to humans in clinical trials.

There is now mounting evidence that IL-6 is involved in the production of fever. IL-6 was first implicated in causing fever by Nijsten et al who found a significant correlation between body temperature and IL-6 levels in 13 patients with severe burns (134). This report was instrumental in the decision to study the relationship between IL-6 and fever as part of this project. These and other workers have since shown that recombinant human IL-6 induced fever after administration to animals

suggesting that IL-6 might be involved in the pyrogenic effect of IL-1 (70,135,136). Le May et al demonstrated that intracerebroventricular injection of recombinant human IL-6 in rats produced a significant increase in core temperature, and that the plasma IL-6 concentration following injection of lipopolysaccharide was closely associated with the degree of fever (137). They concluded that IL-6 was a mediator of lipopolysaccharide induced fever. Further indirect support for this hypothesis was provided by the same group when they showed that lipopolysaccharide injected intravenously into dogs produced dose-dependent increases in both plasma IL-6 and rectal temperature, the peak temperature occurring after the peak IL-6 concentration (138).

In conclusion, evidence exists for the involvement of IL-6 in the production of fever, but it is still unclear whether it, or any of these other cytokines, is the major 'endogenous pyrogen' of the acute phase response.

Metals

The hypercupraemia, hypozincaemia and hypoferraemia which occur during the acute phase response are well documented (139,140).

Hypercupraemia occurs due to increased hepatic synthesis of caeruloplasmin - a copper containing protein which is the principal circulating form of copper (141). Since IL-6 has been shown to stimulate the production of caeruloplasmin by a human liver tumour cell line (114), it is likely that IL-6 is a mediator of the hypercupraemia which occurs during the acute phase response.

Iron is transported in plasma bound to the carrier protein transferrin and in body secretions bound to lactoferrin (142). Non-functional iron is stored intracellularly in a soluble form (ferrihydroxyphosphate crystals) with ferritin - a large apoprotein (molecular weight 400,000 kd) which may contain up to 4500 iron atoms (143). Iron is essential for microbial growth, and hypoferraemia may contribute to host defence mechanisms by limiting the supply of available iron to invading microbes

(144-146). Plasma iron levels reach their nadir approximately 12 hours post trauma (140) but the mechanism by which this occurs is still poorly understood.

Two main hypotheses have been proposed:

i. ferritin is an acute phase protein, the hepatic synthesis and plasma concentration of which increase during inflammation. Ferritin is thought to strip iron from transferrin in plasma and divert it to the liver (147-152).

ii. during inflammation, neutrophils release lactoferrin which strips iron from transferrin and is then selectively taken up by the cells of the reticulo-endothelial system (153).

If the first hypothesis is correct, then IL-6 may well be involved in the production of hypoferraemia as a major inducer of acute phase protein synthesis, although no direct evidence exists that IL-6 stimulates ferritin synthesis. Several workers have reported hypoferraemia in animals following injection of IL-1 and TNFα (127, 154–156). One explanation for these findings is that the effects of IL-1 and TNFα are mediated via IL-6. However, at present the role of IL-6 in controlling plasma iron concentration remains open to speculation.

Like iron, zinc is essential for microbial growth (157), and so hypozincaemia may confer protection on the host. Also like iron, trough levels of zinc occur approximately 12 hours post trauma (140). About 90% of plasma zinc is loosely bound to albumin. The remainder is tightly bound to alpha-2 macroglobulin and is metabolically unavailable (158). Metallothionein is a low molecular weight cytosolic protein which selectively binds heavy metals such as zinc and copper, and the hepatic synthesis of which increases during the acute phase response (159). During this response, metallothionein can extract zinc from plasma leading to hypozincaemia and increased hepatic zinc stores (158). Consequently, it seemed possible that if IL-6 were a major inducer of hepatic protein synthesis, it might well be involved in the production of hypozincaemia. It has been proposed that zinc

and/or metallothionein may act as intracellular antioxidants protecting hepatocytes and other cells when host-generated cytotoxic oxygen species are produced in large amounts during infection (160-162). After this project began, strong in vitro evidence emerged that IL-6 stimulates metallothionein synthesis in the liver. In 1990, Schroeder and Cousins reported that IL-6 in the presence of glucocorticoids regulated metallothionein gene expression by rat hepatocytes in a dose-dependent manner (163). Consequently, it appears probable that IL-6 is involved in causing hypozincaemia during the acute phase response.

SUMMARY

The biological actions of IL-6 described above and summarised in figure 1 suggest that IL-6 has a role in orchestrating host defence mechanisms. Injury or infection causes production of IL-6 which may stimulate haematopoeisis, the immune system, ACTH release, acute phase protein synthesis and other manifestations of the systemic inflammatory response.

It is likely that both IL-1 and TNFα act locally to stimulate IL-6 production during the acute phase response. Consequently, many of the activities previously ascribed to IL-1 and TNFα directly are now thought to be due to IL-1 or TNFα-mediated IL-6 release. Moreover, IL-6 appears to act synergistically with IL-1, TNFα and other cytokines. Thus it is apparent that IL-6 does not act in isolation but forms an important part of the cytokine network which controls the co-ordinated response of the body to injury.

Clinical Studies of IL-6

As it became apparent through in vitro and in vivo work that IL-6 was an important inflammatory mediator, attempts were made to measure it in body fluids in different clinical situations. While IL-1 and TNFα have also been implicated as important inflammatory mediators, increased circulating concentrations have not been detected consistently in infected patients. Increased levels of TNFα have

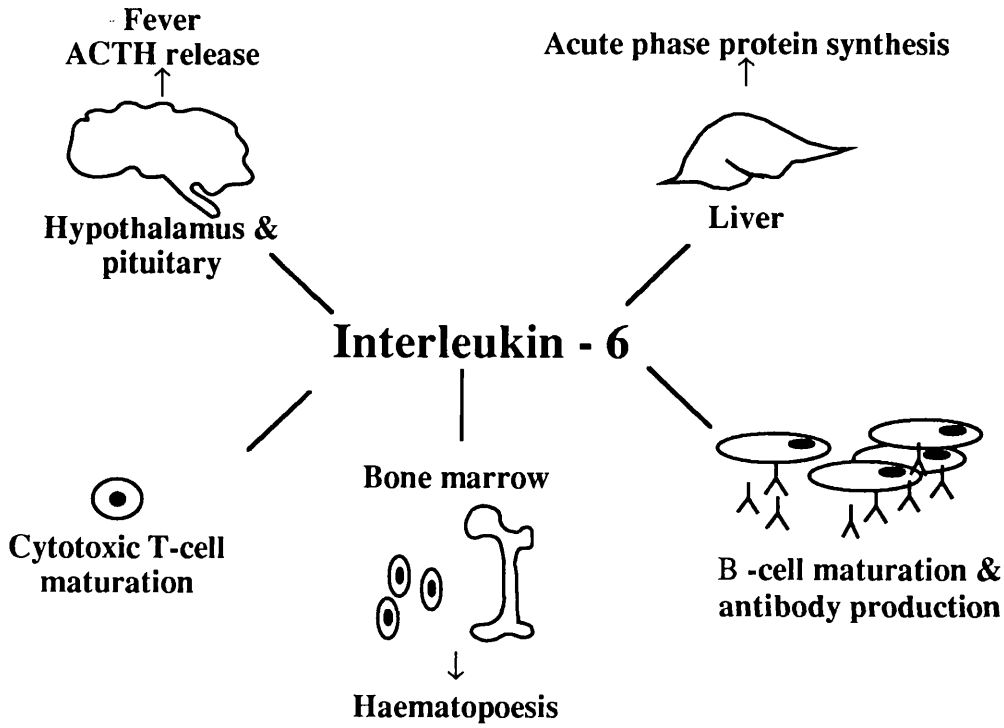


Figure 1 : The principal biological actions of IL-6

been reported in some patients with sepsis, although many more septic patients have been found to have undetectable levels (164-168). In general, however, high TNFα concentrations occurred in critically ill patients and were associated with poor outcome. Few of these patients had detectable plasma IL-1ß concentrations (165,167,168). Workers have failed to detect consistent increases in plasma TNFα (169,170) and IL-1ß (171) following surgery, although a recent report of 6 surgical patients described a small transient rise in plasma IL-1ß post-operation (170). Administration of endotoxin to human volunteers was found to increase circulating levels of TNFα but not IL-1ß (172,173).

The earliest clinical studies involving IL-6 included those of Nijsten et al who found elevated levels of IL-6 in the sera of patients with severe burns (134). The same group reported high levels in the sera and urine of patients with renal transplants during acute rejection episodes (174). Indeed, other workers have since suggested that sequential determinations of serum IL-6 may be useful in discriminating acute rejection from nephrotoxicity in transplant recipients (175).

Another early study by Houssiau et al reported high levels of IL-6 in the CSF of patients with acute central nervous system infections (176), an observation confirmed independently (177). Elevated levels of Il-6 have also been found in the CSF of patients with systemic lupus erythematosus with central nervous system involvement (178), but not in the CSF of patients with neurosyphilis or multiple sclerosis (176). Other workers reported that meningicoccal infection of the meninges caused an inflammatory response during the initial phase of which TNFα, IL-1 and IL-6 were sequentially produced (179).

Raised concentrations of IL-6 have been found in the sera of patients suffering from septic shock (168,180-182), and circulating concentrations of IL-6 have been shown to be significantly associated with plasma TNFα levels (182) and plasma lactate concentrations (180,181). It is likely that IL-6 has a role in the

pathophysiology of shock but whether it is directly involved in causing lethal complications or whether it mediates these effects indirectly remains unclear. To date, there are no reports of the effects of administration of anti-IL-6 antibody to patients with septic shock. Several studies have shown that injection of endotoxin into humans causes peak plasma TNF levels 60 to 90 minutes post-injection followed by peak IL-6 levels 2 to 3 hours post-injection (173,183,184). Other studies have demonstrated that patients receiving infusions of recombinant human TNFα develop increased levels of IL-6 in plasma peaking 3 to 6 hours post-infusion (185,186), providing further evidence that TNFα is an important factor in inducing systemic IL-6 release.

More recently, Baigrie et al confirmed previous reports (169,187) when they reported increases in serum IL-6 concentrations following major surgery (170). The rise in IL-6 was preceded by a small rise in serum IL-1 concentration supporting the in vitro observation that IL-1 induces IL-6 synthesis.

Increased plasma levels of IL-6 have been reported in a variety of other clinical conditions including HIV infection (188), plasma cell dyscrasias (189,190), alcoholic liver disease (191), contact hypersensitivities (192) and Crohn's disease (193).

Although there are now numerous reports of raised serum concentrations of IL-6 in patients with a wide variety of clinical conditions, most of these have been published in the last 3 years. They have been included in this introduction for the sake of completeness. At the time this project was conceived, very few clinical studies had been published (132,174,176) and so the nature of the pathophysiological role of IL-6 in the human acute phase reponse and the clinical value of IL-6 as an inflammatory marker were open to question.

AIMS

Virtually all the workers cited in this thesis have used bioassays to measure IL-6 and, indeed, until very recently, biological assay was the only available methodology. Commercial immunoassay tests are now available but these are prohibitively expensive for large-scale studies.

IL-6 and the Acute Phase Response

By validating a bioassay, I hoped to measure IL-6 in the serum of elective surgical patients to assess its value as a marker of tissue damage and to determine its role in the metabolic response to injury. CRP has been measured extensively as a marker of tissue damage and inflammation although levels have not been found to be associated with the extent of tissue trauma (194). If IL-6 were an important inflammatory mediator, then surgical trauma should produce a rise in serum IL-6, the magnitude of which might be associated with the degree of trauma as well as with any subsequent change in serum CRP concentration, body temperature or plasma metal concentrations. Morever, I wished to study the effect of surgery on circulating concentrations of IL-1β and TNFα.

Clinical Value of IL-6

Assuming that IL-6 proved promising as a marker of tissue damage, it was possible that its measurement might be useful in certain clinical situations.

SURGICAL COMPLICATIONS

It would be highly advantageous to be able to predict which surgical patients were liable to develop post-operative complications. It seemed likely that if serum IL-6 were a major mediator of the systemic inflammatory response, then the occurrence of clinical complications in the post-operative period might be associated with elevated circulating IL-6 concentrations.

SUSPECTED MYOCARDIAL INFARCTION

Myocardial infarction remains the greatest single cause of premature death in the United Kingdom, accounting for 35% of deaths in men, and 30% amongst women. The use of the available thrombolytic compounds, streptokinase (195), tissue plasminogen activator (196) and anistreplase (197) has been shown to reduce the morbidity and mortality by up to 50% in patients treated early after clinical presentation. The object of the use of thrombolytic therapy is to achieve coronary artery patency to improve left ventricular function and reduce mortality. About one half of all deaths after myocardial infarction are within two hours of onset, and it is in the early phase of presentation that the biggest impact can be achieved. Early introduction of therapy leads to more rapid reperfusion in a higher percentage of patients (198). Consequently, early diagnosis is of paramount importance and this has promoted much interest in serological markers of myocardial infarction. The tests most commonly used to diagnose myocardial infarction in the acute situation are total creatine kinase (CK) and creatine kinase isoenzyme MB (CKMB), but false negative results are common (199). Moreover, enzyme levels have been shown not to have independent predictive value of mortality after myocardial infarction (200). Patients presenting with chest pain might be expected to have high IL-6 levels if they had sustained significant myocardial injury, in which case IL-6 might prove useful in discriminating myocardial infarction from unstable angina in the acute situation, or as a prognostic indicator. To my knowledge, there are no reports in the literature concerning serum IL-6 levels during episodes of acute chest pain. Certainly, workers have reported increased CRP levels in myocardial infarction, but peak levels occurred as late as 50 hours after the event (201), and an earlier diagnostic aid is required if it is to influence management during the critical period following a suspected infarct. The aim of this study was to compare the value of IL-6, CK and CRP in the early diagnosis of myocardial infarction, and also their value as prognostic indicators by studying the association

between these variables and left ventricular ejection fraction which has been shown to be an important predictor of survival in patients with coronary heart disease (202).

ACUTE PANCREATITIS

Another acute clinical situation where a non-specific indicator of disease severity could prove valuable is acute pancreatitis. Acute pancreatitis may be classified on clinical or morphological grounds, and usually there is good correlation between clinical severity and morphological severity. Clinically, mild acute pancreatitis has been defined as an acute condition, typically presenting with abdominal pain and usually associated with raised pancreatic enzymes in blood, in which there is no multisystem failure and an uncomplicated recovery. Severe pancreatitis is considered to be present if local complications develop or if one or more body systems become deranged. Morphologically, an attack is severe when parenchymal necrosis and haemorrhage is present. If only oedema is present, then the attack is considered to be mild morphologically (203). It is notoriously difficult to predict the severity of an attack at presentation. Patients with life-threatening disease may appear mild clinically and so not receive appropriate treatment or monitoring. McMahon (204) and Corfield (205) reported that the percentages of severe attacks correctly predicted at presentation using clinical assessment alone were a disappointing 39% and 34% respectively. Consequently, there is a requirement for a reliable test to predict severe attacks of acute pancreatitis as early as possible. CRP has been shown to be an indicator of disease severity (206) but its measurement has not been adopted widely because it is a poor discriminant during the first 24 hours following the onset of symptoms. Several multifactorial screening systems exist for severity assessment in acute pancreatitis (207-211), but their complexity limits their use routinely. One such system is the modified Glasgow system which uses 8 prognostic factors to assess disease severity (210). The aim of this part of the project was to determine if IL-6 might prove more useful

as an early severity indicator than CRP or the modified Glasgow multifactorial scoring system.

RHEUMATOID ARTHRITIS

The aim of the final part of the project was to compare IL-6 with more conventional markers of disease activity in a chronic inflammatory condition such as rheumatoid arthritis.

The characteristic feature of rheumatoid arthritis is an inflammatory synovitis which is associated with cartilage destruction and bone erosions with subsequent joint destruction. T, and, to a lesser extent, B lymphocytes proliferate in synovial fluid. Hypertrophy and hyperplasia of synovial lining cells produce an invasive pannus which spreads to cover articular cartilage and precedes cartilage and bone destruction. There is now abundant evidence that cytokines play an important role in this inflammatory process. Firestein et al estimated the concentrations of various cytokines in synovial membrane affected by rheumatoid arthritis according to the percentage of cells positive for cytokine messenger RNA. IL-6 was found in the highest concentrations followed by IL-1ß and TNFα (212). In contrast, cytokines originating from lymphocytes (interleukin-2, interleukin-3 and interferon gamma) were present only in low concentrations (212) or not at all (213), in spite of the cellular predominance of lymphocytes. Both IL-1 (214) and TNFα (215-217) have been detected in synovial fluid and are reported to be produced by synovial tissue (215,218).

In 1988, Houssiau et al reported that IL-6 was present in the synovial fluid and serum of patients with rheumatoid arthritis (219). This report prompted us to investigate the potential of serum IL-6 concentration as an inflammatory marker in rheumatoid arthritis.

Synoviocytes have since been shown to produce IL-6, both spontaneously and when stimulated by IL-1 and TNFα (49,220) , and there have been further reports

of IL-6 in the synovial fluid of patients suffering from rheumatoid arthritis and other inflammatory arthropathies (49,221-223). Miltenburg et al found a correlation between synovial IL-6 levels and clinical parameters of local inflammation (222). Moreover, patients with rheumatoid arthritis have recently been reported as having elevated serum IL-6 concentrations (223,224).

Quantitative assessment of disease activity in RA has been an elusive goal. There remains a need for a simple, objective test which quantitatively assesses disease activity and which responds quickly to changes in that activity, even before clinical changes are apparent. Two of the most widely used tests are ESR (225,226) and CRP (226,227). The main difficulty in evaluating any of these tests is the lack of a 'gold standard' of disease activity against which to compare the test. In the absence of such a standard we chose to use the Ritchie Articular Index (228) which is recognised as being a good, objective, quantitative clinical assessment of the degree of synovitis as a reference against which to compare the value of IL-6, CRP and ESR as markers of disease activity in rheumatoid arthritis.

Summary

The aims of this work were as follows:

1. To validate a bioassay for the measurement of IL-6 in serum.

2. To characterise the serum IL-6, IL-1β and TNFα responses in patients undergoing elective surgery of varying degrees of severity. By using elective surgical patients as models of controlled tissue damage, I hoped to answer the following questions:

   (a) Does IL-6 have potential as a marker of tissue damage compared to CRP?

   (b) Do IL-6, IL-1β or TNFα play a role in the following three aspects of the acute phase response:

(i) serum CRP response?

(ii) increased body temperature?

(iii) hypozincaemia and hypoferraemia?

3. To assess the value of serum IL-6 measurement in four clinical situations by answering these questions:

(a) Can it be used as a predictor of complications post-operation?

(b) Is it a useful diagnostic or prognostic aid in patients with suspected myocardial infarction?

(c) Has it any value as a severity indicator in acute pancreatitis?

(d) Has it any value as a marker of disease activity in rheumatoid arthritis?

VALIDATION OF A BIOASSAY FOR THE MEASUREMENT OF IL-6 IN SERUM

Bioassay Principle

Cells which are dependent on the presence of IL-6 for growth are incubated in appropriate conditions with serial dilutions of standard material or patient serum/plasma for a fixed time. After this incubation period the number of living cells present is evaluated colorimetrically, that number being dependent on the IL-6 activity present in the standard or patient sample.

Equipment and Materials Used

Incubator (VSL, Scotlab)

Laminar flow work unit (Gelaire, Flow Laboratories)

Adjustable micropipettes - sterilised (Gilson)

Disposable tips - sterilised (Gilson)

7TD1 cell line (Dr J Van Snick, University of Leuven, Leuven, Belgium)

Dulbeccos Modified Eagles Medium (Northumbria Biologicals Ltd)

Medium additives - Foetal calf serum (Northumbria Biologicals Ltd)
L-asparagine (Gibco Biocult)
L-glutamine (Gibco Biocult)
L-arginine (Sigma)
2-mercaptoethanol (Sigma)
Hypoxanthine/thymidine (Gibco Biocult)
Amphotericin (Gibco Biocult)
Penicillin/streptomycin (Gibco Biocult)

Hybridoma growth factor (HGF) (Janssen)

Microscope - Bactil 60 (Watson Barnet)

Counting chamber (Hawksley)

Trypan blue (Northumbria Biologicals Ltd)

Phosphate buffered saline (PBS) without calcium and magnesium - pH 7.2 ± 0.1 - sterilised (Gibco Biocult)

96 flat-bottomed well microtitre plates (Cel-cult, Sterilin)

Automatic dispenser (BCL)

Multi-tip pipette (12-pette, Costar)

Recombinant human interleukin-6 (Dr L Aarden, University of Amsterdam, Amsterdam, The Netherlands)

P-nitrophenol-N-acetyl-β-D-glucosaminide (Sigma)

Triton-X (Scintran grade, BDH Chemicals Ltd)

MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma)

Isopropanol (AnalaR grade, BDH Chemicals Ltd)

Sodium dodecyl sulphate (AnalaR grade, BDH Chemicals Ltd)

Hydrochloric acid (AnalaR grade, BDH Chemicals Ltd)

Microtitre plate reader (Titertek Multiskan)

Cells

CULTURE

The cells used in the assay are the IL-6 dependent mouse-mouse B cell hybridoma 7TD1 line (30) - a generous gift from Dr Van Snick, University of Leuven, Leuven, Belgium. The cells are grown in an atmosphere of 8% CO_2 and at a temperature of 37°C using Dulbeccos Modified Eagles Medium (DMEM) as growth medium in plastic cell culture flasks. The medium is supplemented with:

10% foetal calf serum

1.5 mM glutamine

0.24 mM L-asparagine

0.55 mM L-arginine

50 μM 2-mercaptoethanol

0.1 mM hypoxanthine

16 μM thymidine

Amphotericin 2.5 μg/ml

Penicillin/streptomycin 1% volume/volume

Cells to be used in the assay are kept below a concentration of 50 x 10^4 cells/ml on the recommendation of Dr Van Snick since above this concentration cell responsiveness to IL-6 is reduced.

The 7TD1 cells are fed with hybridoma growth factor (HGF) at an approximate concentration of 400 units/ml. 50 μl added per 5 ml of cell suspension has been found to produce good cell growth. The growth characterisitics of the 7TD1 cells following addition of 50 μl of HGF (400 units/ml) to 5 ml of cell suspension containing approximately 4 x 10^4 cell per ml are shown in figure 2. Cell viability is determined by staining with trypan blue - failure to incorporate the dye implies that a cell is viable (229). Total and viable cell numbers are assessed by microscopy using a counting chamber. From figure 2 it can be seen that the cell doubling time is 15 to 20 hours. The percentage viability of 7TD1 cells is extremely good, being greater than 95% up to 71 hours, then falling to virtually zero by 138 hours.

USE IN ASSAY

When the cells are to be used in the assay, they are counted and the required number of cells are washed three times by centrifugation and resuspension in phosphate buffered saline (PBS) to remove any medium containing HGF (IL-6). The cells are then resuspended in medium (which contains no IL-6) at a concentration of 4 x 10^4 cells/ml. 96 well microtitre plates are prepared containing 50 μl volumes per well of serial dilutions of standard/serum. To each well a 50 μl volume of cells is added (approximately 2 x 10^3 cells) which produces satisfactory numbers of cells post-incubation. Fewer than 2 x 10^3 cells per well result in low numbers of cells post-incubation and larger numbers (eg. 2 x 10^4 per well) give very high cell concentrations post-incubation with loss of sensitivity i.e. a flat dose

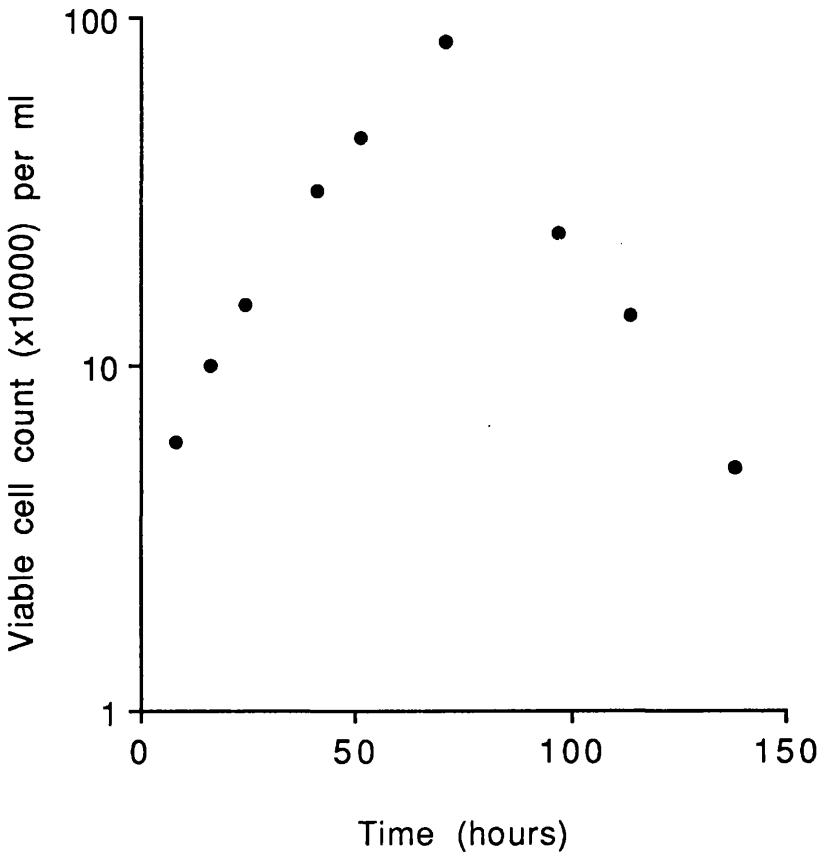


Figure 2 : 7 TD1 cell growth curve.

response curve. The plates are then incubated for 4 days at 37°C in 8% CO_2 prior to evaluation of cell numbers.

Setting Up Plates of Standard Dilutions

Serial two-fold dilutions of standard material in assay medium are performed using 96 well microtitre plates (8 rows x 12 columns). 100 µl of top standard is added to each of the 12 wells in the top row. 50 µl medium is added to all the other rows. 50 µl two-fold dilutions are then performed (using a multi-tip pipette) down the eight rows starting at the top row. This produces a final volume of 50 µl in each well to which 50 µl of cells can be added. Thus eight different standard concentrations are generated and can be used to construct a dose response curve.

Standard Material

Recombinant human IL-6 was a generous gift from Dr L Aarden in Amsterdam (29). Dr Aarden has assigned this material a potency in units/ml - the unitage used in this work. He defines one unit as that amount of recombinant IL-6 which produces half maximal growth (measured by tritiated thymidine incorporation) in the B9 hybridoma cell line. The specific activity of this recombinant material is quoted as 10^6 units/µg so that 1 unit approximates to 1 pg of recombinant IL-6.
I have recently assessed the accuracy of this material's designated specific activity by comparing its potency with that of the National Institute of Biological Standard and Controls' (NIBSC) recombinant IL-6 of known mass concentration. 1 unit of Dr Aarden's standard has the same potency in the 7TD1 bioassay as 1.4 pg of the NIBSC material. Thus the approximate specific activity designated, ie. 1 unit = 1 pg, appears to be accurate.

Evaluation of Cell Numbers

My aim was to develop a colorimetric detection method. Previously, tritiated thymidine incorporation has been used extensively to evaluate cell numbers.

However, this method is cumbersome, imprecise and has the obvious disadvantage of necessitating handling and storage of radioisotopes. I hoped that colorimetry might provide a fast and reliable means of measuring cell growth.

Two types of colorimetric assay were studied. Both assays used chromogenic substrates to measure the activity of specific cellular enzymes. The absorbance of each well was measured using a Titertek Multiskan microtitre plate reader.

HEXOSAMINIDASE ACTIVITY

The first detection method involved determination of hexosaminidase activity (230). N-acetyl-β-D-hexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) is a ubiquitous lysosomal enzyme involved in the degradation of glycosylated cellular constituents. Its activity can be measured using the chromogenic substrate p-nitrophenol-N-acetyl-β-D-glucosaminide. The method is described below.

The above-named substrate was dissolved at a concentration of 7.5 mM in 0.1 mM citrate buffer, pH = 5. This solution was mixed with an equal volume of 0.5% Triton X in water and then stored at -20°C. Following a 4-day incubation period, cells were washed 3 times with PBS by centrifuging the plates at 250 x g for 5 minutes for each wash to remove spent medium. If spent medium was not removed, the indicator phenol red present in the medium was turned yellow by the acid pH of the substrate causing interference with absorbance readings. The washing step also removed serum, which might have contained high levels of hexosaminidase. After washing, 60 µl of substrate solution was added to each well and the plates were incubated for a further 4 hours at 37°C in 8% CO_2. 90 µl of 0.1 M glycine, pH 10.4 was then added to each well to block the enzyme activity and develop the yellow colour reaction. Absorbance of each well at a wavelength of 405 nm was determined using a blank reading at a wavelength of 620 nm.

The hexosaminidase detection method as used in the IL-6 assay is summarised below:

1. Using 96 well microtitre plates, 2000 washed cells per well were incubated with serial dilutions of samples or standards for 4 days at 37°C in 8% CO_2.

2. Plates were washed three times with PBS to remove spent medium and serum.

3. Substrate was added (60 µl/well) and plates were incubated for a further 4 hours.

4. Reaction was blocked and yellow colour was developed by the addition of glycine buffer (90 µl/well).

5. Absorbance was read at 405 nm with a blank reading at 620 nm.

To assess the linearity of the hexosaminidase detection method, serial dilutions of 7TD1 cells were performed in triplicate on 96 flat-bottomed well microtitre plates. Steps 2 to 5 of the above protocol were then carried out. The relationship between cell numbers and absorbance reading is shown in figure 3 which demonstrates a near linear relationship using a semi-logarithmic plot when more than 3×10^4 cells are present per well.

Figure 4 shows the dose response curve obtained when serial dilutions of recombinant IL-6 were assayed as per the above protocol. A semi-logarithmic plot gives a sigmoid shaped curve with an acceptable slope and with the linear portion extending over 0.4 absorbance units. The flattening out at the top of the curve indicates that maximal cell growth has been achieved.

Thus, the hexosaminidase method produces a satisfactory dose response curve. However, a major problem of this detection system is the limitation to sample

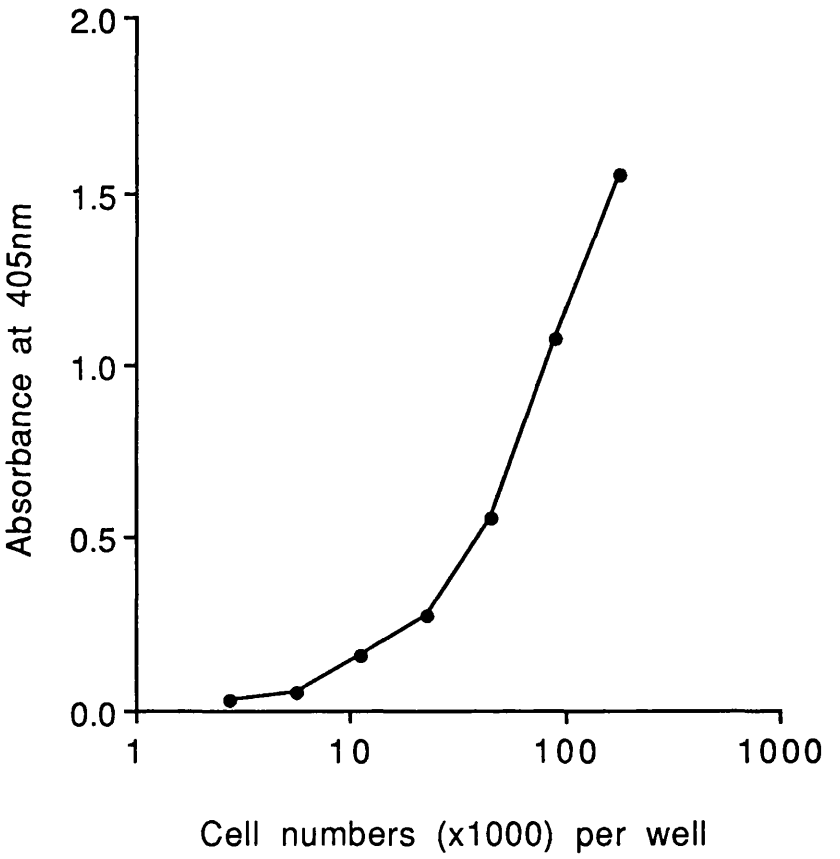


Figure 3 : 7 TD1 cell dilution curve using the hexosaminidase detection method.

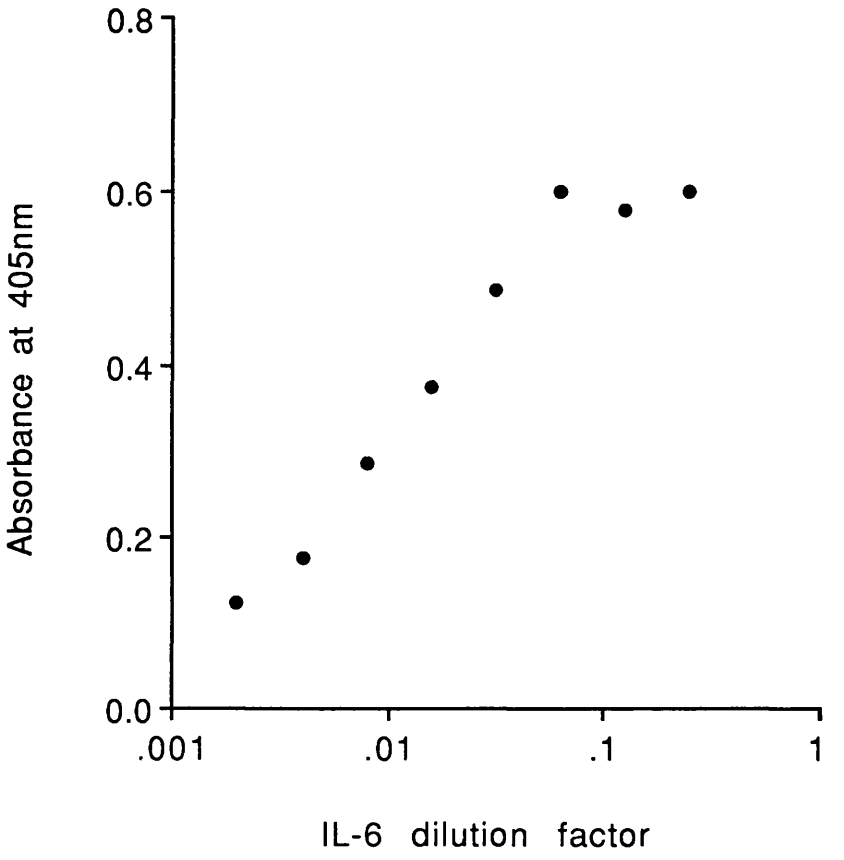


Figure 4 : Dose response curve using the hexosaminidase detection method.

throughput caused by the washing steps which are both time consuming and labour intensive.

DEHYDROGENASE ACTIVITY

The curtailment of sample throughput by the washing steps in the hexosaminidase method prompted me to look at more convenient methods. One such method measures dehydrogenase activity to evaluate cell numbers using the tetrazolium salt, MTT (3-(4, 5-dimethylthiazol-2-yl) - 2, 5-diphenyl tetrazolium bromide), as a chromogenic substrate (the action of dehydrogenase turns the yellow MTT salt purple) (231). The tetrazolium ring is cleaved by dehydrogenase enzymes in active mitochondria and so the reaction occurs only in living cells. After incubation with MTT, a detergent is added to produce uniform colour formation. MTT is made up at a concentration of 5 mg/ml in PBS and filtered to sterilise prior to use. No washing steps are required.

Three possible detergents were evaluated using the protocol shown below. The detergents used were acidified isopropanol (231) 10% sodium dodecyl sulphate (SDS) in 0.04 M HCl and 10% Triton-X in 0.5 M HCl (232).

1. Using 96 well microtitre plates, 2000 washed cells (50 µl) per well were incubated with serial dilutions of sample or standard (50 µl) for 4 days at 37°C in 8% CO_2.

2. 10 µl of MTT (5 mg/ml in PBS) was added to each well and plates were incubated for a further 4 hours.

3. 50 µl of detergent was added to each well.

4. Absorbance was read at 540 nm with a blank reading at 690 nm.

Acidified isopropanol was found to cause turbidity by precipitating serum proteins. When it was added to wells containing 15 different sera at dilutions similar to that

used in the IL-6 assay but containing no cells, absorbance readings obtained ranged from 0.183 to 0.341, median 0.276. Thus patient samples were producing falsely elevated absorbance readings when acidified isopropanol was used as a detergent.

Similar experiments conducted using both Triton-X and SDS as detergents demonstrated negligible contribution to absorbance by serum at similar dilutions to that used in assay. Thus neither serum protein precipitation nor interference caused by dehydrogenase activity present in serum appeared to be a problem when MTT was used with either SDS or Triton-X as detergent.

To assess linearity of the MTT/SDS and MTT/Triton-X detection methods, serial dilutions of 7TD1 cells were performed and steps 2 to 4 of the above protocol were carried out. Solubilisation and colour formation with both methods were better when the plates were left for approximately 16 hours at room temperature following detergent addition. Thus absorbance readings were made after plates had been left overnight.

The relationship between cell numbers and absorbance is illustrated in figure 5. Both plots demonstrate near linearity but the sensitivity of the MTT/Triton-X detection system appears superior to that of MTT/SDS. Consequently the MTT/Triton X system was chosen to evaluate cell numbers and adopted in the final assay protocol.

Optimal Incubation Time

Following addition of the 7TD1 cells, the assay plates are incubated for a fixed period of time at 37°C in 8% CO_2.

In order to determine the optimal incubation period, 3 identical plates were set up, each containing 12 x 8 serial dilutions of recombinant IL-6. Following the addition of the 7TD1 cells, one plate was incubated for 3 days, one for 4 days and one for 5

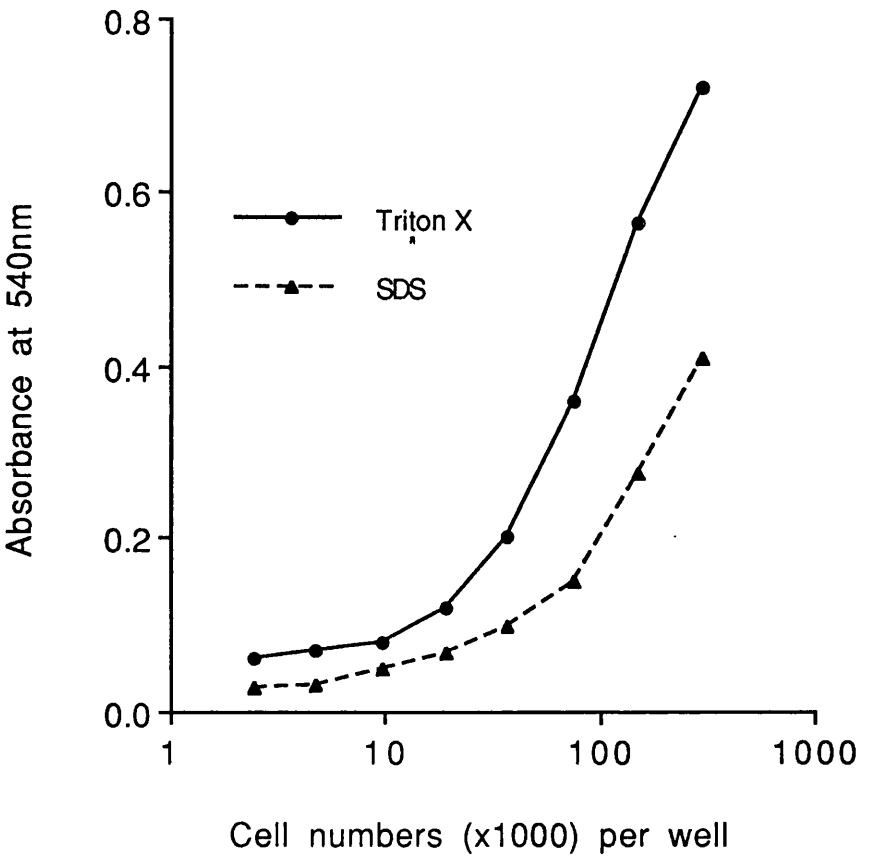


Figure 5: 7 TD1 cell dilution curves using MTT with either Triton X or SDS as detergent.

days. The number of cells present at the end of each incubation period was evaluated colorimetrically and the resulting dose response curves plotted for the 3 plates (figure 6). The 3 days curve remains flat. The 4 and 5 days curves both have acceptable sensitivities although the 5 days curve flattens out at a lower dose than the 4 days curve. Thus, because the 4 days curve had a larger linearity range than the 5 days curve and because it was preferable to have as short an incubation stage as possible, 4 days was chosen as the assay incubation period.

Samples

COLLECTION

Blood may be collected in either a plain or lithium heparin tube as serum and heparinised plasma have been found to give equivalent results in the assay (table 3). EDTA plasma should not be used as it was found to inhibit cell growth (table 4).

STABILITY

Blood should be separated as soon as possible after collection to minimise any in vitro IL-6 production, although I have found that the IL-6 activity in serum left for up to 6 hours before separation from blood cells does not differ from that in serum separated immediately.

Serum/plasma samples may be stored at 4°C for at least 4 days with no loss of IL-6 activity (table 5a). Frozen samples may be stored at -20°C for at least 7 months with no loss of IL-6 activity (table 5b).

INHIBITORS

It was noted that the serum dilution dose response curve seen when serum dilution factor was plotted against absorbance reading exhibited a hook effect, i.e. higher concentrations of serum (lower dilutions) produced lower absorbance readings in

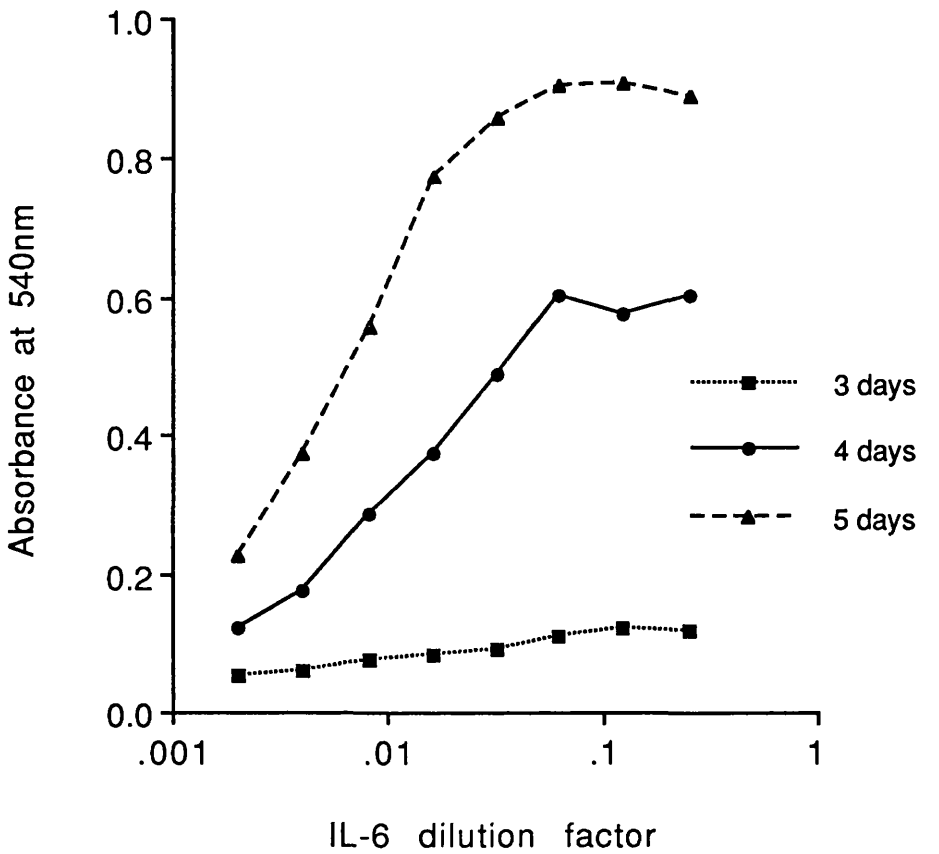


Figure 6 : Dose response curves obtained after 3 day, 4 day and 5 day incubation periods.

	IL-6 concentration (units/ml)	
Sample	**Serum**	**Heparinised Plasma**
1	18	20
2	<14	<14
3	552	597
4	364	335
5	204	203
6	<14	<14
7	84	98
8	154	150
9	28	29
10	578	558
11	426	412
12	84	99
13	106	108
14	94	97
15	395	363

$t = -0.07$; $p = 0.94$, paired t-test

Table 3: Comparison of IL-6 results obtained from serum and heparinised plasma. Each pair consists of aliquots from the same blood sample

	Absorbance	
Sample	**Serum (1/4 dilution)**	**EDTA Plasma (1/4 dilution)**
1	0.042	0.040
2	0.288	0.066
3	0.646	0.061

Table 4: Examples of the effect of EDTA plasma on cell proliferation determined colorimetrically.

Each sample was divided into EDTA anticoagulated and clotted tubes and then separated. Serum and EDTA plasma were assayed together.

	IL-6 concentration (units/ml)	
Serum	**Baseline**	**After storage for four days at 4°C**
1	28	27
2	464	420
3	244	256
4	1845	1892
5	224	196
6	1094	1158

t = -0.02; p = 0.98, paired t-test

	IL-6 concentration (units/ml)	
Serum	**Baseline**	**After storage for 7 months at -20°C**
1	529	537
2	125	134
3	112	105
4	64	48
5	258	295
6	112	105

t = -0.04; p = 0.97; paired t-test

Tables 5a and 5b: Effect of storage on IL-6 activity in serum.

the assay (figure 7). This was thought to be due to the presence of inhibitors in serum which might be inhibiting cell growth either specifically or non-specifically.

The experiment described below was performed to determine if this effect could be abolished by heating serum prior to analysis.

Serum from a healthy volunteer was divided into two aliquots and one aliquot was heated for 30 minutes in a waterbath at 56°C. (This procedure has been recommended by Dr Aarden as a method for removing inhibitors which did not influence IL-6 activity (174)). A fixed amount of IL-6 standard was added to wells containing varying dilutions of both the heated and unheated aliquots of serum. The plate was then analysed and the absorbance of each well measured (table 6). The degree of dilution of heated serum did not affect the absorbance readings obtained for the fixed amounts of IL-6. However, it was apparent that the presence of unheated serum in the assay inhibited cell growth (confirmed by microscopy) and produced very small absorbance readings. This inhibitory effect of unheated serum did not disappear until serum was present at a dilution factor of 1 in 128 or more.

This experiment supported the hypothesis that serum contains factors inhibiting IL-6 dependent cell growth which can be destroyed by heating. Consequently all plasma/serum samples are heated for 30 minutes at 56°C prior to analysis.

PREPARATION OF SAMPLES FOR ANALYSIS

Either serum or heparinised plasma may be used. 25 µl of sample is added to each of the 12 wells in the top row of a 96 well microtitre plate. 75 µl of medium is also added to each well in the top row producing a 1 in 4 dilution of serum. Thereafter 50 µl of medium is pipetted into all the other wells in the plate. 50 µl twofold dilutions are then performed starting from the top row down the eight rows on the plate. Consequently each of the twelve 8-well columns on a plate contains eight

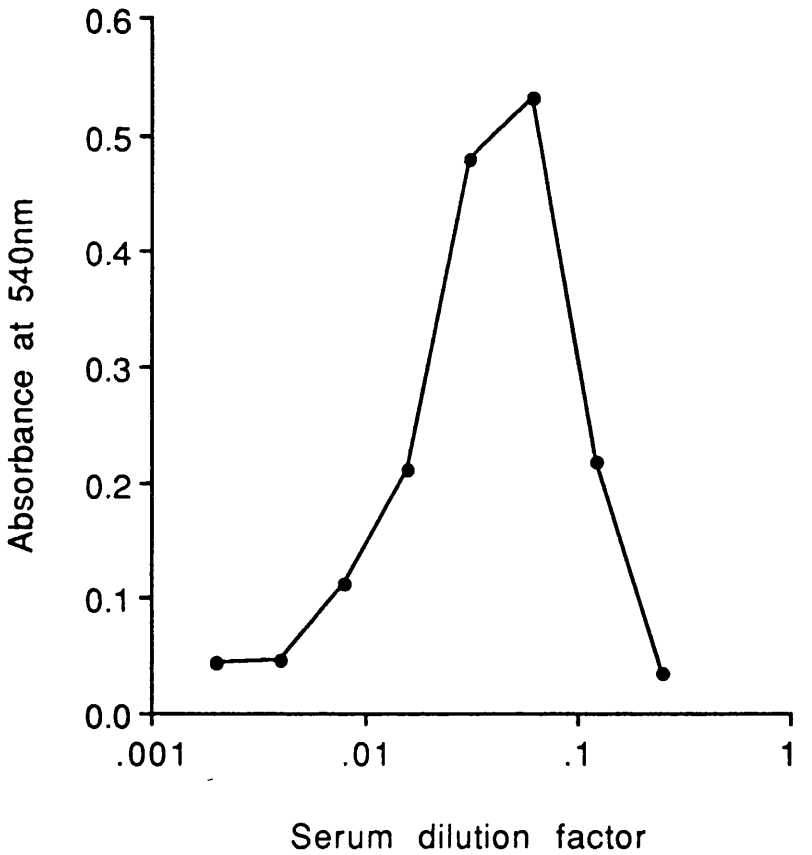


Figure 7 : Dose response curve of unheated serum showing hook effect.

Absorbance when serum not heated	Serum dilution	Absorbances when serum heated
0.07	1 in 2	0.53
0.08	1 in 4	0.54
0.07	1 in 8	0.52
0.05	1 in 16	0.52
0.12	1 in 32	0.47
0.42	1 in 64	0.57
0.54	1 in 128	0.51

Table 6 : Effect of heating serum on absorbance readings in IL-6 assay.

Each well contains a fixed amount of IL-6

two-fold dilutions of serum, ranging from 1 in 4 to 1 in 512. All samples are run in duplicate and so every sample analysed generates 16 assay points (8 dilutions in duplicate).

This sample dilution process is essential in order to obtain a detection signal which can be read off the standard curve, and to demonstrate parallelism of sample and standard dose response curves (page 73).

Final IL-6 Assay Protocol

1. Duplicate serial dilutions of serum (starting at 1 in 4 dilution) and standard (top standard concentration = 100 units/ml) are carried out in 96 well microtitre plates giving a volume of 50 µl/well.

2. 50 µl containing approximately 2 x 10^3 washed 7TD1 cells are added to each well.

3. Plates are incubated for 4 days at 37°C in 8% CO_2.

4. 10 µl MTT (5 mg/ml in PBS) is added to each well.

5. Plates are incubated for a further 4 hours at 37°C in 8% CO_2.

6. 50 µl Triton-X (10% in 0.5 M HCl) is added to each well.

7. Plates are left at room temperature overnight.

8. Absorbances are read at a wavelength of 540 nm with a blank reading at 690 nm.

Plate 1 shows a microtitre plate which has reached step 8 of the above protocol. Figure 8 shows the sigmoid shaped dose response curve obtained when the final assay protocol described above is followed. The curve is linear from about 0.1 to

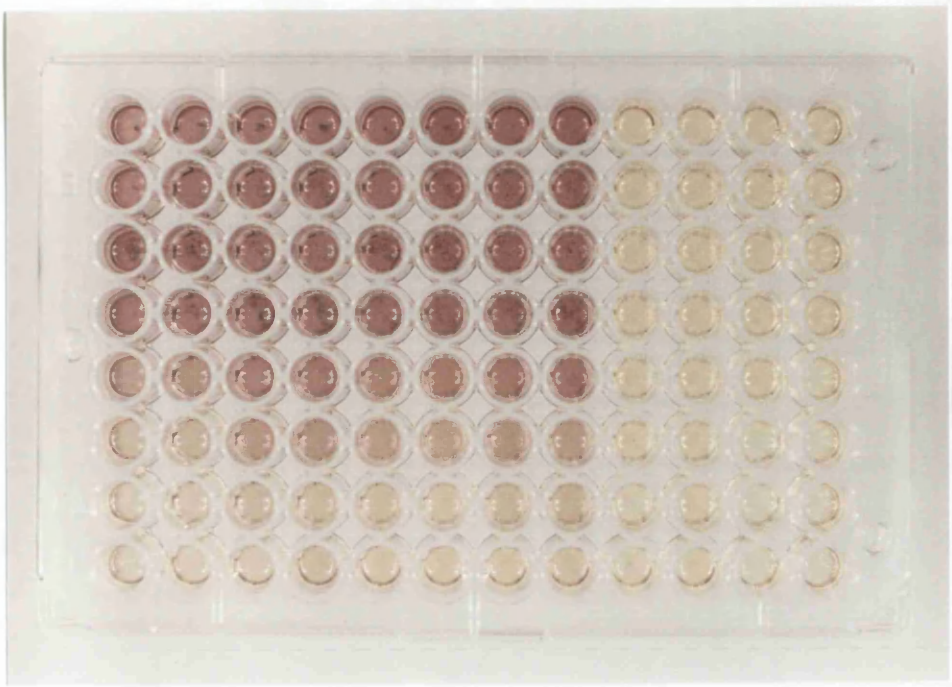


Plate 1 : 96 well microtitre plate which has been analysed according to the final IL-6 assay protocol and is ready for the absorbance of each well to be read.

The first eight columns contain serial dilutions of recombinant IL-6 standard starting from the top row. The wells in the last four columns contain medium only (reagent blanks).

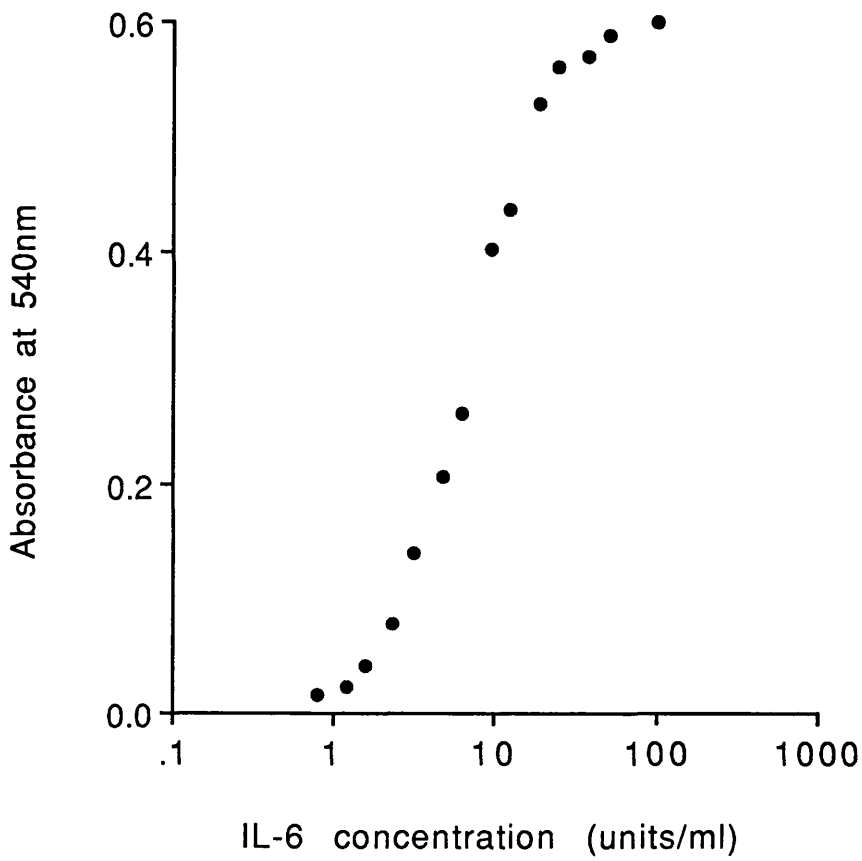


Figure 8 : IL-6 standard curve using final assay protocol.

0.5 absorbance units and flattens out thereafter indicating that maximal cell growth occurs at an IL-6 concentration of less than 50 units/ml.

Blanks

SERUM

Originally, all samples were analysed in triplicate, the third test being used as a sample blank (50 µl medium added instead of 50 µl of cells). Blank absorbances including those from icteric and haemolysed samples were low (<0.05) and were unaffected by the degree of serum dilution - suggesting that these blanks represented background absorbance. This was accounted for by the use of reagent blanks and so sample blanks were dispensed with.

REAGENT

Reagents blanks are run in every assay. These are prepared by adding 50 µl of washed cells to 50 µl medium (instead of sample). After incubation, virtually no living cells should be visible by microscopy. Following MTT/Triton-X addition, absorbances should be less than 0.05, reflecting background absorbance. Absorbances greater than 0.1 or significant numbers of cells on microscopy imply that the cells have lost their dependence on IL-6 (or have not been washed properly) thus invalidating the assay.

Reagent blank absorbances are subtracted from mean absorbance value for each sample/standard duplicate.

Calculation of Unknown Results

All serum/plasma samples are set up in duplicate at 8 different dilutions (twofold from 1 in 4 to 1 in 512). The initial dilution of 1 in 4 was chosen to minimise any possible serum effects and because I had found that serum present at this dilution caused no interference with the assay detection method (page 59).

Serial dilutions of patient samples are necessary for two reasons: firstly, to demonstrate parallelism with the standard curve; secondly, to obtain absorbance readings from at least 2 different sample dilutions which may be read from the linear portion of the standard curve. Failure to demonstrate parallelism between sample and standard material invalidates the IL-6 results obtained since factors other than IL-6 in such samples would thus appear to be stimulating cell growth. Only by performing multiple dilutions of all samples is one likely to obtain several absorbance readings on the linear portion of the standard curve.

A standard curve is constructed by plotting log IL-6 concentration against absorbance. Absorbances obtained from 2 or ideally 3 different dilutions of each sample run in duplicate (4 or 6 assay points) are averaged for each duplicate and the blank subtracted. The resulting mean absorbance value for each sample dilution is then read from the standard curve and the resulting IL-6 concentration multiplied up according to the appropriate dilution factor. These concentrations are then averaged to give the IL-6 concentration in units/ml.

Figure 9 shows the dilution curves for 3 patient sera which exhibit acceptable parallelism with the recombinant IL-6 standard curve. In each case, the top 3 absorbance values are read from the standard curve and the mean of the resulting concentrations (after correction for dilution) is used to provide the IL-6 concentration in each sample.

Quality Control

Two pools of patient sera (high and low) were heated and frozen to be used as control material. These QCs are run in every assay at intervals of approximately 20 samples. QC results are discussed in the 'Assay Parameters' section (page 75).

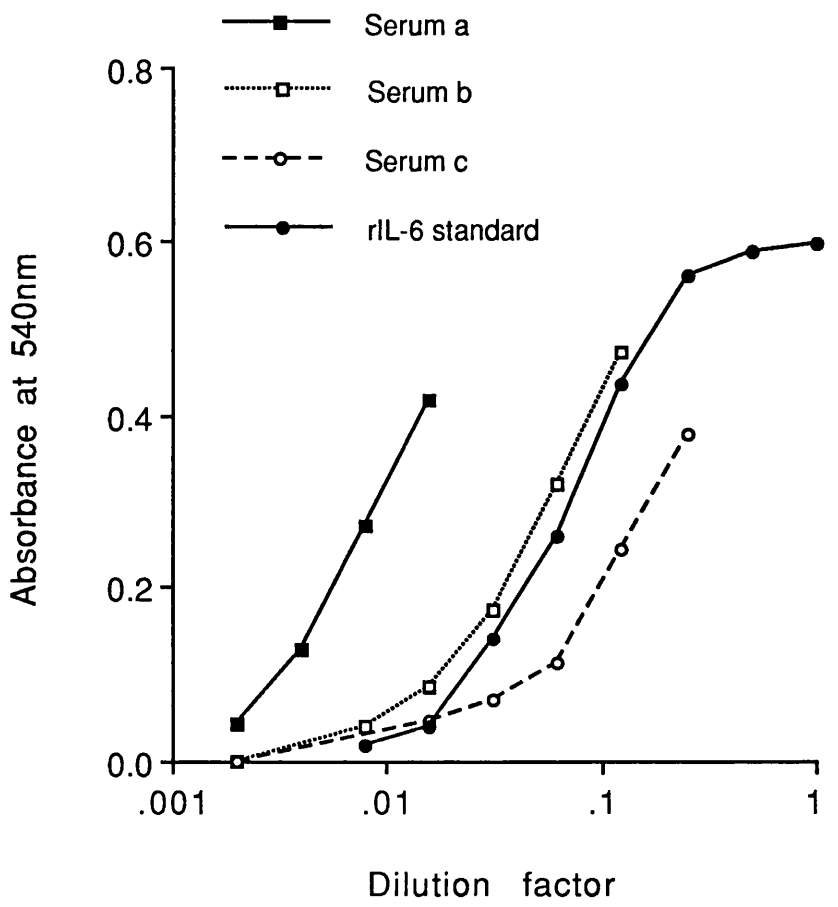


Figure 9 : Parallelism of patient sera and recombinant IL-6 dose response curves.

Assay Parameters

IMPRECISION

High and low control materials were analysed in twenty consecutive assays performed over a two month period. Overall assay imprecision was calculated using all the high QC results and all the low QC results. Each QC result was determined from a minimum of 4 assay points. The low QC results had a CV of 19% (n = 32, mean = 75.7, SD = 14.7 units/ml), and the high QC results had a CV of 23% (n = 26, mean = 1426, SD = 327 units/ml). Thus overall assay imprecision is approximately 20% - unacceptably high for more conventional assays, but a satisfactory level of imprecision for a bioassay (233,234).

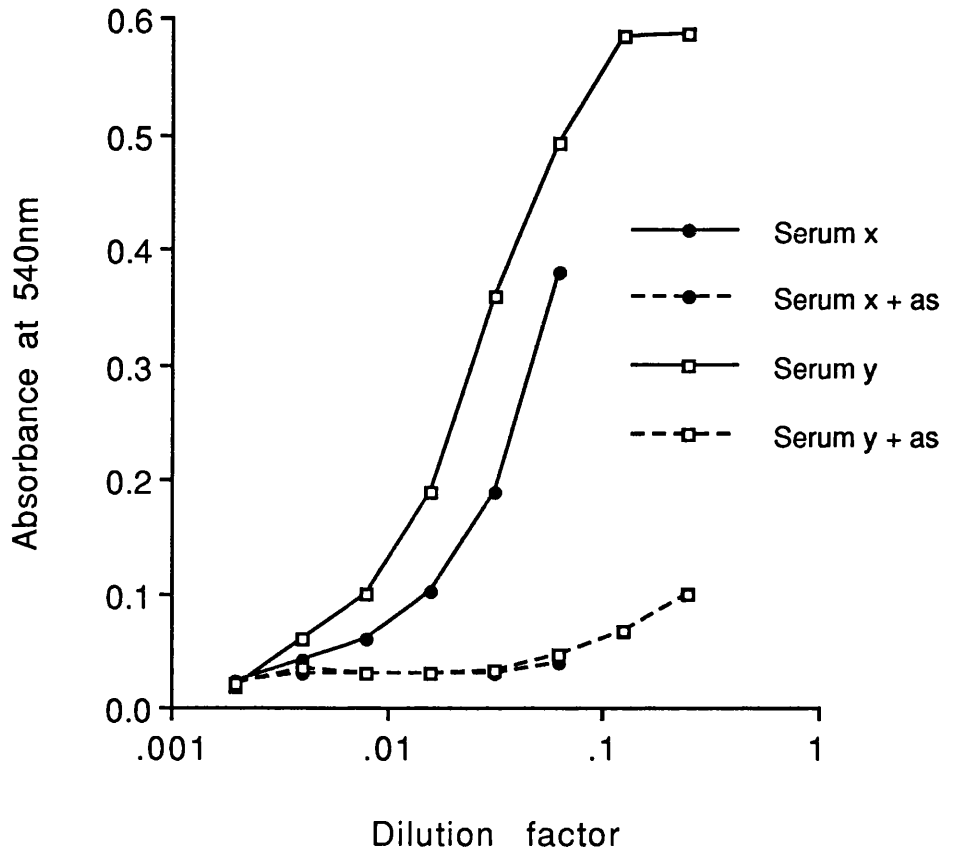
SPECIFICITY

Assay specificity was confirmed in three ways.

First, serial dilutions of samples were performed to demonstrate parallelism with the standard dose response curves (page 73). Parallelism makes assay specificity more likely.

Second, recombinant IL-1α, IL-1β,TNFα and interleukin-2 were also used to test specificity. No cell growth resulted from the use of these materials in assay at high concentrations (0.75 mg/ml, 1.7 mg/ml, 1 μg/ml and 20 units/ml respectively).

Third, rabbit polyclonal anti-human IL-6 antiserum heated for 30 minutes at 56°C to remove non-specific cytotoxicity was used in the IL-6 assay. 50 μl of antiserum at a dilution of 1 in 300 was added to wells containing either sample or standard material. In all cases the addition of antiserum abolished cell growth. The dose response curves of 2 patients' samples in figure 10 illustrate the effect of the addition of antiserum.



Key : as = antiserum.

Figure 10 : Effect of addition of heated rabbit polyclonal anti-human IL-6 antiserum on dose response curves of sera from 2 patients.

RECOVERY

Recovery experiments performed by adding 100 units/ml of IL-6 to different patient samples gave recovery values ranging from 86% to 129%.

ACCURACY

Accuracy is a function of specificity and standardisation. Specificity is discussed above and the evidence indicates that the assay is specific. Standardisation is discussed earlier in the section 'Standard Material' (page 53). Calibration against the IL-6 standard from the NIBSC did suggest that the designated specific activity of our standard material is accurate. However, no external quality control material is yet available to assess accuracy.

WORKING RANGE

The working range of the assay is 14 to 5000 units/ml based on a standard curve linear from 3.5 to 10 units/ml (figure 8) and sample dilutions from 1 in 4 to 1 in 512.

REFERENCE RANGE

IL-6 was measured in serum from 20 healthy volunteers. The range of results obtained was <14 to 18 units/ml, the median being <14 units/ml.

Summary

A bioassay for the measurement of IL-6 in serum which uses colorimetry to evaluate cell proliferation has been validated. The colorimetric detection system chosen involves the use of MTT as a substrate for dehydrogenase activity, and Triton-X as a detergent. This system is preferable to the colorimetric determination of hexosaminidase activity because it requires no washing steps. Addition of MTT and Triton-X to 1000 assay points (approximately 10 plates) each takes less than 10

minutes. In contrast, the washing steps necessary prior to the addition of hexosaminidase substrate to 10 plates take well over an hour.

The simplicity of the MTT/Triton-X detection system also compares favourably with tritiated thymidine incorporation. Following incubation with tritiated thymidine, cells are harvested and added to counting vials with scintillation fluid. The harvesting process takes up to 10 minutes per plate, is exceedingly operator-dependent and requires a harvester. To count each assay point using tritiated thymidine incorporation takes in excess of 1 minute (usually 3 minutes) whereas the Titertek Multiskan takes approximately 1 second to read each assay point. Furthermore, few laboratories have beta counting facilities available. In terms of reagent cost, the MTT/Triton-X detection system compares extremely well - 78 pence per 1000 assay points as against £34.00 per 1000 assay points using tritiated thymidine.

The bioassay described here performs well in relation to other bioassays with respect to reproducibility. MTT/Triton-X evaluation of cell numbers is fast, simple and economic. Either serum or heparinised plasma may be used - the minimum volume required is 60 µl. It takes approximately 3 hours for one person to set up an assay of 100 serum samples and the assay turnaround time is 5 days.

METHODS

STUDY DESIGN

IL-6 and the Acute Phase Response

PATIENTS

The project was approved by the Ethical Committee of the Royal Infirmary, Glasgow, and informed consent was obtained from all patients taking part in the study.

39 patients in 6 broad surgical categories were studied prospectively between February 1988 and July 1989 (table 7). No patient was on drug treatment known to affect the acute phase response. Patients underwent routine general anaesthesia: pre-medication with papaveretum or temazepam; anaesthesia with thiopentone, suxamethonium or vecuronium, nitrous oxide, enflurane or halothane, atropine, neostigmine; post-operative analgesia with morphine or papaveretum. All of these agents were received by patients in each surgical category with the exception of halothane which was not given to any patients in the minor surgery or vascular surgery groups.

CLINICAL

Each patient's post-operative clinical course was monitored for the development of complications. Axillary temperatures were recorded from the routine temperature charts.

Patients were classified as complicated or uncomplicated retrospectively. Patients were considered to have had complicated post-operative courses if their axillary temperature increased above 38°C, or if they developed clinical problems in the first seven days post-operation. In the absence of these, they were considered

Surgical Category	Number of Patients
Minor (varicose veins, lumpectomy, thyroidectomy)	7
Moderate abdominal (vagotomy, cholecystectomy)	7
Hip replacement	6
Colorectal	11
Vascular (aneurysm resection, bypass grafts)	6
Major vascular (aneurysm resection plus aortic bifurcation graft)	2
Total	39

Table 7: Elective surgical patients studied in 'IL-6 and Acute Phase Response' study.

to have made uncomplicated recoveries. Only patients who made uncomplicated recoveries were included for the purpose of studying the acute phase response.

SAMPLES

Venous blood samples were collected from each patient prior to surgery and at 2-hourly intervals until 6 hours (hourly in certain patients), 8-9 hours, 12 hours, 24 hours, 48 hours, 72 hours and 96 hours post-incision. Samples were separated and sera stored at -20°C prior to analysis for IL-6, CRP, IL-1β, TNFα, iron, transferrin, zinc and albumin.

ASSAYS

IL-6 was measured in all samples using the bioassay described in the Chapter 'Validation of a Bioassay for the Measurement of IL-6 in Serum' (page 49).

CRP was measured in all samples by fluorescence polarisation immunoassay (TDX, Abbot, Nottingham, Berks, UK) which had a detection limit of 10 mg/l and a CV of less than 5%.

IL-1ß was measured in samples from the first fifteen patients studied (none of whom developed complications) using an enzyme linked immunosorbent assay (Interleukin-1β ELISA kit, Cistron Biotechnology, Pine Brook, New Jersey, USA) which had a detection limit of 20 pg/ml.

TNFα was measured in samples from the first fifteen patients studied using an immunoradiometric assay (TNFα IRMA, Medgenix Diagnostics, Brussels, Belgium) which had a detection limit of 14 pg/ml.

Iron and transferrin were measured in samples taken prior to surgery and at 12 hours and 48 hours post-surgery from 22 patients who made uncomplicated recoveries post-operation. Transferrin was measured by immunoturbidimetry using antisera and standard from Atlantic Antibodies (Maine, USA) and the Encore

Chemistry System (Baker Instruments, Windsor, Berkshire, UK) (CV less than 7.5%), and iron was measured by colorimetric assay (Ferrozine) on a Hitachi 704 analyser (Boehringer Mannheim, Germany).

Zinc and albumin were measured in samples taken prior to surgery and at 12 hours and 48 hours post-surgery from 25 patients who made uncomplicated post-operative recoveries. Zinc was measured by atomic absorption spectrometry and albumin was measured by bromocresol green colorimetric assay on a BM Hitachi 704 analyser.

OUTCOMES

The time course and magnitude of the responses of serum IL-6, TNFα, and IL-1ß to surgery were compared and related to the serum CRP response and to the changes in body temperature which occurred. The magnitude of each response was determined by measuring the area under the response curve.

Iron and zinc are mostly bound to transferrin and albumin respectively in serum. Since serum concentrations of both transferrin and albumin decrease during the acute phase response, iron/transferrin and zinc/albumin ratios were calculated to correct for any changes in iron and zinc concentration which occurred due to decreases in concentrations of their binding proteins. Iron and zinc concentrations reach their nadir approximately 12 hours post-incision (140). The associations between decreases in iron/transferrin and zinc/albumin ratios during the first 12 hours post-incision and serum IL-6 response were studied.

Moreover, in order to study the temporal relationship between changes in serum IL-6 concentration and changes in iron/transferrin and zinc/albumin ratios, the IL-6 responses in 5 uncomplicated cholecystectomy patients were related to changes in the iron/transferrin ratio and zinc/albumin ratio which occurred following surgery in 9 different cholecystectomy patients previously studied by our group (140).

Clinical Value of IL-6

SURGICAL COMPLICATIONS

IL-6 and CRP responses in surgical patients who were classified retrospectively as complicated were related to their clinical course, and serum IL-6 and CRP concentrations in patients with complications were compared with those in patients who did not develop complications.

SUSPECTED MYOCARDIAL INFARCTION

Patients

The project was approved by the Ethical Committee of the Royal Infirmary Glasgow, and informed consent obtained from all patients.

15 patients admitted to hospital with suspected myocardial infarction were studied prospectively between January and June of 1989. All patients were seen at presentation by a cardiologist who made the diagnosis of suspected or probable myocardial infarction.

Clinical

The time between the onset of symptoms and presentation was recorded as was the clinical course. The diagnosis of myocardial infarction or unstable angina pectoris was made retrospectively based on the WHO criteria (235).

Left ventricular performance was assessed on the third hospital day. Apical biplane cross-sectional echocardiography was performed and a Simpson's rule algorithm used to calculate end-diastolic volume and end-systolic volume (236) from which the left ventricular ejection fraction (LVEF) was calculated.

Samples

Venous blood was taken on admission from each patient. Samples were separated and stored at -20°C prior to analysis for IL-6 and CRP. Serum was also stored at 4°C and CK activity measured the following day.

Assays

IL-6 and CRP were measured as described previously.

CK was measured, using an enzymatic assay (CK NAC activated, Boehringer Mannheim, Germany) on a Hitachi 737 analyser (Boehringer Mannheim, Germany).

Outcomes

Admission serum IL-6, CRP and CK concentrations in patients with unstable angina were compared with those in patients who had myocardial infarctions. Moreover, the associations between these three variables and left ventricular performance as assessed by LVEF were studied.

ACUTE PANCREATITIS

Patients

The project received Ethical Committee approval and informed consent was obtained from all patients.

23 patients admitted with acute pancreatitis were prospectively entered into the study between December 1987 and July 1989. The diagnosis of acute pancreatitis was made on the basis of a serum amylase greater than 720 IU/l in the presence of a compatible clinical picture.

Clinical

The delay between the onset of symptoms and presentation was noted. The clinical course of the disease and a modified Glasgow score were recorded for each patient. (One point was scored for each of the 8 prognostic factors shown in table 8 present during the 48 hours following admision. A score of three or more suggests a severe attack of pancreatitis (210)).

Definitive classification of disease severity was performed retrospectively depending upon the clinical outcome. Patients were considered to have had a severe attack if one or more of the following was present: pancreatic pseudocyst; pancreatic sepsis; respiratory failure (pO_2 < 8 kPa requiring oxygen for more than 5 days or assisted ventilation); renal failure (urine output less than 400 ml per 24 hours despite adequate fluid replacement); shock (systolic blood pressure less than 90 mmHg in the presence of an adequate circulating volume); hospital stay of more than 14 days (unless discharge was delayed for cholecystectomy or for social reasons); or death. If none of these was present, then the patient was considered to have suffered a mild attack.

Samples

Venous blood samples were taken on admission, 6-hourly for 48 hours and then twice daily for a further 3 days. Samples were separated and stored at -20°C prior to analysis for IL-6 and CRP.

Assays

IL-6 and CRP were measured as previously described in all samples from the 23 patients. Amylase was measured using an enzymatic assay (α amylase PNP, Boehringer Mannheim, Germany) on a BM Hitachi 737 analyser.

Arterial partial pressure of oxygen	<8.0 kPa
Serum albumin	<32 g/l
Serum calcium	<2.0 mmol/l
White cell count	>15 x 10^9/l
Aspartate transaminase (AST)	>200 IU/l
Lactate dehydrogenase (LDH)	>600 IU/l
Plasma glucose	>10 mmol/l (in the absence of diabetes mellitus)
Serum urea	>16 mmol/l

Table 8: The modified Glasgow criteria for severity prediction in acute pancreatitis. A severe attack is indicated by the presence of 3 or more criteria.

Outcomes

Serum concentrations of IL-6 and CRP following admission and modified Glasgow scores were compared between those patients classified as having mild disease and those classified as having severe disease.

RHEUMATOID ARTHRITIS

Patients

33 patients with rheumatoid arthritis who attended a rheumatology out-patient clinic at Glasgow Royal Infirmary were studied.

Clinical

Disease activity was scored using the Ritchie Articular Index (228).

Samples

Venous blood from each patient was collected into Westergren and clotted tubes. The clotted samples were separated and sera stored at -20°C prior to analysis for IL-6 and CRP.

Assays

IL-6 and CRP were measured as described previously. ESR was measured by Westergren's method (237).

Outcomes

The associations between serum IL-6, CRP and ESR and clinical disease activity as measured by the Ritchie Articular Index were studied.

DATA ANALYSIS AND PRESENTATION

Results are expressed as median and range throughout this work. Statistical analyses have been performed using MINITAB statistical softwear (Minitab Inc, Pennsylvania, USA).

The Mann Whitney test has been applied to test differences between groups, and the W statistic (the sum of the ranks corresponding to the observations in the first sample), p value and 95% confidence interval (CI) for differences are quoted.

Associations between variables have been studied using the Pearson product moment correlation coefficient (r) and linear regression analysis. The regression equation is presented as $y = a + bx$ along with the values of t and p for the null hypothesis that b does not equal 0 ie, that there is statistically significant evidence of an association between the variables x and y. Where appropriate the 95% confidence interval for the value of b is also quoted.

For the sake of clarity, error bars in line graphs have been omitted, and these graphs are used only to illustrate time courses. Where differences between groups have been analysed, data points are presented in the form of scatter plots. Likewise, most associations between variables are presented in the form of scatter plots showing the regression line.

Diagnostic sensitivity of a test is calculated as

$$\frac{\text{the number of true positive results}}{\text{number of people with the disease}} \times 100\%$$

Diagnostic specificity of a test is calculated as

$$\frac{\text{the number of true negative results}}{\text{number of people without the disease}} \times 100\%$$

Positive predictive value of a test is calculated as

$$\frac{\text{the number of true positive results}}{\text{total number of positive results}} \times 100\%$$

Negative predictive value of a test is calculated as

$$\frac{\text{the number of true negative results}}{\text{total number of negative results}} \times 100\%$$

Efficiency of a test is calculated as

$$\frac{\text{the number of true results}}{\text{the total number of results}} \times 100\%$$

RESULTS

IL-6 and the Acute Phase Response

Of the 39 elective surgical patients studied, 29 made uncomplicated recoveries post-operation (table 9). Of these, one medical student who underwent partial thyroidectomy has been excluded from the minor group because her surgery which was particularly painstaking was not representative. The remaining 28 patients have been used as models to study the role of IL-6, TNFα and IL-1 in the acute phase response.

The duration of surgery in the minor group was shorter than that in the other 5 groups (table 10). The moderate group tended to have shorter operations than the more major groups but the differences failed to meet statistical significance. There was considerable overlap in duration of surgery among the 4 more major surgical groups.

None of the first 15 patients studied had detectable serum levels of either TNFα or IL-1ß at any time post-surgery.

Serum IL-6 rose within 2 to 4 hours of incision in all 28 patients, peaking at 6 to 12 hours post-incision (slightly later in the hip surgery patients) (figure 11). The magnitude of the IL-6 response (measured as the area under the response curve up to 48 hours post-incision) differed among the various surgical groups (table 11, figure 12). Patients undergoing minor procedures had a significantly smaller response than any of the other patients. Similarly, the moderate abdominal group demonstrated a significantly smaller IL-6 response than the 4 more major surgical groups. Considerable overlap existed among the responses of the hip, vascular and colorectal groups. Peak IL-6 levels were closely associated with integrated IL-6 response (figure 13) ($r = 0.95$, $y = -78.2 + 1.1x$, $t = 15.12$, $p < 0.001$, 95% CI = (1.0, 1.2)) and so may be considered to be representative of the overall response.

	Number of patients having:	
Surgical Category	**Uncomplicated Recovery**	**Complicated Recovery**
Minor	6	0
Moderate	5	2
Hip	6	0
Colorectal	6	5
Vascular	4	2
Major Vascular	1	1
Total	28	10

Table 9: Post-operative course in 38 of the 39 surgical patients studied (one patient has been excluded from the minor group (see text)).

Surgical Category	Duration of Surgery (minutes)
Minor (n = 6)	45 (15-55)[a]
Moderate (n = 5)	75 (45 - 135)[b]
Hip (n = 6)	120 (60 - 190)[c]
Vascular (n = 4)	85 (75 - 160)[d]
Colorectal (n = 6)	110 (75 - 270)[e]
Major Vascular (n = 1)	225

a<b, W = 24.0, p = 0.034, 95% CI = (-90, 0)
a<c, W = 21.0, p = 0.008, 95% CI = (-90, -15)
a<d, W = 21.0, p = 0.013, 95% CI = (-115, -30)
a<e, W = 21.0, p = 0.005, 95% CI = (-215, -30)

(all differences tested using Mann Whitney test)

Table 10: Duration of surgery in 6 surgical categories. Results are expressed as median (range).

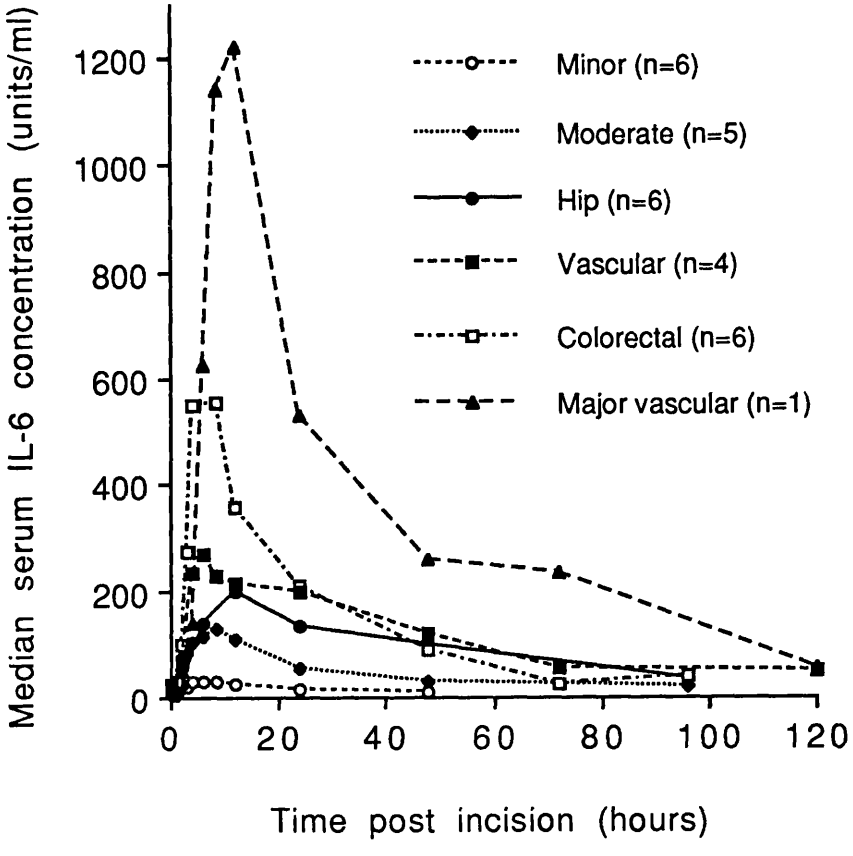


Figure 11 : Serum IL-6 response to 6 categories of elective surgery.

Surgical Category	Area under IL-6 response curve up to 48 hours post-incision*	Peak serum IL-6 concentration (units/ml)
Minor (n = 6)	46 (35-54)[a]	36 (28-46)
Moderate (n = 5)	164 (76 - 210)[b]	130 (56 - 225)
Hip (n = 6)	291 (246 - 681)[c]	208 (122 - 395)
Vascular (n = 4)	367 (262 - 628)[d]	272 (192 - 527)
Colorectal (n = 6)	562 (438 - 1500)[e]	569 (346 - 2000)
Minor (n = 1)	1292	1220

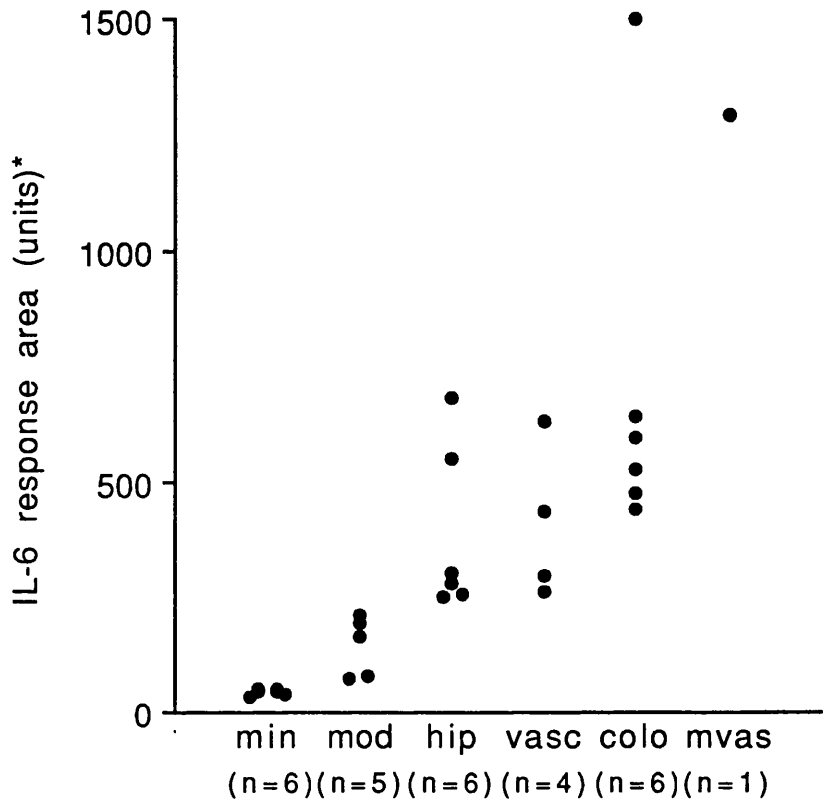
* One unit of area is equivalent to 20 units/ml.hour.

a<b, W = 21.0, p = 0.008, 95% CI = (-164, -30)
a<c, W = 21.0, p = 0.005, 95% CI = (-627, -205)
a<d, W = 21.0, p = 0.014, 95% CI = (-582, -216)
a<e, W = 21.0, p = 0.005, 95% CI = (-1446, -404)
b<c, W = 15.0, p = 0.008, 95% CI = (-490, -63)
b<d, W = 15.0, p = 0.020, 95% CI = (-548, -70)
b<e, W = 15.0, p = 0.008, 95% CI = (-1308, -274)

(all differences tested using Mann Whitney test)

Table 11: IL-6 response and peak serum IL-6 concentration in 6 surgical categories.

Results are expressed as median (range).



Key: min = minor; mod = moderate; vasc = vascular; colo = colorectal; mvas = major vascular.

*one unit of area is equivalent to 20 units/ml.hour.

Figure 12 : Comparison of the magnitude of the serum IL-6 response among 6 surgical categories.

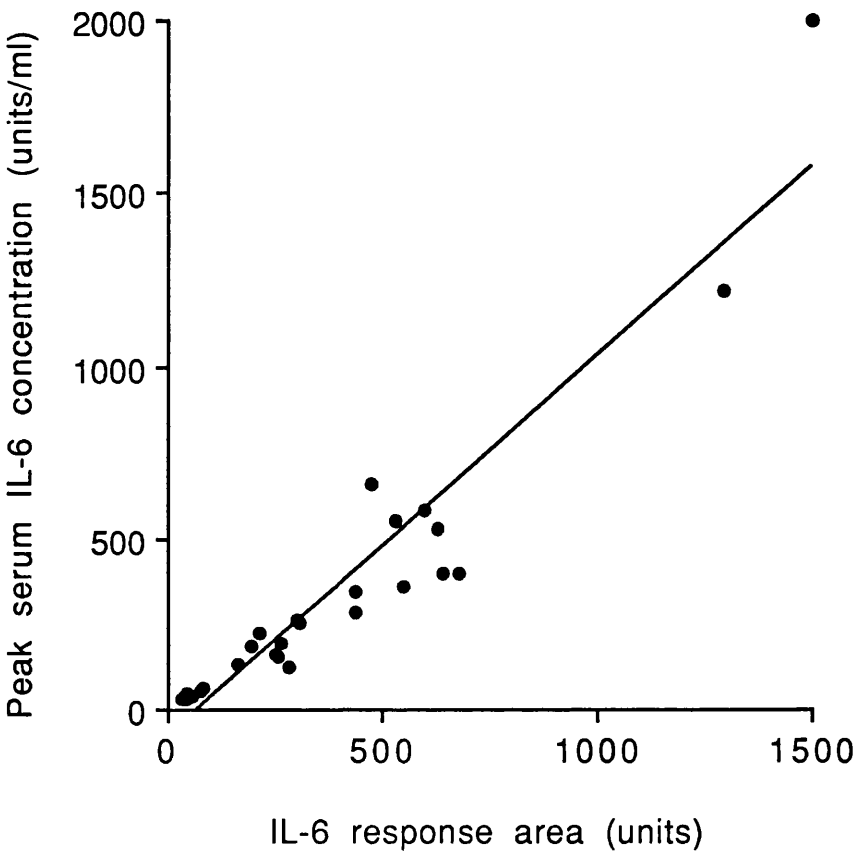


Figure 13 : The association between integrated IL-6 response and peak serum IL-6 concentration in 28 surgical patients.

($r = 0.95$, $y = -78.2 + 1.1\,x$, $p < 0.001$)

The differences in IL-6 response among the surgical categories prompted me to examine the relationship between integrated IL-6 response over 48 hours and the length of operation (intended to reflect the degree of tissue trauma sustained during surgery). Figure 14 illustrates the highly significant correlation between integrated IL-6 response and length of operation ($r = 0.85$, $y = -139.0 + 5.1x$, $t = 8.37$, $p < 0.001$, 95% CI = (3.9, 6.4)) which does suggest that the overall response is related to magnitude of tissue damage. As expected, peak IL-6 levels were also closely associated with the duration of surgery ($r = 0.81$, $y = -231.0 + 5.7x$, $t = 7.0$, $p < 0.001$, 95% CI = (4.1, 7.3)) and may therefore be considered to reflect extent of injury.

Figure 15 shows the CRP response to surgery in the six surgical categories. Unlike IL-6, there was no detectable increase in CRP in the group of minor surgical patients. In the other four groups, CRP rose 8 to 12 hours post-incision peaking 24 to 48 hours post-incision. However, no significant differences were observed in integrated CRP responses 12 to 72 hours post-incision among these four groups (figure 16). As was the case for IL-6, there was good correlation between peak CRP and integrated CRP response ($r = 0.96$, $y = 8.0 + 0.5x$, $t = 17.81$, $p<0.001$, 95% CI = (0.4, 0.6)). The integrated CRP response was less closely associated with length of operation (figure 17) ($r = 0.63$, $y = 76.4 + 1.8x$, $t = 4.12$, $p < 0.001$, 95% CI = (0.9, 2.7)) than was the integrated IL-6 response.

Figures 18a-d illustrate the temporal relationship between serum IL-6 and CRP responses in 4 of the 6 surgical groups. The minor and major vascular groups are not shown since there was no CRP response in the former, and the latter consisted of only 1 patient. In general, IL-6 and CRP responses follow the same pattern with peak CRP levels occurring 20-40 hours after peak IL-6 levels. The integrated IL-6 response was associated weakly but significantly with the integrated CRP response (figure 19) ($r = 0.63$, $y = 143.0 + 0.3x$, $t = 4.15$, $p < 0.001$, 95% CI = (0.2, 0.4)).

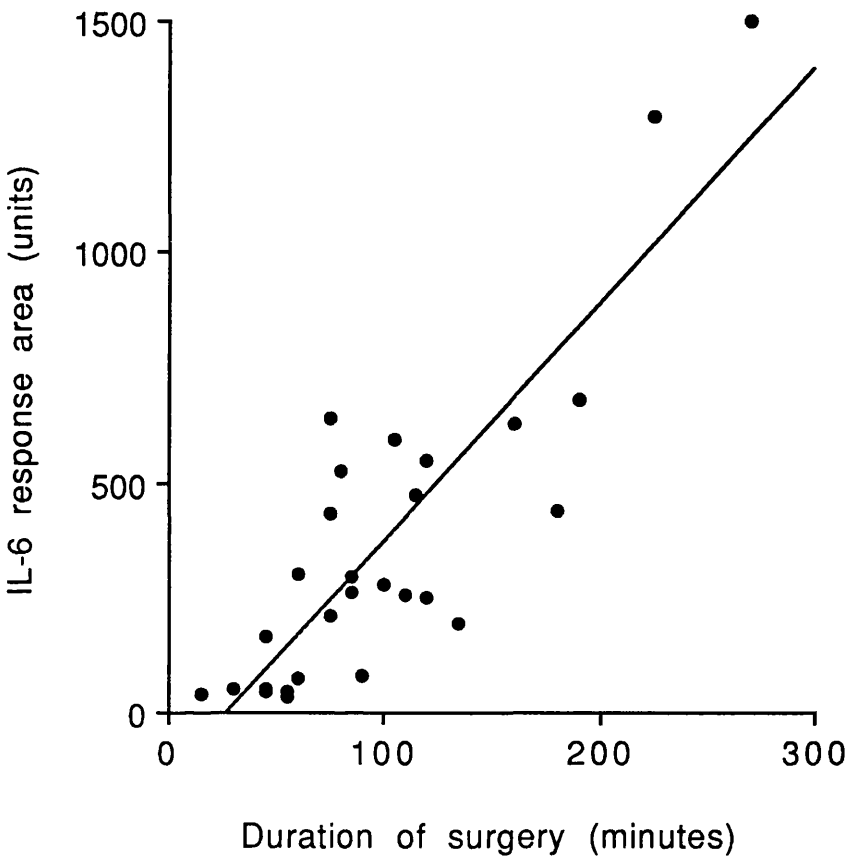


Figure 14 : The association between duration of surgery and integrated IL-6 response in 28 surgical patients.

($r = 0.85$, $y = -139.0 + 5.1\ x$, $p < 0.001$)

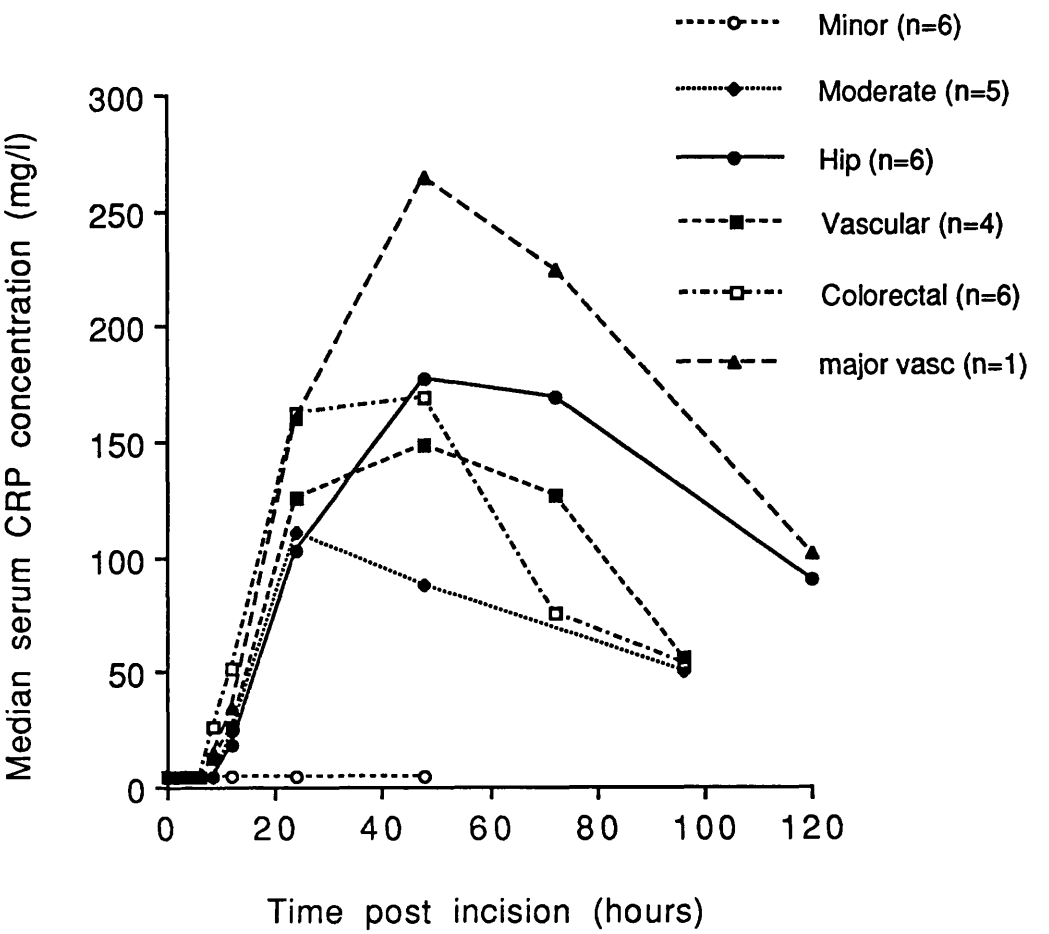
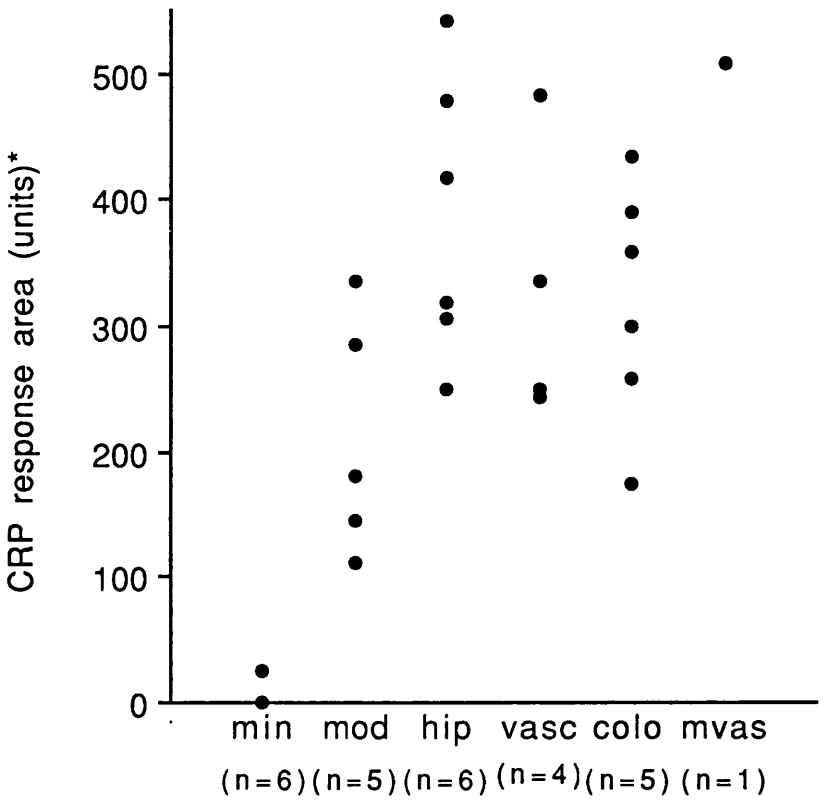


Figure 15 : Serum CRP response to 6 categories of elective surgery.



Key: min = minor; mod = moderate; vasc = vascular; colo = colorectal; mvas = major vascular

* one unit of area is equivalent to 20 mg/l. hour.

Figure 16 : Comparison of the magnitude of the serum CRP response among 6 surgical categories.

(5 of the 6 minor patients had no detectable CRP response)

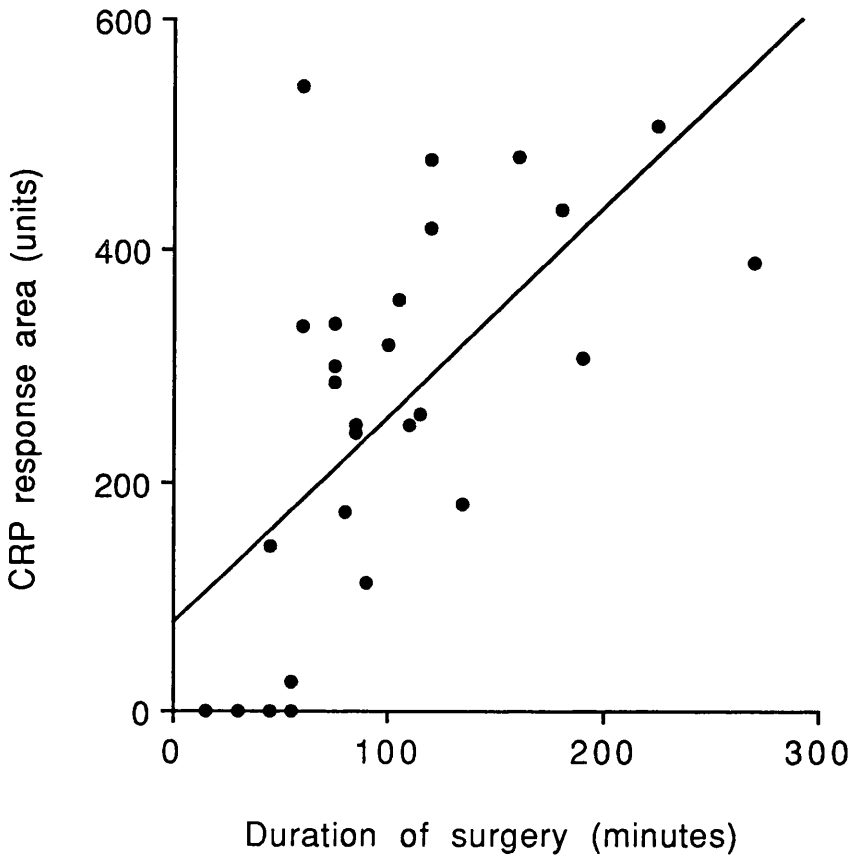
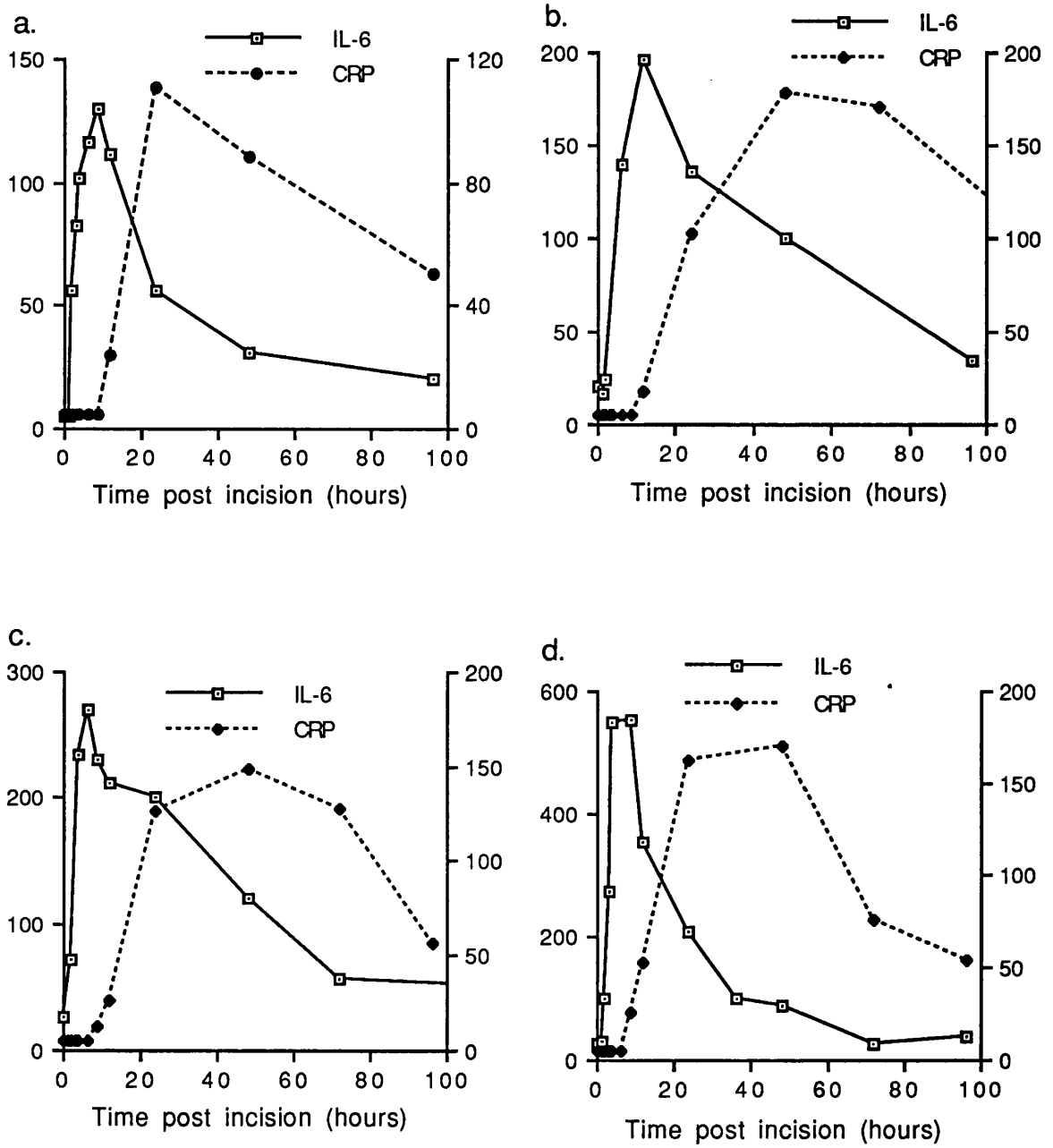


Figure 17 : The association between duration of surgery and integrated CRP response in 28 surgical patients.

($r = 0.63$, $y = 76.4 + 1.8\,x$, $p < 0.001$)



Key: a = moderate (n=5); b = hip (n=6); c = vascular (n=4); d = colorectal (n=6)

Figures 18a-d:The temporal relationship between serum IL-6 and CRP concentrations after surgery in 4 surgical categories.

(in each figure, the left-hand vertical axis represents median serum IL-6 concentration (units/ml) and the right-hand vertical axis represents median serum CRP concentration (mg/l))

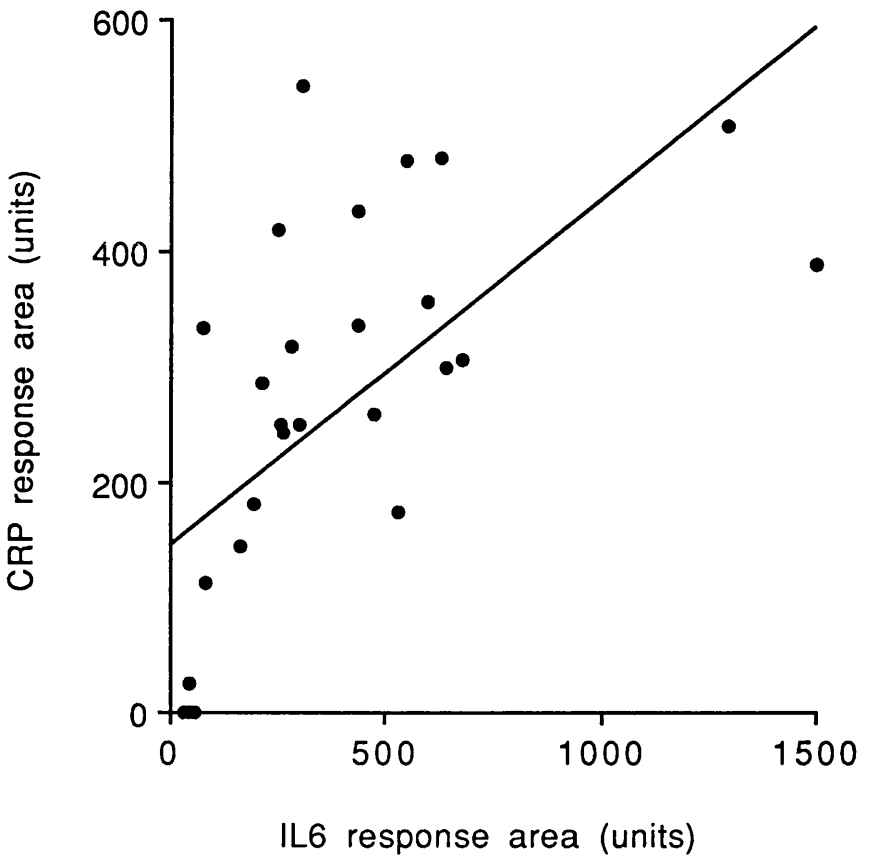


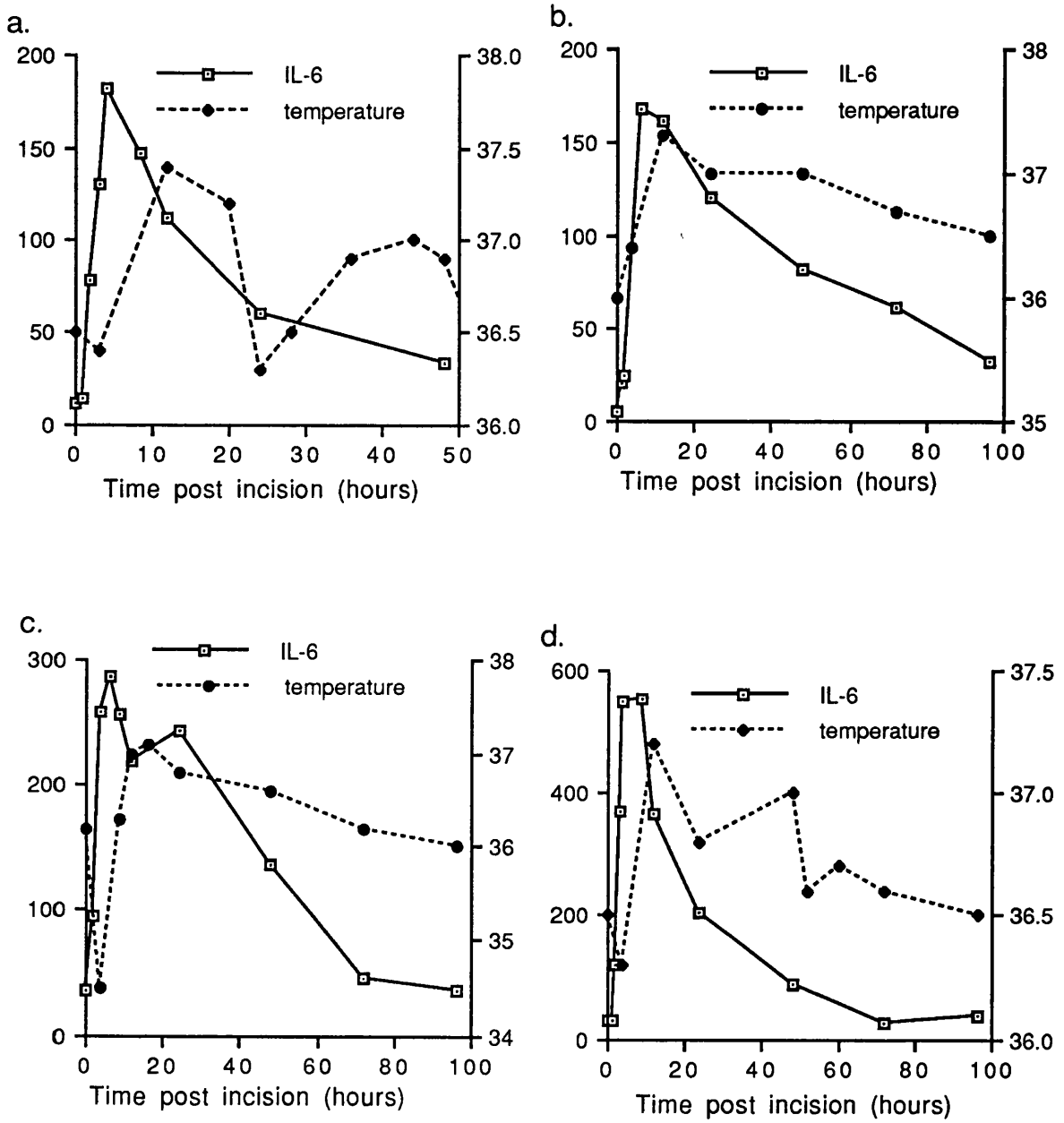
Figure 19 : The association between integrated IL-6 and CRP responses in 28 surgical patients.

($r = 0.63$, $y = 143.0 + 0.3\,x$, $p < 0.001$).

Temperature charts were available from 19 of these 28 patients. Axillary temperature rose in all 19 patients and peaked approximately 12 hours post-incision. Figures 20a-d illustrate the time course of the changes in axillary temperature relative to serum IL-6 in four of the six surgical groups (for ease of presentation the minor group is not shown). In each group, changes in IL-6 concentration parallel those in temperature, and the peak serum IL-6 and the peak temperature occur within a few hours of each other. Moreover a significant association exists between peak serum IL-6 and peak axillary temperature in these 19 patients (figure 21) ($r = 0.53$, $y = 37.0 + 0.001x$, $t = 2.59$, $p = 0.019$).

The changes in serum iron concentration and iron/transferrin ratio are shown in figures 22a and b, and can be seen to mirror one another. Iron concentration and iron/transferrin ratio fell between 0 and 12 hours post-incision in all 22 patients except the major vascular patient (who exhibited no change in iron levels). By 48 hours, iron and iron/transferrin ratio had fallen substantially in the major vascular patient, and remained low in all the other patients. Indeed, the hip group and the vascular group exhibited further falls in iron and iron/transferrin ratio between 12 and 48 hours post-incision. The extent of the reductions in iron and iron/transferrin ratio appears not to depend on the severity of the surgical procedure performed - an observation confirmed in figure 23, which shows considerable overlap in reduction in iron/transferrin ratio among the groups. Figure 24 illustrates the poor association between serum IL-6 response area and reduction in iron/transferrin ratio over the first 12 hours post-incision ($r = -0.33$, $y = 4.9 - 0.003x$, $t = -1.59$, $p = 0.128$).

Serum zinc and zinc/albumin ratio had fallen in all 25 patients by 12 hours post-incision in all groups (figure 25a and b). By 48 hours zinc levels were returning to normal in all groups other than the hip group. Unlike iron/transferrin ratio, reductions in the zinc/albumin ratio tended to be smaller in the groups undergoing more minor surgery (table 12, figure 26). Figure 27 illustrates the significant



Key: a = moderate (n=3); b = hip (n=3); c = vascular (n=3);
d = colorectal (n=4)

Figures 20a-d : The temporal relationship between serum IL-6 concentration and axillary temperature in four surgical categories.

(in each figure the left-hand vertical axis represents median serum IL-6 concentration (units/ml) and the right-hand vertical axis represents median axillary temperature (°C)).

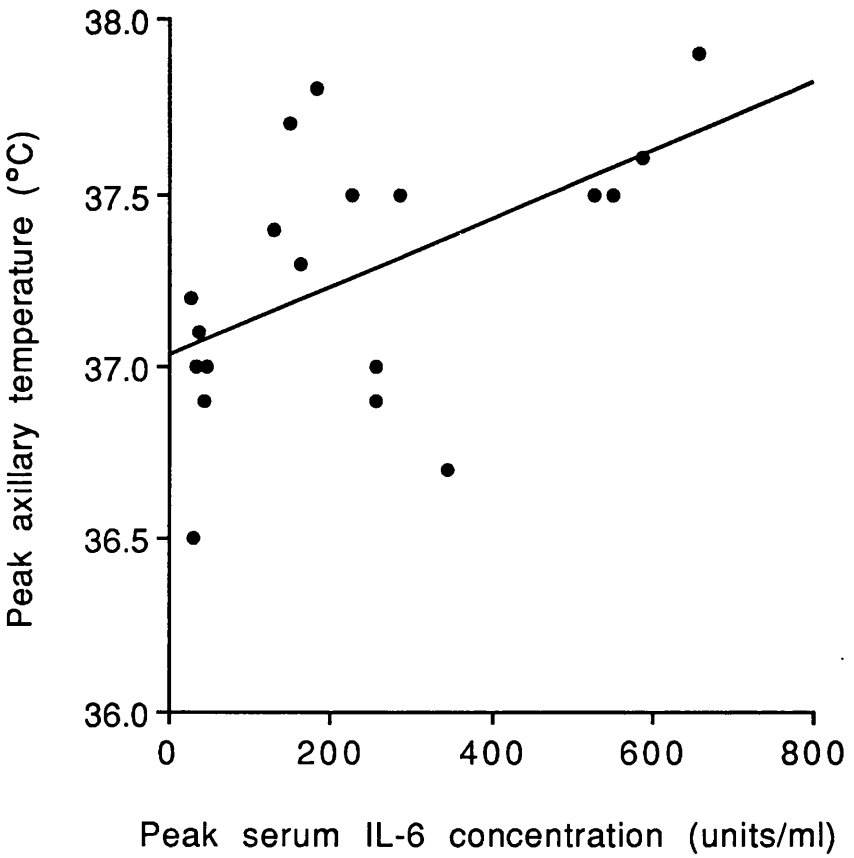
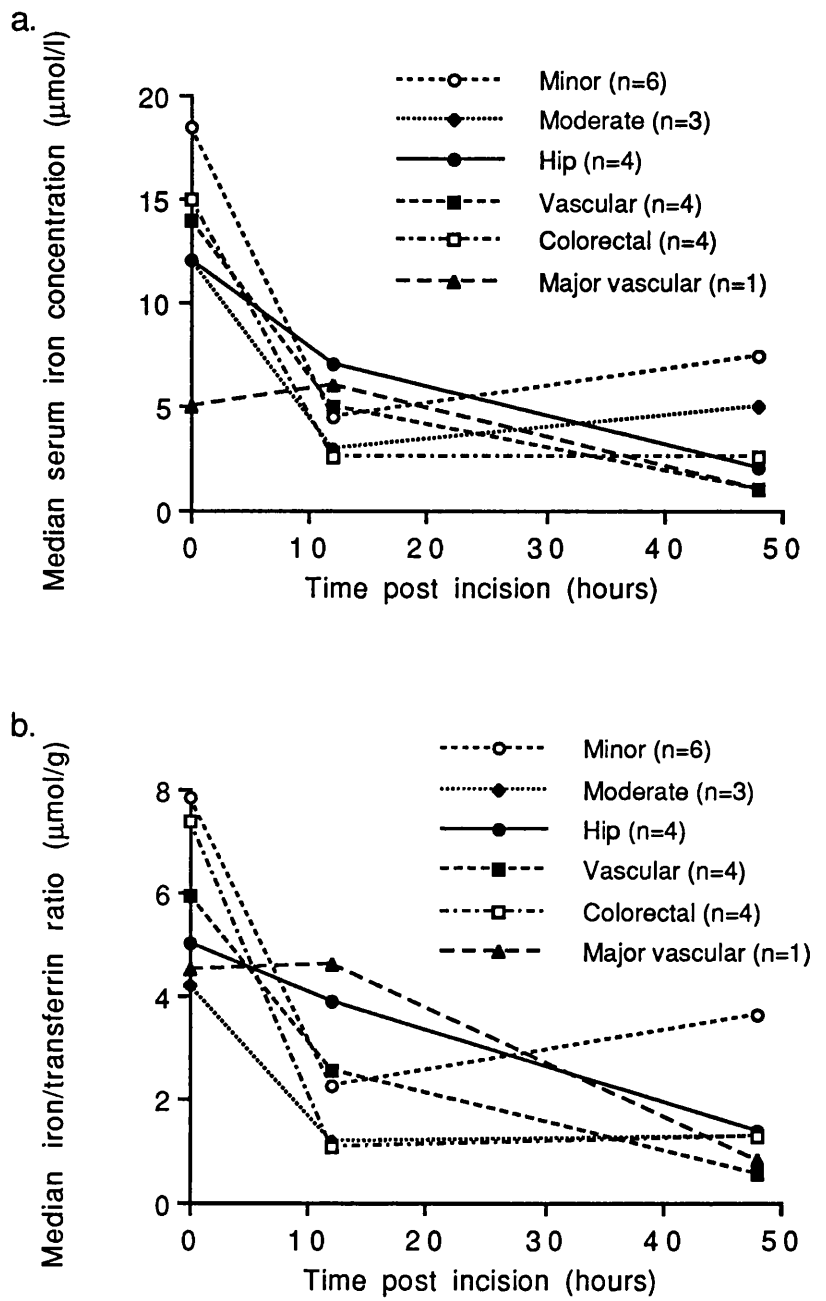
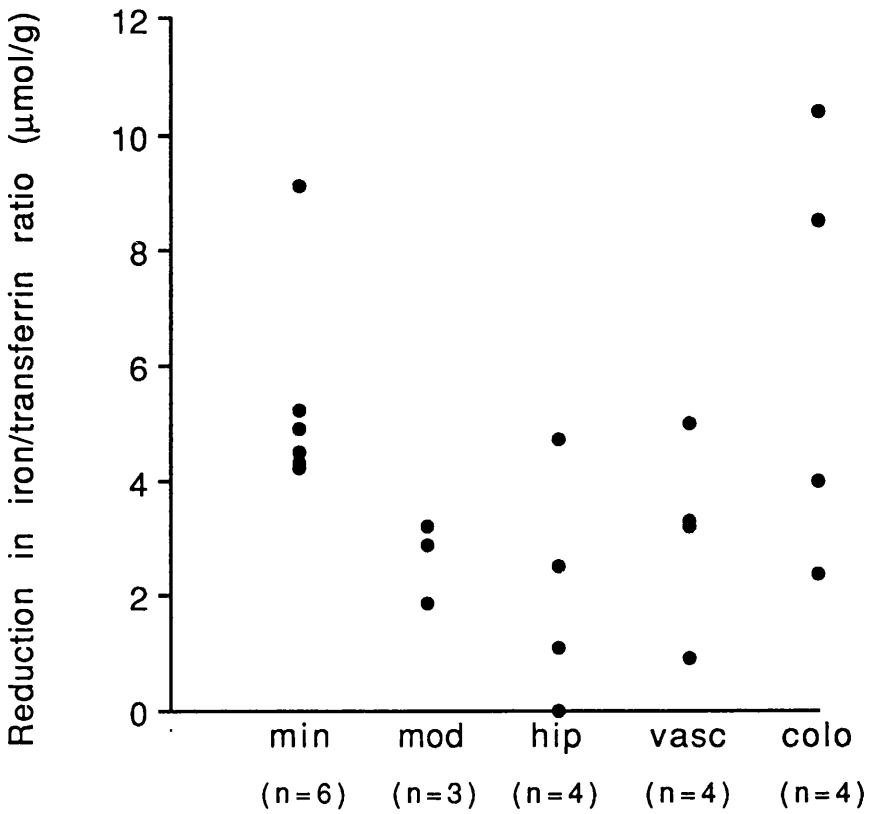


Figure 21 : The association between peak serum IL-6 concentration and peak axillary temperature in 19 surgical patients.

($r = 0.53$, $y = 37.0 + 0.001\ x$, $p = 0.019$)



Figures 22a,b : The changes in (a) serum iron concentration and
(b) iron/transferrin ratio following surgery in 6 surgical categories.



Key: min = minor; mod = moderate; vasc = vascular; colo = colorectal

Figure 23 : Comparison of the magnitude of the reductions in iron/transferrin ratio in the first 12 hours post-operation among 5 surgical categories.

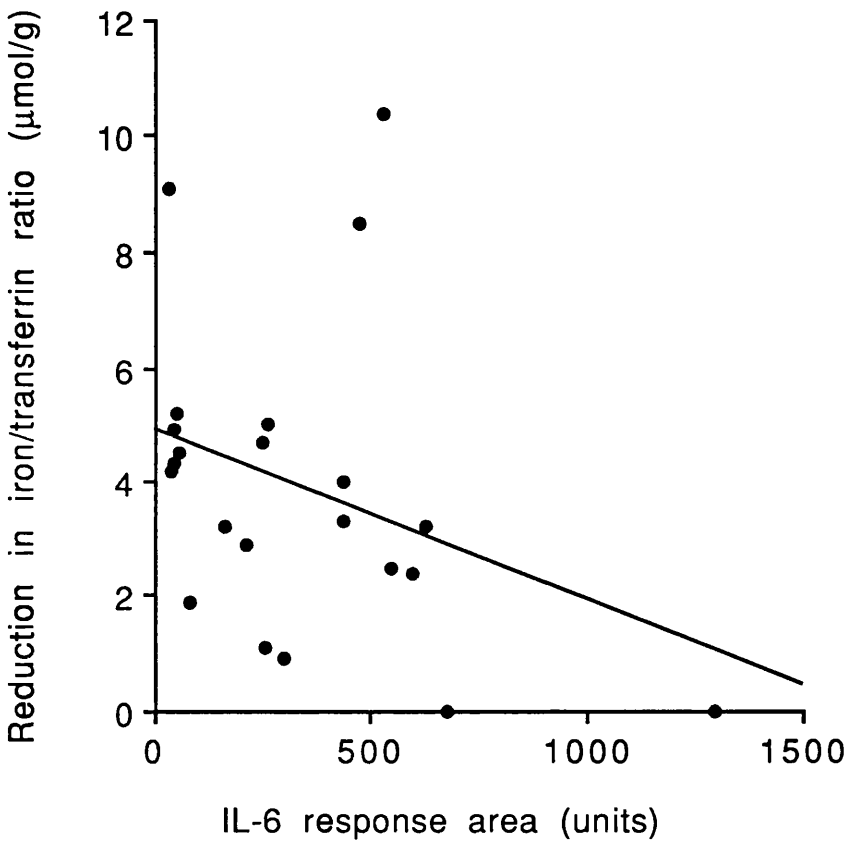
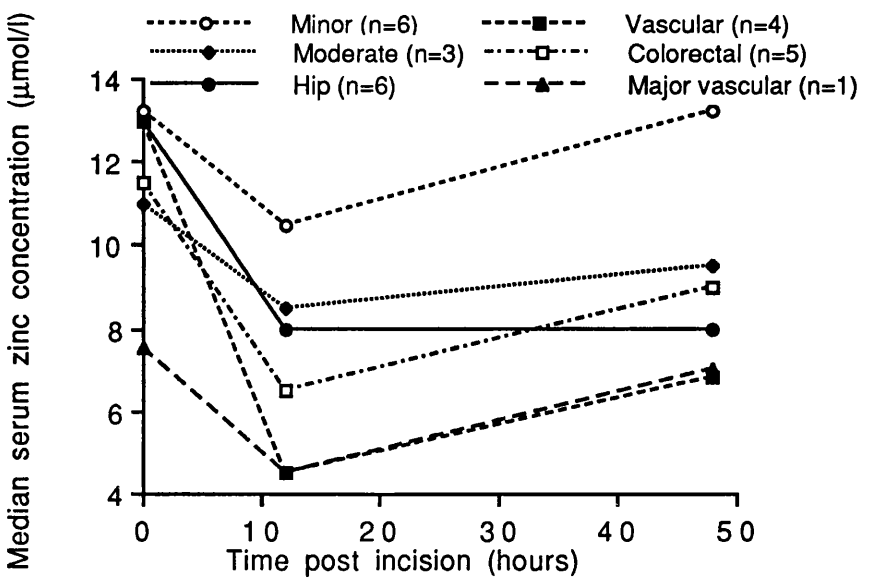


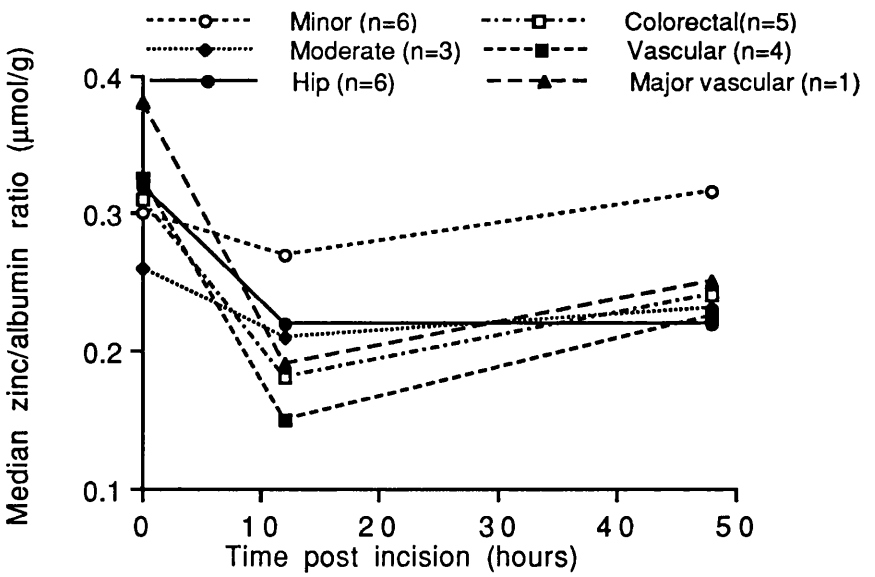
Figure 24 : The association between integrated IL-6 response and reduction in iron/transferrin ratio in the first 12 hours post-surgery in 22 surgical patients.

($r = -0.33$, $y = 4.9 - 0.003\,x$, $p = 0.128$)

a.



b.



Figures 25a,b : The changes in (a) serum zinc concentration and (b) zinc/albumin ratio following surgery in 6 surgical categories.

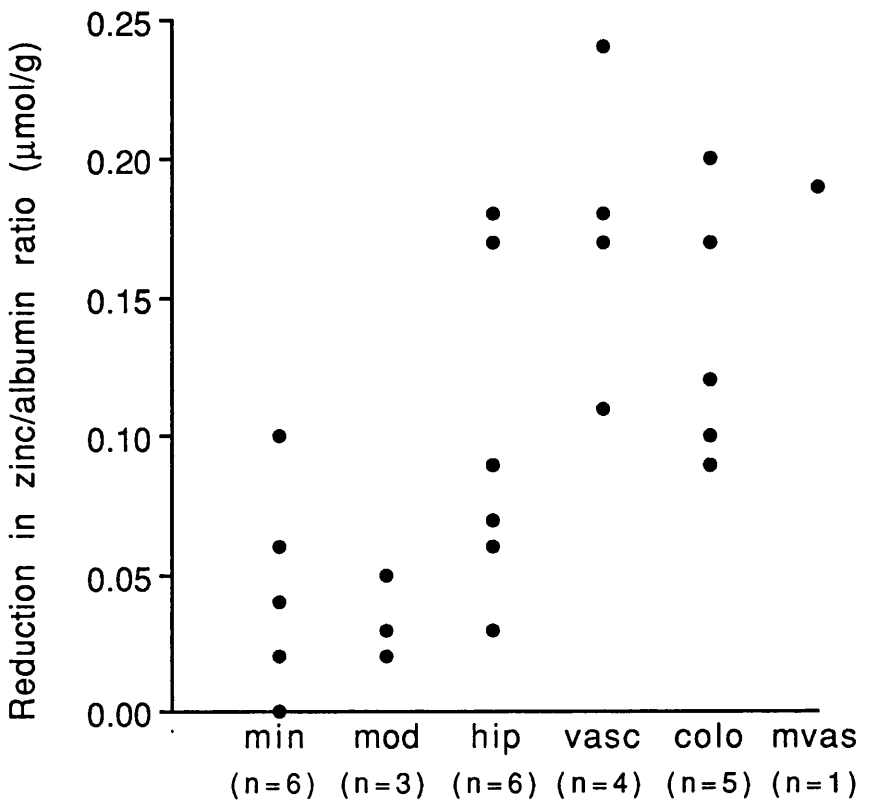
Surgical Category	Reduction in zinc/albumin ratio in first 12 hours post-operation (μmol/g)
Minor (n = 6)	0.03 (0.0 - 0.1)[a]
Moderate (n = 3)	0.03 (0.02 - 0.05)[b]
Hip (n = 6)	0.08 (0.03 - 0.18)
Vascular (n = 4)	0.18 (0.11 - 0.24)[c]
Colorectal (n = 5)	0.12 (0.09 - 0.20)[d]
Major Vascular (n = 1)	0.19

a<c, W = 21.0, p = 0.014, 95% CI = (-0.22, -0.07)
a<d, W = 22.5, p = 0.017, 95% CI = (-0.17, -0.03)
b<d, W = 6.0, p = 0.037, 95% CI = (-0.18, -0.04)

(all differences tested using Mann Whitney test)

Table 12: Reduction in zinc/albumin ratio in the first 12 hours post-operation in 6 surgical categories.

Results are expressed as median (range).



Key: min = minor; mod = moderate; vasc = vascular;
colo = colorectal; mvas = major vascular

Figure 26 : Comparison of the magnitude of the reduction in zinc/albumin ratio in the first 12 hours post-operation among 6 surgical categories.
(2 patients in the minor group showed no reduction in zinc/albumin ratio)

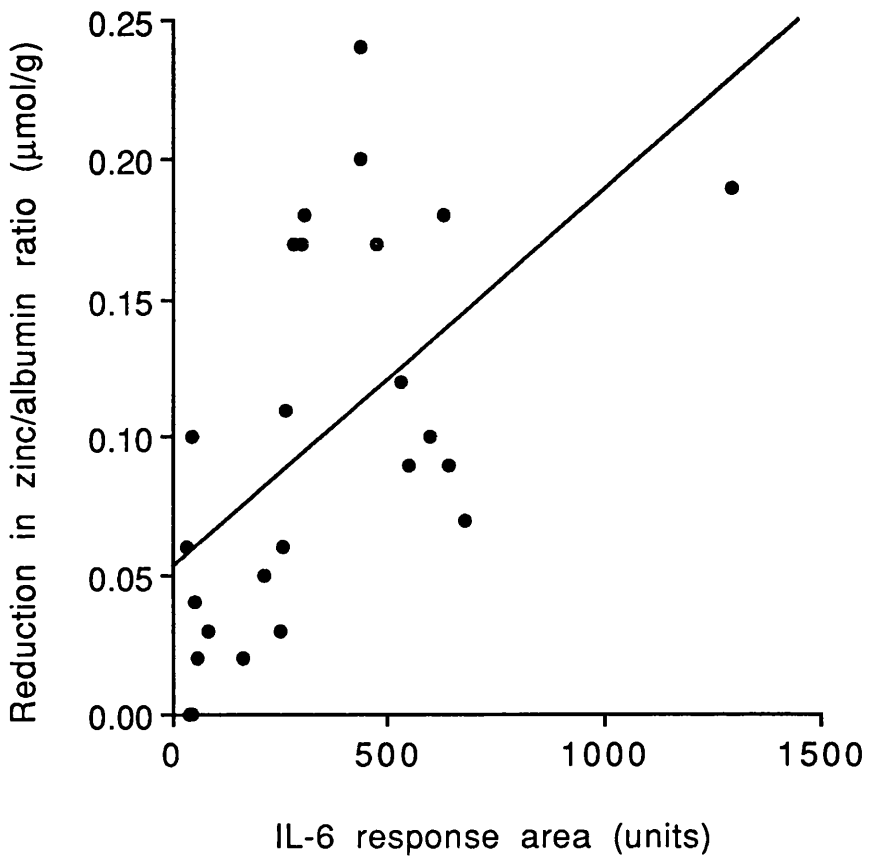


Figure 27 : The association between integrated IL-6 response and reduction in zinc/albumin ratio in the first 12 hours post-surgery in 25 surgical patients.

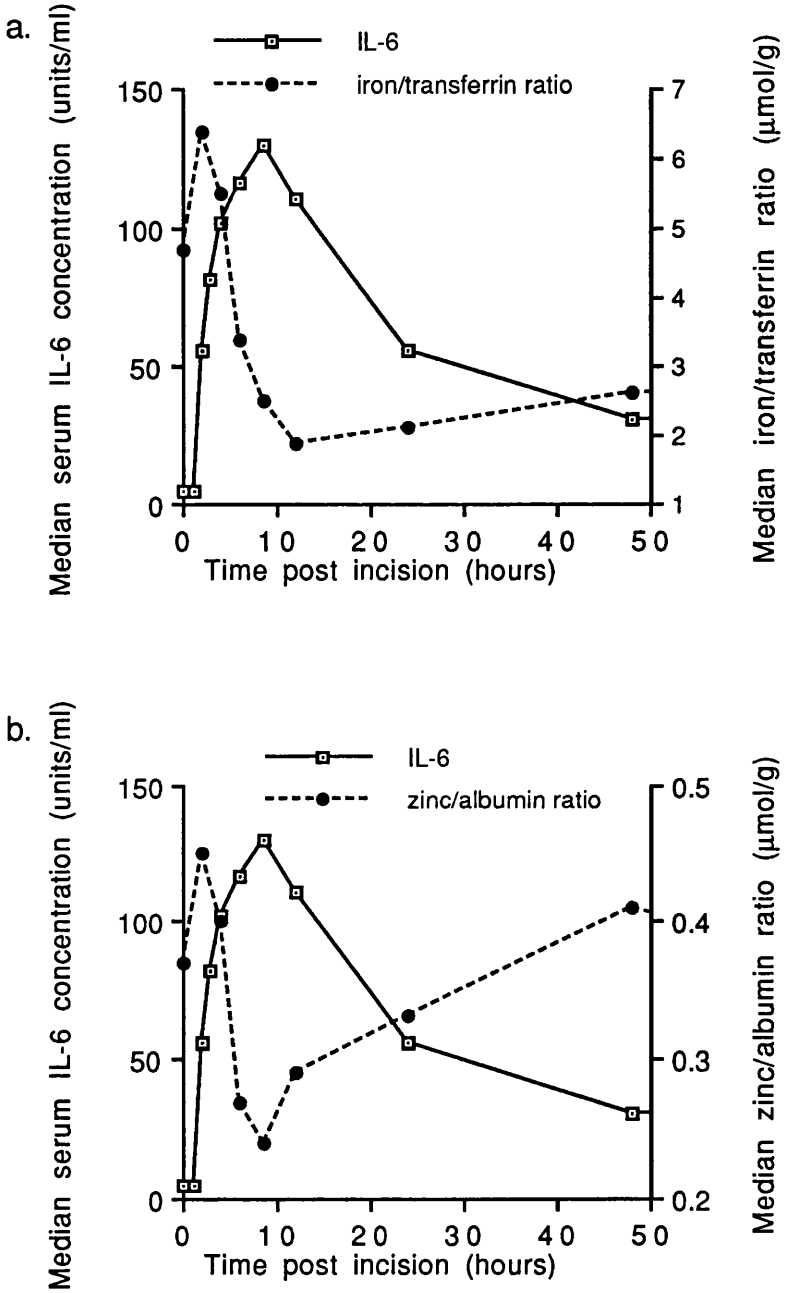
($r = 0.56$, $y = 0.05 + 0.001\ x$, $p = 0.003$)

correlation between integrated serum IL-6 response and reduction in zinc/albumin ratio ($r = 0.56$, $y = 0.05 + 0.001x$, $t = 3.26$, $p = 0.003$).

In figures 28a and b, the timecourse of the IL-6 response in our 5 cholecystectomy patients is compared with that of the changes in iron/transferrin and zinc/albumin ratios in 9 cholecystectomy patients studied previously (140). Iron/transferrin ratio reaches its nadir approximately 12 hours post-incision (3 hours after peak IL-6 levels) but the ratio remains low despite a fall in circulating IL-6 levels. Peak serum IL-6 concentrations occur at approximately the same time as the trough of zinc/albumin ratio (9 hours post-incision), and there is a strikingly close inverse relationship between these two variables.

SUMMARY

Serum IL-1 and TNFα levels remained undetectable in the 15 patients in whom they were measured. In contrast, serum IL-6 rose in all 28 patients and was strongly associated with duration of surgery. Serum CRP was less closely associated with duration of surgery. Serum IL-6 was significantly associated with serum CRP, with peak axillary temperature, and with reduction in zinc/albumin ratio. However, there was poor correlation between serum IL-6 and reduction in iron/transferrin ratio. Peak serum IL-6 levels occurred 20-40 hours before peak CRP levels, approximately 3 hours before peak temperature and at around the same time as lowest zinc/albumin ratio.



Figures 28a,b : The temporal relationship between serum IL-6 concentration and (a) iron/transferrin ratio, (b) zinc/albumin ratio.

(The IL-6 data are from the 5 cholecystectomy patients studied here; the metal data are from 9 cholecystectomy patients studied previously)

Clinical Value of IL-6

SURGICAL COMPLICATIONS

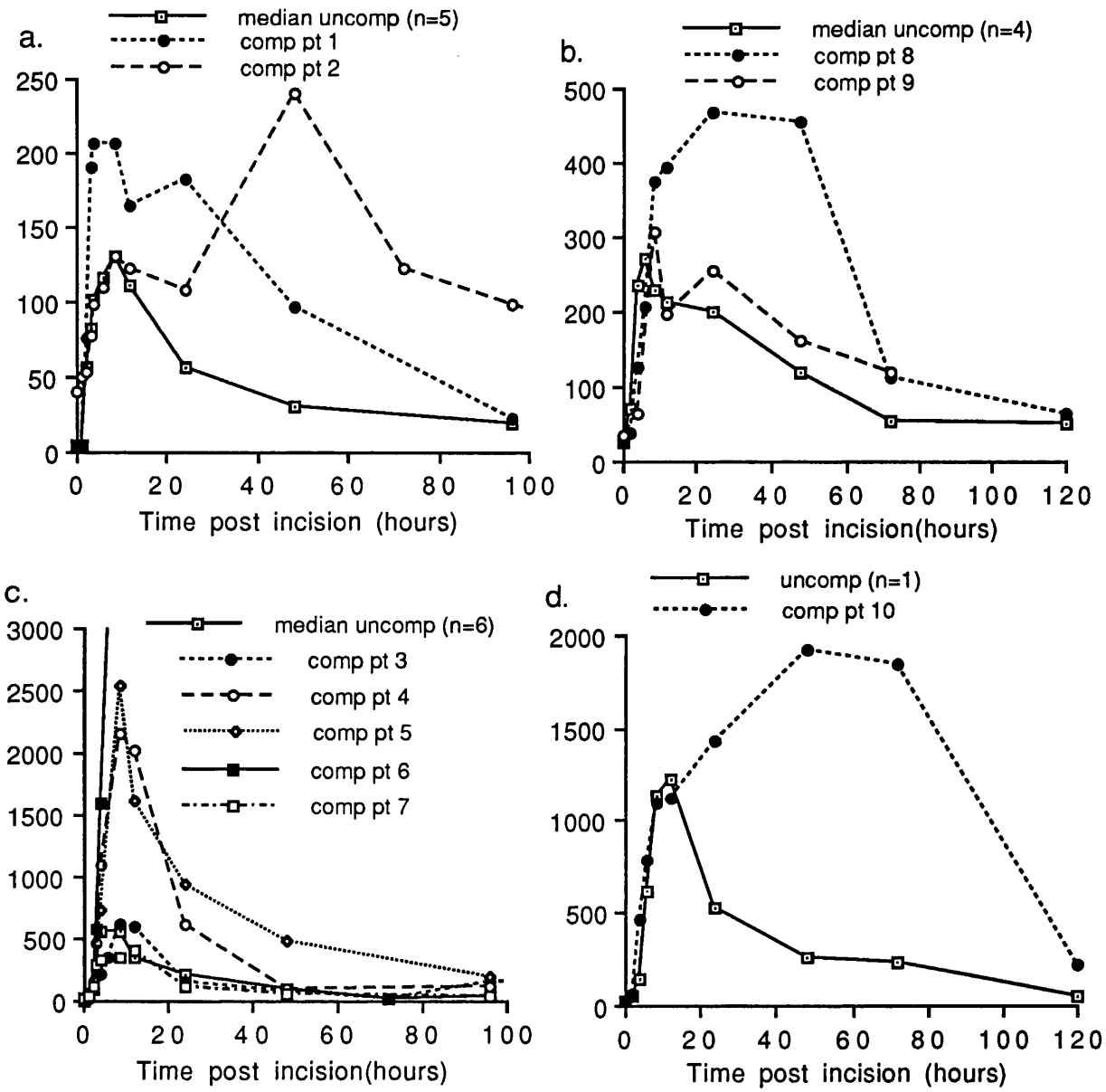
Ten of the 39 surgical patients studied developed complications post-operation (table 9). These 10 patients came from 4 of the 6 surgical categories and details of the complications are shown in table 13. Complications are divided into those which occurred within the first 4 post-operative days and those which developed on days 5 to 7.

The serum IL-6 responses in patients who developed complications are compared with the median response of those who did not in the same four surgical groups in figures 29a-d. The initial increase in all the patients with complications parallels that in the patients without complications. Patients without complications exhibit peak IL-6 levels at around 9 hours post-operation and 5 of the patients who developed complications had much higher IL-6 concentrations at 9 hours post-operation. By 24 hours, 8 of the 10 patients who developed complications had IL-6 levels which were higher than the median concentrations in the relevant groups. Interestingly, the 2 patients (patients 3 and 7, table 13) whose 24 hour IL-6 concentrations were similar to those in the patients without complications were the 2 patients in whom complications did not develop until the 6th and 7th day post-operation. One of these patients exhibited an increase in IL-6 96 hours post-operation (unfortunately, a 96 hour sample was not obtained from the other patient).

The corresponding data for CRP are presented in figures 30a-d. It is apparent that CRP discriminates less well between complicated and uncomplicated patients and that differences, if they occur at all, do so only 48 hours post-operation.

Surgical Category	Patient	Nature of Complication		Outcome at 6 weeks
		First 4 days post-surgery	Days 5 to 7 post-surgery	
Moderate	1	Unexplained pyrexia 48 hours post-op.		Alive
	2	Pyrexia 60 hours post-op. Chest infection		Alive
Colorectal	3		Pyrexia day 7. DVT and PTE	Alive
	4	Unexplained pyrexia 12 hours post-op.		Alive
	5	Dyspnoea 48 hours post-op but apyrexial. Pleural effusion		Dead
	6	Pyrexia 36 hours post-op. Septic shock		Dead
	7		Pyrexia and abdominal pain day 6. Bowel infarction.	Alive
Vascular	8	Unexplained pyrexia 48 hours post-op.		Alive
	9	Wound infection 48 hours post-op.		Alive
Major vascular	10	Pyrexia and hypoxia 24 hours post-op. Bronchopneumonia		Alive

Table 13: Clinical details of the 10 patients who developed complications post-operation.

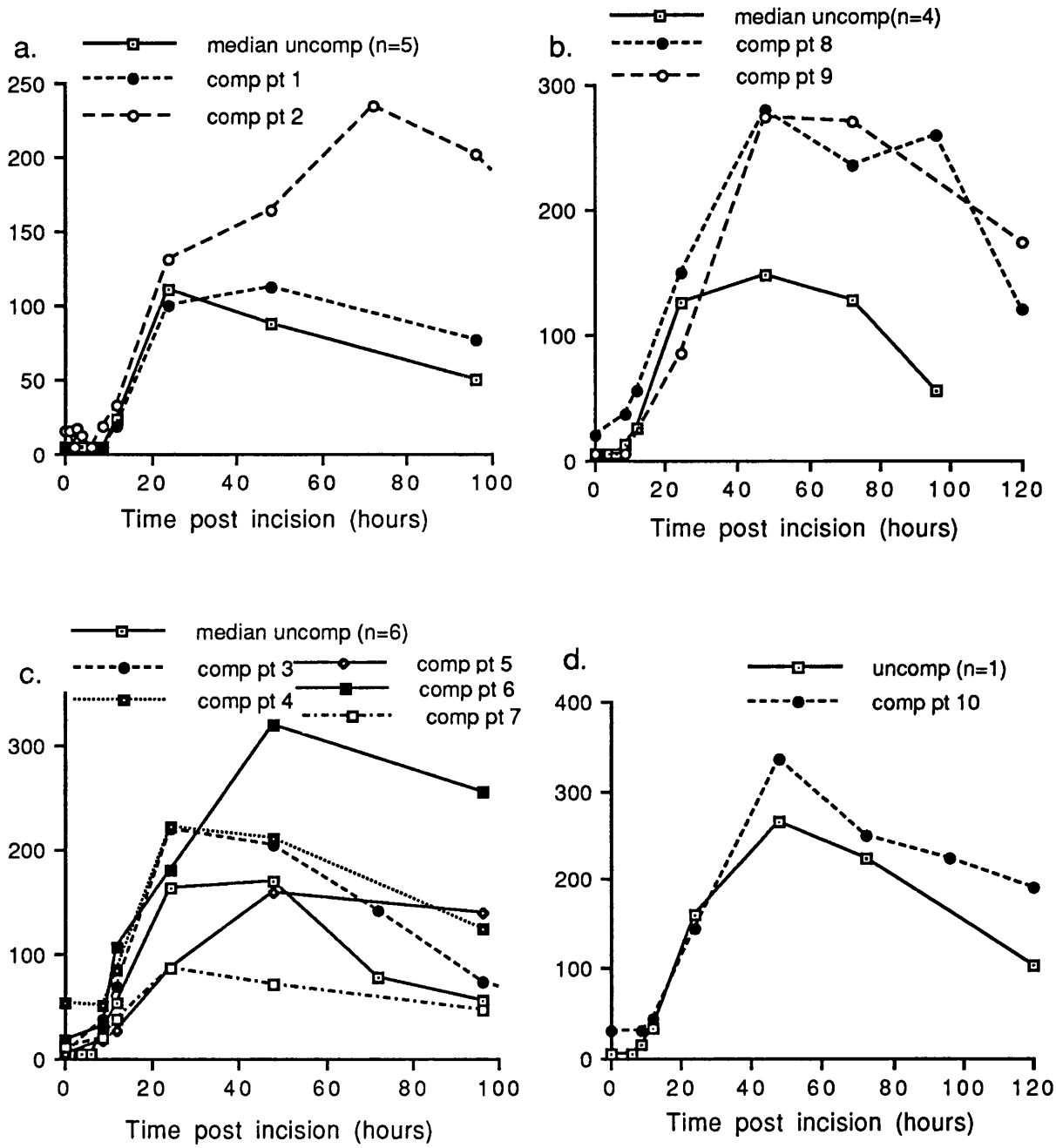


Key: uncomp = patients without complications; comp pt = patient with complications (table 13)

Figures 29a-d : Comparison of the serum IL-6 response in patients who developed complications with the median serum IL-6 response in uncomplicated patients from the same surgical category:
a = moderate; b = vascular; c = colorectal; d = major vascular.

(the vertical axis represents serum IL-6 concentration (units/ml))

(Serum IL-6 levels in complicated patient 6 rose to >5000 units/ml and remained very high)



Key: uncomp = patients without complications; comp pt = patient with complications (table 13)

Figures 30a-d: Comparison of the serum CRP response in patients who developed complications with the median serum CRP response in uncomplicated patients from the same surgical category:
a = moderate; b = vascular; c = colorectal; d = major vascular

(the vertical axis represents serum CRP concentration (mg/l))

Table 14 compares the 24 hour IL-6 and CRP results in patients having early complications (i.e. within 4 days) with those in the patients without complications, along with details of the length of operation and length of hospital stay post-operation. There was no difference in the lengths of operation between patients with and without complications. Post-operative stay in the 8 patients with complications (median (range): 14 (5-26) days) was longer overall (Mann Whitney test, W = 196.0, p = 0.001, 95% CI = (2,14)) than in the 16 patients who made uncomplicated recoveries (8.5 (4-12) days). There was no difference in 24 hour CRP levels between patients with and without complications. However, all patients with early complications had 24 hour IL-6 concentrations which were higher than those of any of the patients undergoing similar surgery (i.e. those in the same surgical group) who did not develop complications. 24 hour IL-6 levels overall in the 8 patients who developed early complications (median (range): 543 (108->5000) units/ml) were significantly higher (Mann Whitney test, W = 141.0, p = 0.013, 95% CI = (52, 925)) than those overall in the 16 patients without complications in the same four surgical groups (181 (22-529) units/ml).

Surgical Group	Duration of surgery (minutes)	Serum IL-6 24 hours post-op. (units/ml)	Serum CRP 24 hours post-op. (mg/l)	Duration of hospital stay post-op (days)	Outcome at 6 weeks post-op.
Moderate					
Uncomp (n = 5)	75(45-135)	56(22-84)	111(41-133)	5(4-5)	All alive
Comp Pt 1	45	183	100	5	Alive
Comp Pt 2	55	108	131	7	Alive
Colorectal					
Uncomp (n = 6)	110(75-270)	207(141-397)	163(103-259)	8.5(7-12)	All alive
Comp Pt 4	140	619	223	24	Alive
Comp Pt 5	125	947	85	26*	Dead
Comp Pt 6	120	>5000	180	17*	Dead
Vascular					
Uncomp (n = 4)	85(75-160)	200(148-244)	126(60-175)	10	All alive
Comp Pt 8	85	467	150	10	Alive
Comp Pt 9	135	256	86	12	Alive
Major Vascular					
Uncomp (n = 1)	225	529	160	11	Alive
Comp Pt 10	240	1434	145	16	Alive

Key: Uncomp = patients without complications (results expressed as median (range)).
Comp Pt = patient with complications

*indicates patient died on that day

Table 14: Comparison of duration of surgery, serum IL-6 and CRP concentrations 24 hours post-operation, and duration of post-operative hospital stay between patients who had uncomplicated post-operative courses and those in the same surgical category who developed complications within the first 4 days post-surgery. (Patients 3 and 7 have been excluded since clinical complications did not occur until after the fourth post-op day).

SUSPECTED MYOCARDIAL INFARCTION

Clinical details for the 15 patients presenting with chest pain are shown in table 15. Five patients were diagnosed as having unstable angina, and ten as having myocardial infarction. The time interval from onset of symptoms to admission to hospital did not differ significantly between the unstable angina pectoris group and the myocardial infarction group. Three of the patients who had sustained infarctions were dead at 6 weeks post-infarction.

The scatter of admission IL-6 concentrations in the 2 groups is shown in figure 31. Admission IL-6 concentrations were higher (Mann Whitney test, $W = 105.0$, $p = 0.003$, 95% CI = (19, 227)) in the infarct patients (median (range) 120 (77-295) units/ml)) than in the angina group (62 (29-72) units/ml). More important clinically, admission IL-6 discriminated completely between those patients with angina pectoris and those who had an acute infarct. In other words, using 75 units/ml as a cut-off, the sensitivity and specificity were 100% albeit on small numbers. Admission CRP concentrations did not differ between the 2 groups (figure 32). Although all patients with angina had undetectable CRP levels, 6 patients with infarcts also had undetectable levels. Some infarct patients had very high CK levels on admission, but overall these levels did not differ significantly between myocardial infarction patients and angina patients (figure 33).

Consequently, admission IL-6 was the best discriminant between those who had and those who had not sustained myocardial infarctions.

Figures 34a-c show the association between LVEF on the third hospital day and admission serum IL-6, CRP and CK concentrations respectively. A good negative correlation exists ($r = -0.87$, $y = 364 - 5.2x$, $t = -6.38$, $p < 0.001$, 95% CI = (3.5, 7.0)) between admission IL-6 and LVEF at 3 days. The correlation for admission CRP is poorer ($r = -0.60$, $y = 56 - 0.9x$, $t = -2.68$, $p = 0.019$, 95% CI = (-1.6, -0.2))

Patient	Diagnosis	Delay between onset of symptoms and admission (hours)	Outcome at 6 weeks
1	Angina	8	Alive
2	Angina	2	Alive
3	Angina	6	Alive
4	Angina	4	Alive
5	Angina	12	Alive
6	MI	8	Alive
7	MI	12	Dead
8	MI	16	Alive
9	MI	2	Alive
10	MI	4	Alive
11	MI	9	Alive
12	MI	21	Alive
13	MI	18	Alive
14	MI	11	Dead
15	MI	24	Dead

Key: MI = myocardial infarction

Table 15: Clinical details in 15 patients presenting with suspected myocardial infarction.

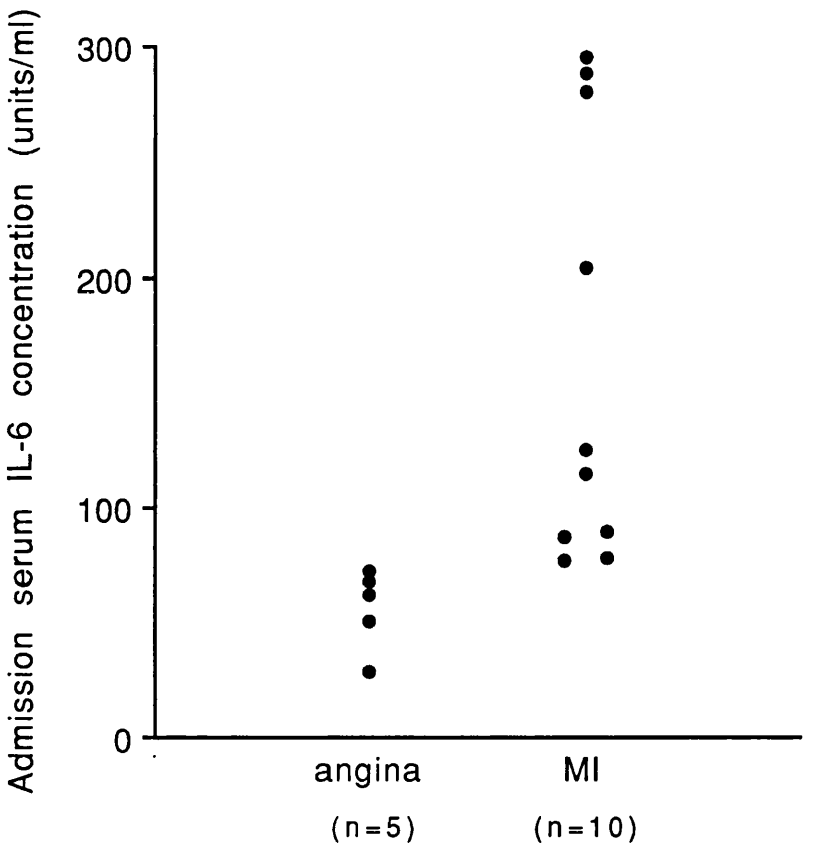


Figure 31 : Comparison of serum IL-6 concentrations on admission between patients with angina and those with myocardial infarction.

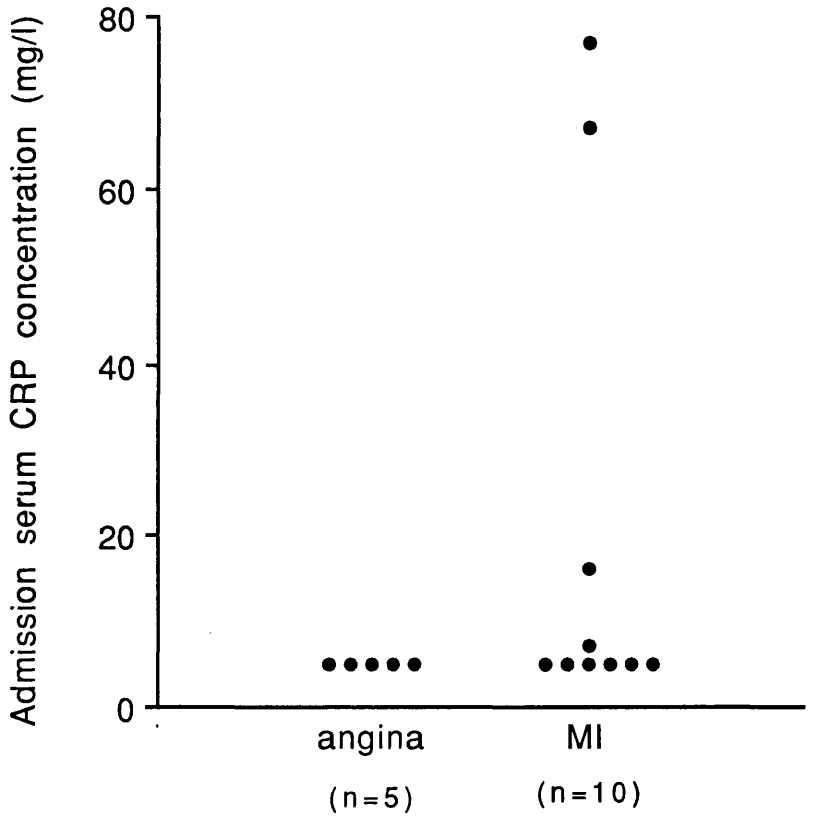


Figure 32 :Comparison of serum CRP concentration on admission between patients with angina and those with myocardial infarction.

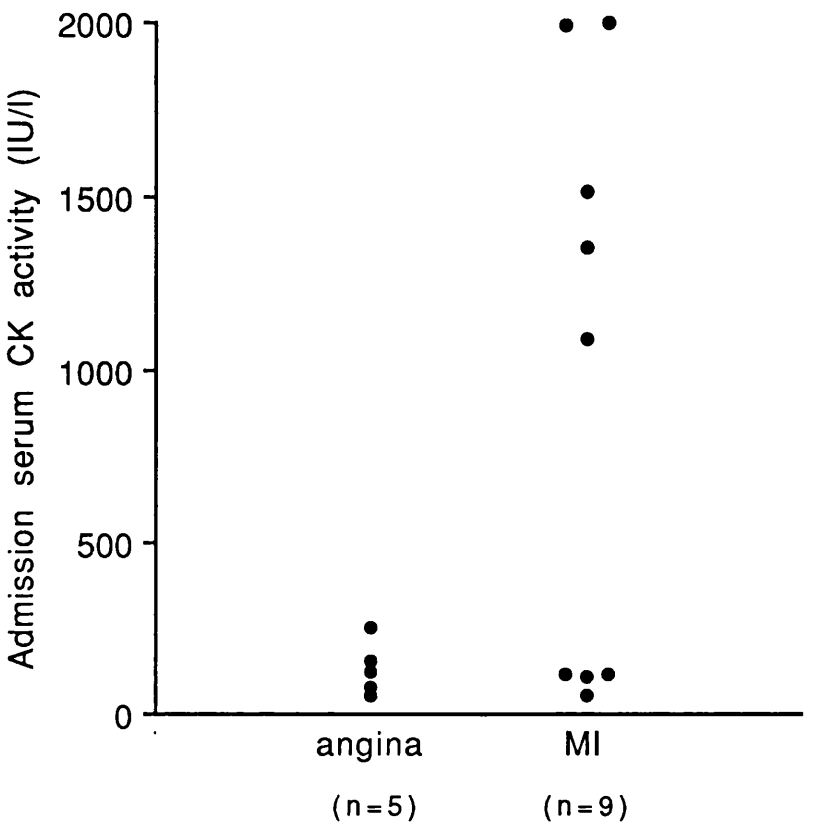


Figure 33 : Comparison of serum CK activity on admission between patients with angina and those with myocardial infarction.

(One myocardial infarction patient had a CK of 4200 IU/l which has not been shown)

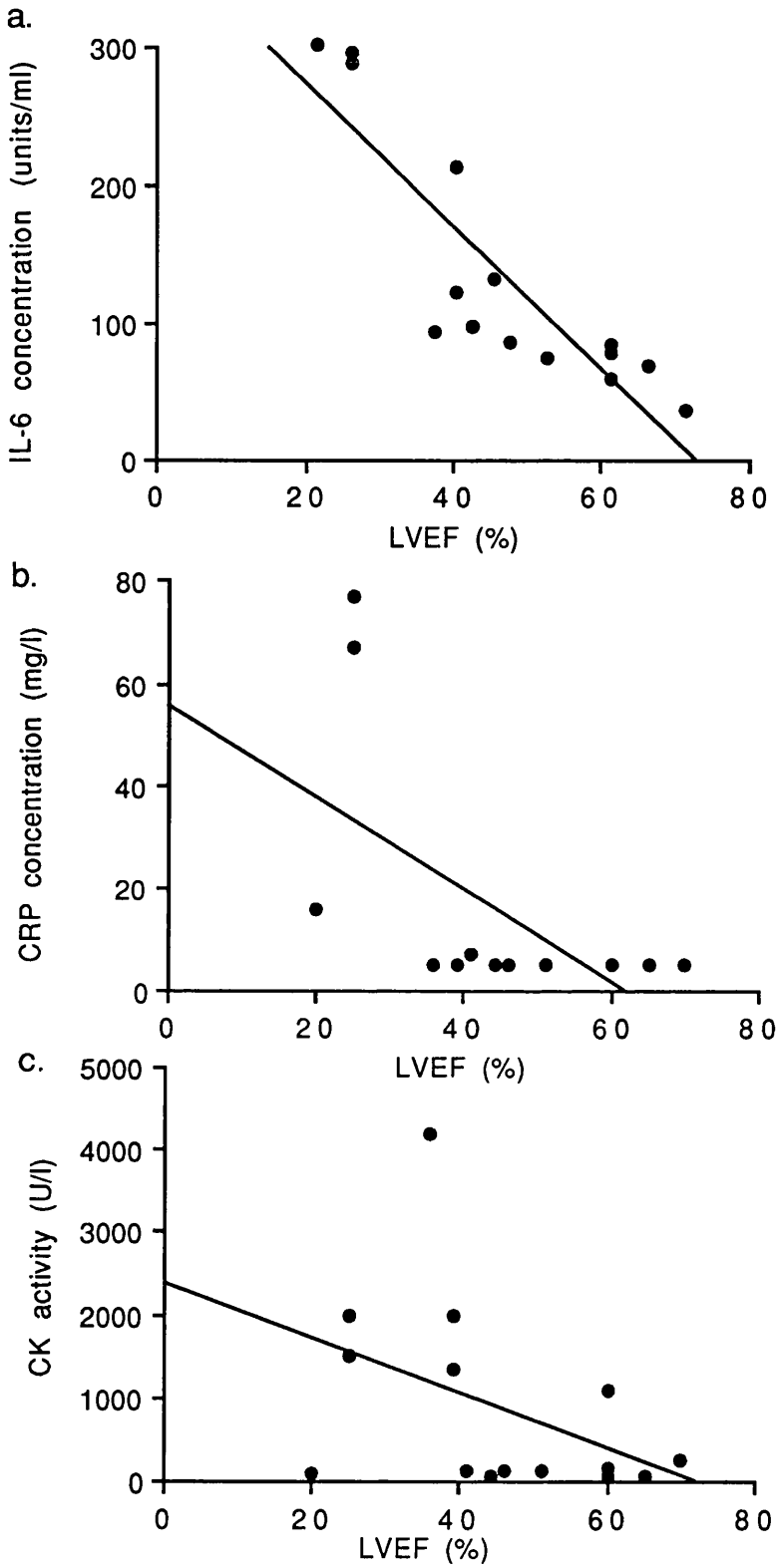


Figure 34a-c : The associations between LVEF on the third hospital day and: (a) admission serum IL-6 concentration ($r = -0.87$, $y = 364 - 5.2\,x$, $p < 0.001$); (b) admission serum CRP concentration ($r = -0.60$, $y = 56 - 0.9\,x$, $p = 0.019$; (c) admission serum CK activity ($r = -0.43$, $y = 2363 - 32.7\,x$, $p = 0.112$) in 15 patients with suspected myocardial infarction.

and that for admission CK is poorer still ($r = -0.43$, $y = 2363 - 32.7x$, $t = -1.70$, $p = 0.112$)), although peak CK levels had a correlation coefficient of -0.67 (data not shown). These results suggest that IL-6 concentration on admission may be a useful predictor of residual left ventricular function. Moreover, the 2 patients with the highest IL-6 levels on admission died although one of these patients also had the highest CK level (and an undetectable CRP), and the third patient who died did not have a particularly high admission IL-6. Nevertheless IL-6 was found to discriminate between myocardial infarction and angina, and to be closely associated with 3-day LVEF.

ACUTE PANCREATITIS

Of the 23 patients with pancreatitis, 10 patients were classified retrospectively as having severe attacks and 13 as having mild attacks. The clinical details of these patients are presented in tables 16a and b.

There were no significant differences between the 2 groups in serum amylase concentration on admission, or in the time interval between the onset of symptoms and admission. Not surprisingly, patients with severe attacks had significantly longer (Mann Whitney Test, W = 176.0, p = 0.006, 95% CI = (6,17)) hospital stays (median (range): 19 (7-27) days) than those with mild disease (8 (1-12) days). Patients with severe attacks had higher (Mann Whitney test, W = 163.5, p = 0.004, 95% CI = (0,3.0)) Glasgow scores (median (range): 2.5 (0-5)) than those with mild (0 (0-3)) (tables 16a and b) but 5 of the 10 severe patients had Glasgow scores less than 3. The diagnostic efficiency of this score was 74% (table 17).

The median serum IL-6 responses in the 2 groups are shown in figure 35. The pattern of the IL-6 response post-admission for each patient obviously depends on the time between onset of symptoms and admission. However, since there was no overall difference in this time between the 2 groups, then it is valid to compare the 2 responses. Peak IL-6 levels occurred approximately 24 hours post-admission in the severe group, and within 20 hours of admission in the mild group. Admission IL-6 concentrations and peak IL-6 concentrations appear higher in severe pancreatitis patients and are shown in more detail in figures 36 and 37. (The highest IL-6 concentration in the severe group (1216 units/ml) has been omitted from figure 36). Admission and peak IL-6 concentrations were significantly higher (Mann Whitney test W = 176.0, p <0.001 for both, 95% CI = (56, 199) and (142, 458) respectively) in the severe group (median (range): 135 (67-1216) units/ml and 370 (163-1408) units/ml respectively) than in the mild group (36 (<14 - 177) units/ml and 84 (25-237) units/ml respectively).

Patient	Delay between onset of symptoms and admission (hours)	Admission Amylase (IU/l)	Glasgow Score	Hospital stay (days)	Complication
1	12	851	0	12	None
2	6	1076	0	8	None
3	58	730	0	6	None
4	4	2298	0	8	None
5	34	1436	1	7	None
6	13	5660	0	5	None
7	12	5040	0	10	None
8	20	2700	3	1	None
9	6	841	0	5	None
10	6	5250	0	12	None
11	8	1283	1	12	None
12	1	4520	0	8	None
13	9	1263	0	6	None

Table 16a: Clinical details of 13 patients with mild acute pancreatitis.

Patient	Delay between onset of symptoms and admission (hours)	Admission Amylase (IU/l)	Glasgow Score	Hospital Stay (days)	Complications
14	15	1975	5	25	Respiratory failure
15	22	3890	0	14	Pancreatic pseudocyst
16	7	4720	2	12	Pancreatic collection
17	72	1276	3	18	Pancreatic collection
18	15	3110	5	27	Slow recovery
19	72	3240	3	24	Slow recovery
20	24	2199	3	24	Slow recovery
21	12	3870	2	20	Pancreatic pseudocyst Respiratory and renal failure
22	10	1608	1	15	Pancreatic pseudocyst
23	12	1740	0	7	Pancreatic pseudocyst

Table 16b: Clinical details of 10 patients with severe acute pancreatitis.

	TEST			
	Admission Serum IL-6 Concentration ≥70 units/ml	**Peak Serum IL-6 Concentration ≥ 200 units/ml**	**Peak Serum CRP Concentration ≥ 150 mg/l**	**Glasgow Score ≥3**
Sensitivity	90%	90%	90%	50%
Specificity	85%	77%	85%	92%
PPV	82%	75%	82%	83%
NPV	92%	91%	92%	71%
Efficiency	87%	83%	87%	74%

Key: PPV = positive predictive value; NPV = negative predictive value.

Table 17: Performance characteristics of 4 indicators of severe acute pancreatitis.

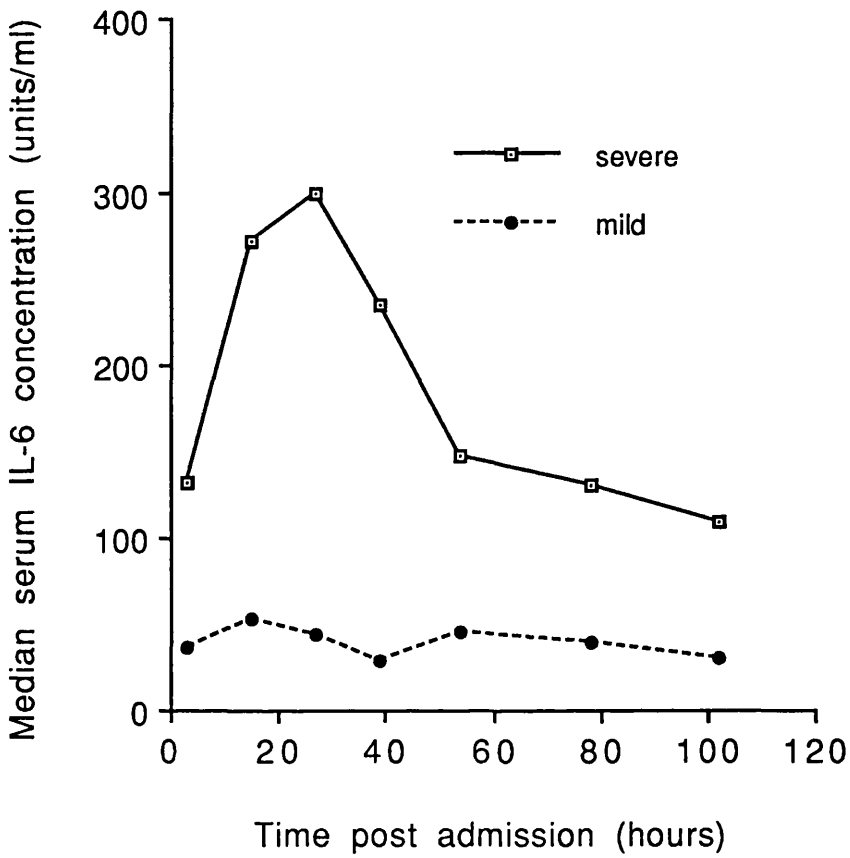


Figure 35 : Serum IL-6 response following admission in patients with mild and patients with severe pancreatitis.

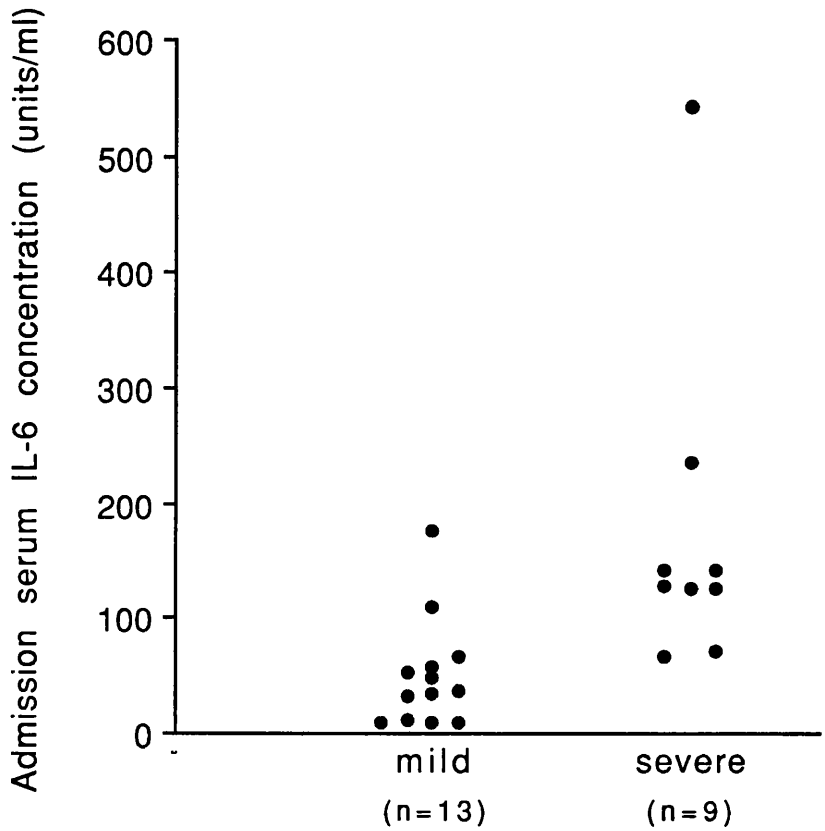


Figure 36 :Comparison of serum IL-6 concentration on admission between patients with mild and patients with severe pancreatitis.

(one patient with severe pancreatitis had a serum IL-6 concentration on admission of 1216 units/ml which has not been shown)

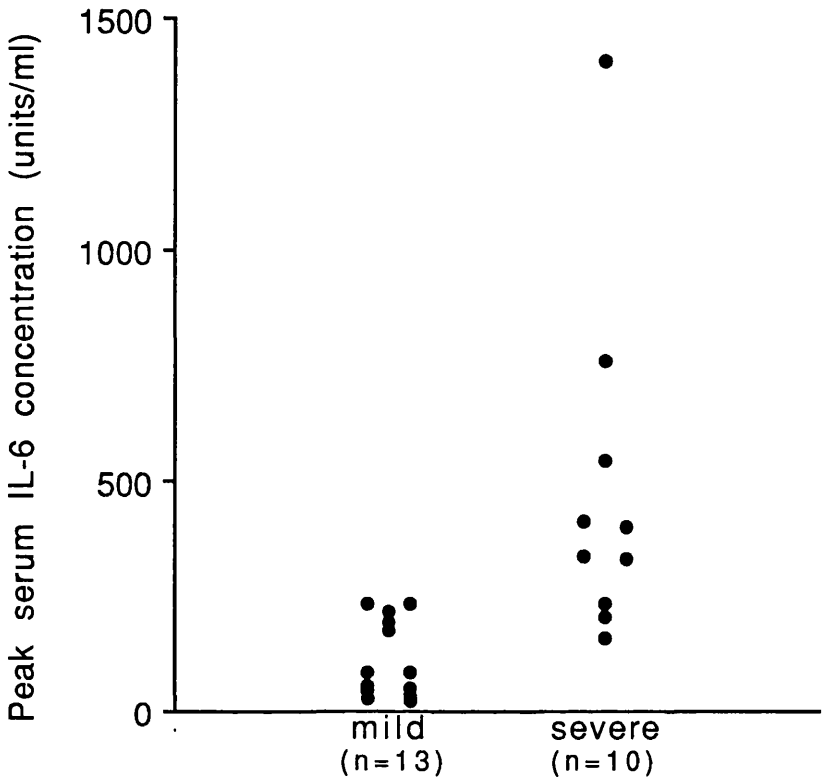


Figure 37 : Comparison of peak serum IL-6 concentration between patients with mild and patients with severe pancreatitis.

More relevant clinically is the diagnostic reliability of serum IL-6 as a predictor of severe pancreatitis. The performance characteristics of admission serum IL-6 concentration and peak IL-6 concentration as tests to diagnose a severe attack of pancreatitis are shown in table17. The cutoff point used for admission IL-6 concentration is 70 units/ml and that for peak IL-6 concentration is 200 units/ml. The overall diagnostic efficiency of admission IL-6 is slightly better than that of peak IL-6. It also has the advantage of being available some 20 hours before peak IL-6 levels occur. Thus serum IL-6 concentrations around the time of admission may prove useful in selecting patients liable to develop complications once the diagnosis of acute pancreatitis has been made.

Peak CRP levels occur approximately 30 hours post-admission in the mild group and later, at approximately 40 hours post-admission, in the severe group (figure 38). In both groups, the peak IL-6 has preceded the peak CRP by 15-20 hours. Differences in serum CRP concentration between the groups do not become apparent until approximately 24 hours post-admission. By the time peak levels have been reached, this difference has become marked. Figures 39 and 40 show admission and peak CRP values in the 2 groups and confirm the lack of discrimination between the groups using admission CRP. However, peak CRP levels are significantly higher (Mann Whitney test, $W = 180.0$, $p < 0.001$, 95% CI = (87, 230)) in the severe pancreatitis patients (median (range): 239 (100-429) mg/l) than in the mild pancreatitis patients (68(16-175) mg/l). Using a cut-off of 150 mg/l, the diagnostic efficiency of this test was 87% (table 17). However, the major disadvantage of this test is that it can only be applied at least 24 hours after admission.

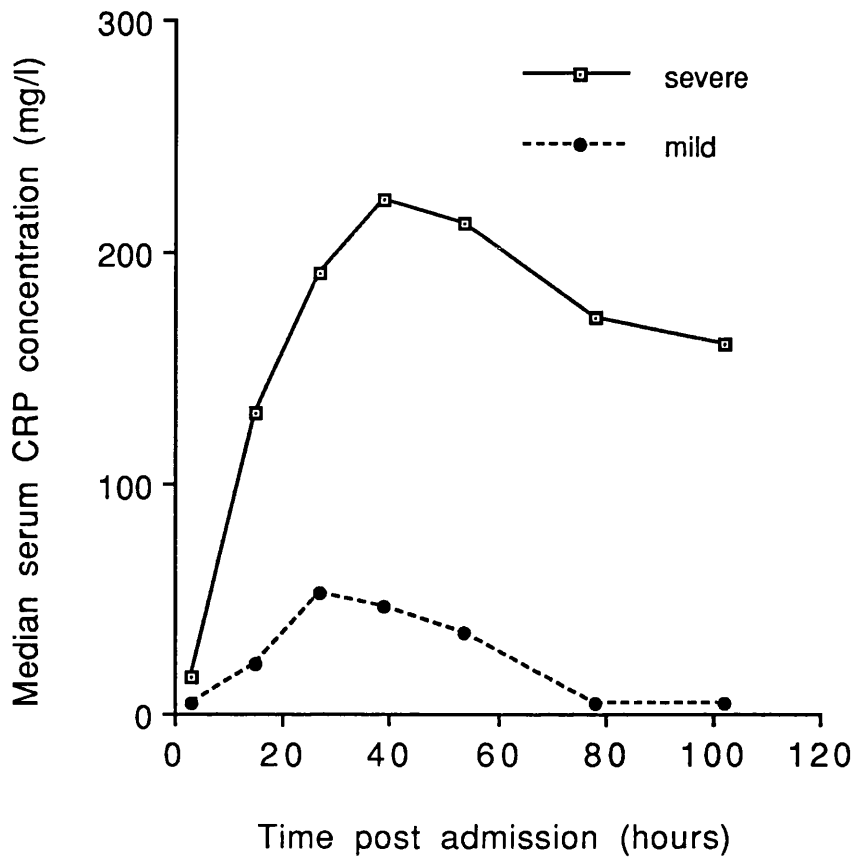


Figure 38 : Serum CRP response following admission in patients with mild and patients with severe pancreatitis.

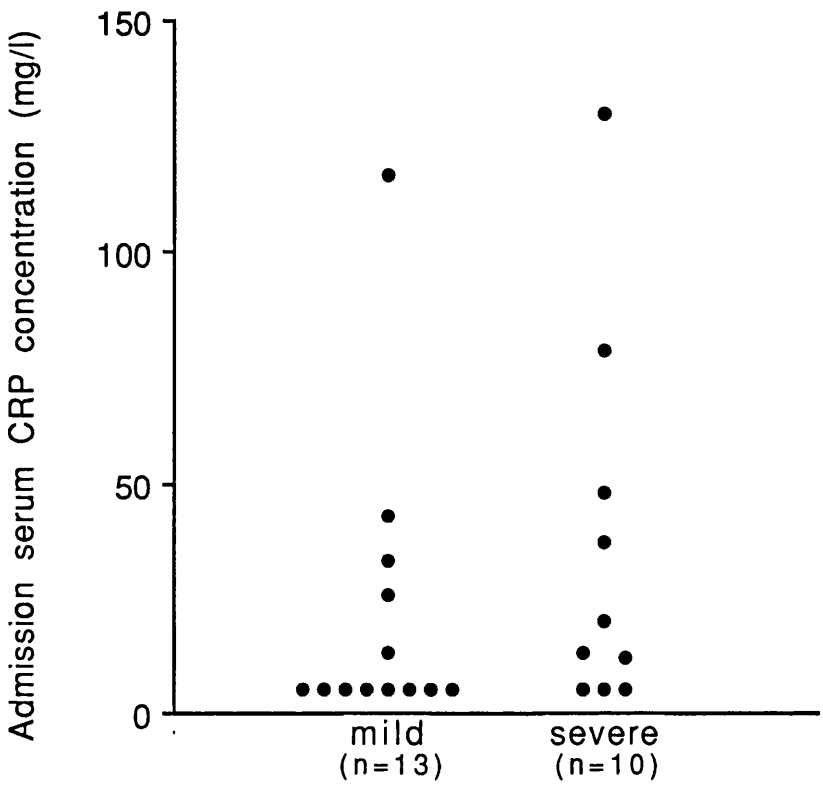


Figure 39 : Comparison of serum CRP concentration on admission between patients with mild and patients with severe pancreatitis.

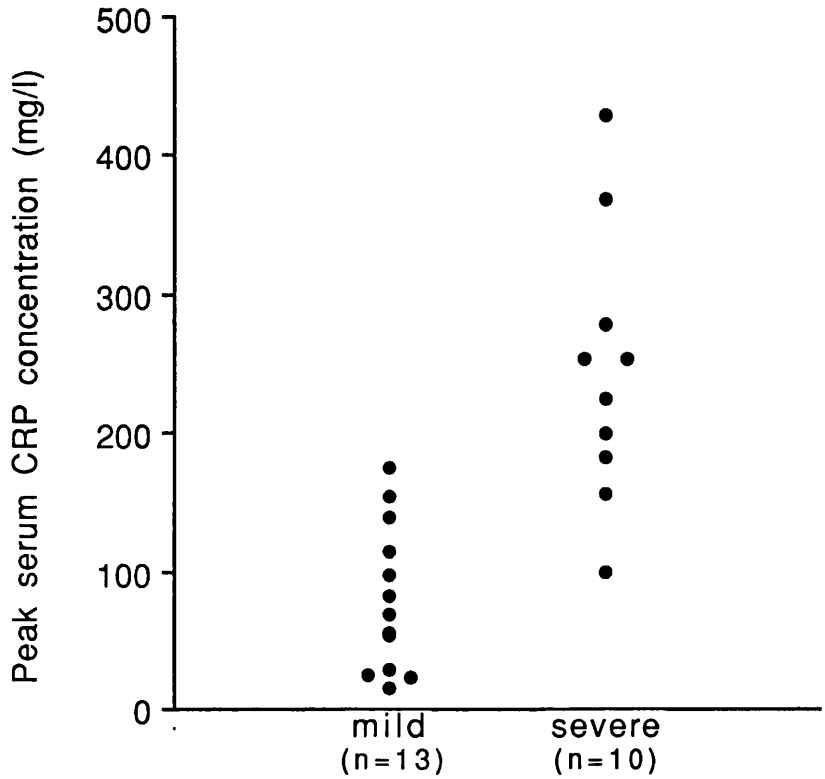


Figure 40 : Comparison of peak serum CRP concentration between patients with mild and patients with severe pancreatitis.

RHEUMATOID ARTHRITIS

Serum IL-6 concentrations were raised in 27 of the 33 patients with rheumatoid arthritis (figure 41). There was a weak but significant correlation between the Ritchie Articular Index and serum IL-6 concentration ($r = 0.35$, $y = 27.6 + 3.4\,x$, $t = 2.10$, $p = 0.044$, 95% CI = (0.1, 6.7)) (figure 42a). There was no association between Ritchie Articular Index and either CRP or ESR (figures 42b and c). Consequently, serum IL-6 appears to reflect disease activity more accurately than serum CRP or ESR.

Interestingly, the correlation between IL-6 and CRP in these patients with chronic disease ($r = 0.65$, $y = 19.3 + 0.3x$, $t = 4.55$, $p < 0.001$, 95% CI = (0.2, 0.4)) (figure 43) was similar to that found in the 28 surgical patients.

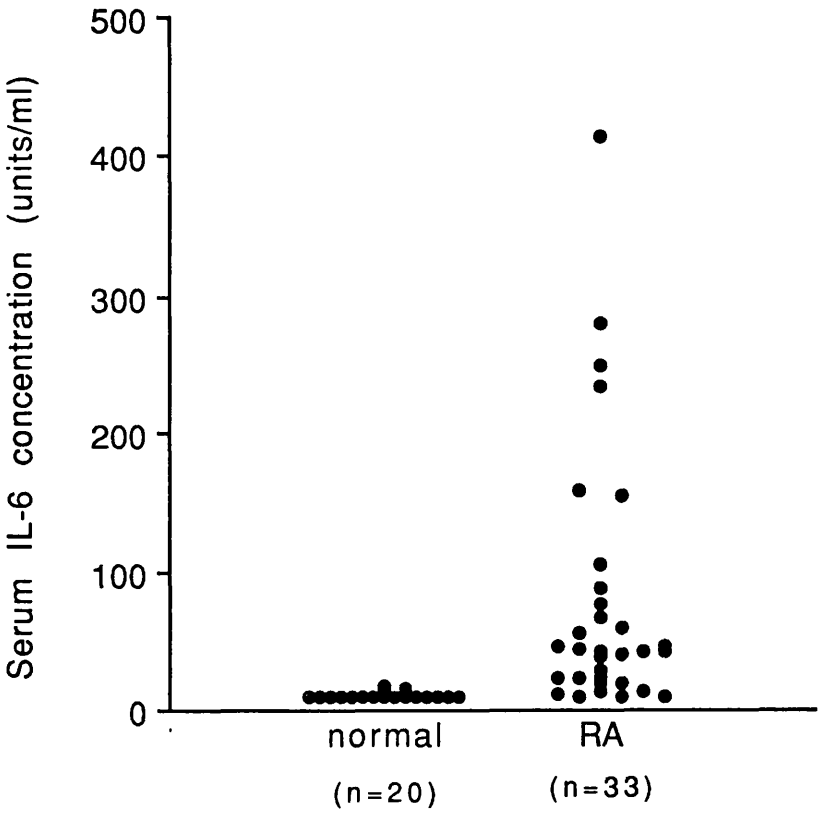
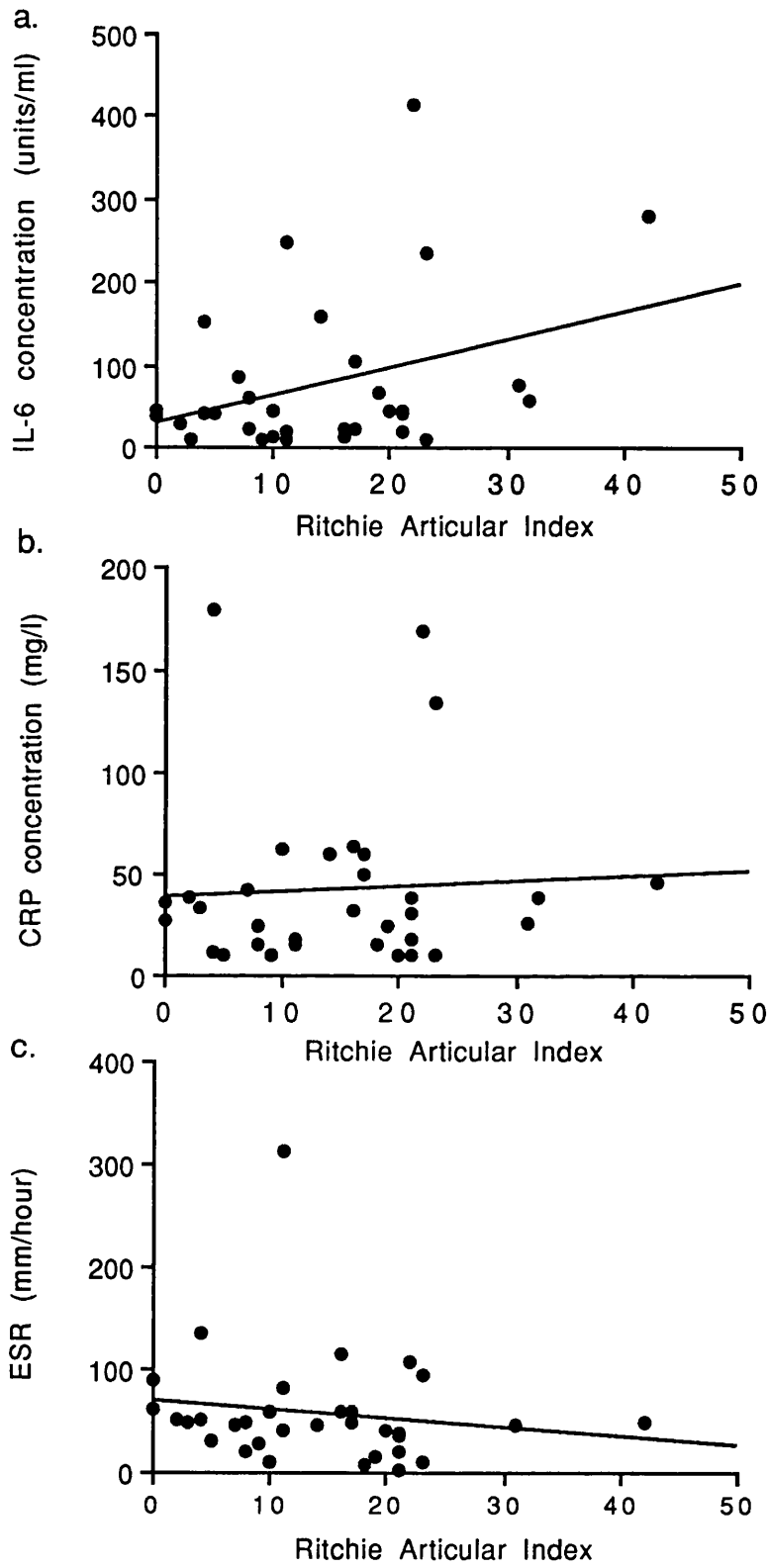


Figure 41 : Comparison of serum IL-6 concentration between healthy volunteers (normal) and patients with rheumatoid arthritis (RA).



Figures 42a-c : The associations between RitchieArticular Index and: (a) serum IL-6 concentration ($r = 0.35$, $y = 27.6 + 3.4\ x$, $p = 0.044$); (b) serum CRP concentration ($r = 0.06$, $y = 37.0 + 0.25\ x$, $p = 0.753$); (c) ESR ($r = 0.15$, $y = 69.6 - 0.83\ x$, $p = 0.411$) in 33 patients with rheumatoid arthritis.

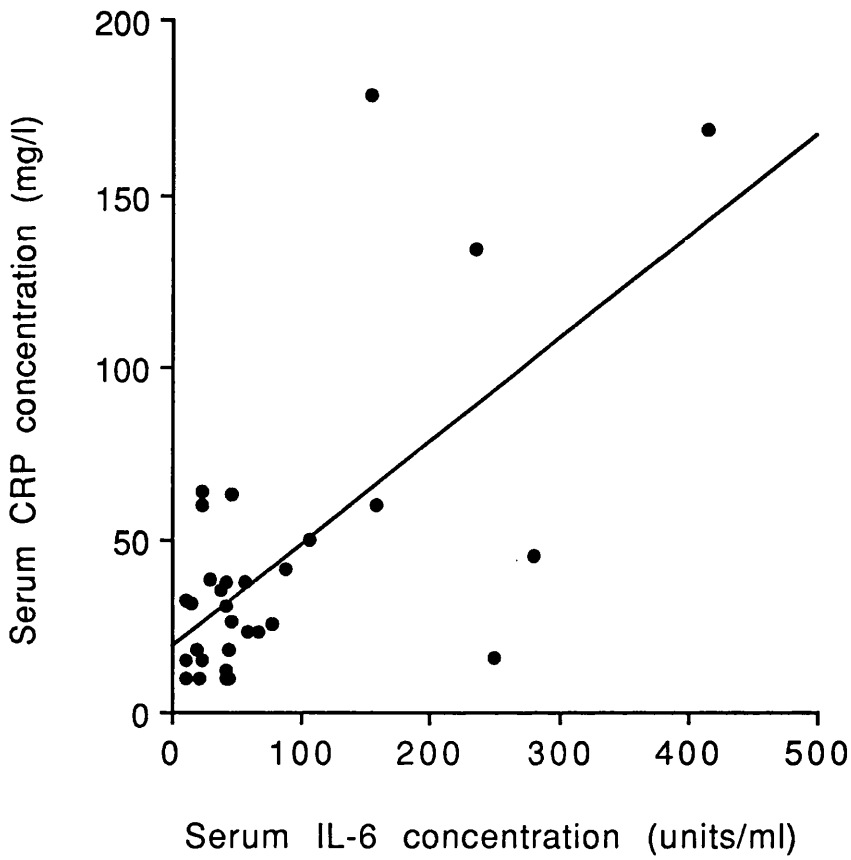


Figure 43 : The association between serum IL-6 and CRP concentrations in 33 patients with rheumatoid arthritis.
($r = 0.65$, $y = 19.3 + 0.3\,x$, $p < 0.001$)

SUMMARY

Serum IL-6 levels were higher 24 hours post-operation in surgical patients who went on to develop clinical complications than in those who did not. There was no difference in CRP levels at this time. Serum IL-6 concentrations discriminated well on admission between patients with angina and those with myocardial infarction, and between patients with mild and those with severe attacks of pancreatitis. Serum CRP on admission was of no value in these situations. Moreover, there was good correlation between admission IL-6 levels and LVEF on the third hospital day in patients with chest pain. There was poor though significant correlation between serum IL-6 and the Ritchie Articular Index in patients with rheumatoid arthritis, while no association was found between the Ritchie Articular Index and CRP or ESR.

DISCUSSION

IL-6 and the Acute Phase Response

IL-1 AND TNFα

Baigrie et al recently reported measurable serum concentrations of IL-1 but not TNFα in the first few hours following surgery in all of six patients studied who underwent elective aortic surgery (170). In spite of intensive sampling during the first few hours we failed to detect either IL-1ß or TNFα in any sample from the 15 patients studied, some of whom underwent major hip and colorectal surgery. The concentrations of IL-1 reported by Baigrie et al are very low, the maximum being approximately 30 pg/ml. It is likely that this discrepancy is due to differing sensitivities of the immunoassays used. The Cistron assay quoted a detection limit of 20 pg/ml whereas the Medgenix assay used by Baigrie et al quoted a detection limit of 4 pg/ml. Consequently, any increases in serum IL-1ß which occurred in our patients may have been too small for the Cistron immunoassay to detect. It is probable that surgery does provoke the release of large amounts of IL-1 locally, which may cause a small, short-lived increase in serum levels. In contrast, no increases in TNFα concentrations in serum were detected following surgery by either ourselves or Baigrie et al. The undetectable serum concentrations of IL-1ß and TNFα found in our patients make it impossible to establish any quantitative relationship between these cytokines and CRP response, changes in plasma metal concentration, or changes in body temperature. Our findings, and those of Baigrie et al are compatible with the hypothesis that neither of these cytokines act systemically themselves to produce the principal manifestations of the acute phase response, and that they play a more local role which includes stimulation of IL-6 production and release.

IL-6 RESPONSE TO SURGERY

The findings described here of increased serum concentrations of IL-6 in all patients post surgical incision make it likely that IL-6 does have an early and important role to play in the systemic inflammatory response. Detectable increases in serum IL-6 in all patients occurred within 2 to 4 hours of incision, in keeping with previous observations that in stimulated monocytes, IL-6 messenger RNA is detectable one hour post-stimulation becoming maximal after three hours (28). The timecourse of the IL-6 response following surgery recently reported by other workers parallels the time course seen in our patients (169,170,187). In general, the extent of the IL-6 response was related to the magnitude of tissue damage as reflected by the type of surgical procedure and the duration of the operation.

The possibility that duration of anaesthesia might have contributed to the response of IL-6 was investigated by studying the serum levels of IL-6 in the medical student (see Results Chapter, page 90) who underwent a particularly painstaking partial thyroidectomy. The peak IL-6 concentration was only 56 units/ml in spite of an operation which lasted 170 minutes, suggesting that duration of anaesthesia is not an important contributory factor.

Peak IL-6 levels were found to be representative of the integrated IL-6 response during the 48 hours post-incision. This implies that the serum IL-6 concentration 6 to 12 hours after an acute event should reflect the overall IL-6 response and so the magnitude of underlying tissue damage. It should be noted however, that the nature of the response may partly depend on the site of injury since extra-abdominal surgery (hip replacement) tended to produce a flatter, more drawn out IL-6 response than intra-abdominal surgery which involves bowel handling and which may be a potent stimulus to IL-6 production. The mechanism for this is unclear but our failure to detect endotoxin in sera of patients (unpublished data) suggests that it is not related to endotoxin release during bowel handling. These

results overall are promising, however, and indicate that IL-6 may be useful as an early, sensitive, quantitative marker of tissue damage.

IL-6 AND C-REACTIVE PROTEIN

The hepatocyte stimulating effect of IL-6 is thought to be mediated mainly at the level of transcription (117), and maximal CRP synthesis in vitro has been shown to occur 20-30 hours after hepatocytes have been stimulated with IL-6 (238). The relative time courses of in vivo IL-6 and CRP responses observed in the present study are in keeping with these in vitro findings, and are consistent with IL-6's being a major inducer of CRP synthesis. However, had this been the case, a stronger correlation between integrated IL-6 response and CRP response might have been expected, although, of course, this would not be proof of a causal role for IL-6 in CRP synthesis. To demonstrate conclusively that IL-6 induces either CRP synthesis or hypozincaemia, hypoferraemia or fever, one must show not only a good correlation between IL-6 levels and those other variables but also that injection of IL-6 into humans produces these effects at IL-6 concentrations comparable to those found here. To date, this has not been possible. Since a rather weak correlation between IL-6 and CRP was found, it may be postulated that IL-6 is only one of several inducers of hepatic acute phase protein synthesis which may interact synergistically. Support for this view is lent by the findings of Morrone et al (117) who found that in Hep 3B cells, recombinant IL-6 only partially activated the CRP gene relative to the degree of activation produced by activated monocyte supernatant. This suggested that factors other than IL-6 are present in monocyte supernatant which are required either alone or in combination with IL-6 to stimulate maximal CRP synthesis. Moreover, Baumann et al (239) have demonstrated that optimal Hep G2 acute phase protein synthesis required stimulation by a combination of IL-6, IL-1 and dexamethasone. Thus it appears unlikely that IL-6 is the only inducer of CRP synthesis. Another explanation is that IL-6 does not act in a dose-dependent manner although in vitro work does not

support this (116). Alternatively, the rather poor association between serum IL-6 and CRP may reflect a poor relationship between serum IL-6 concentration and the local concentration at the physiologically active site.

IL-6 AND BODY TEMPERATURE

The relationship between IL-6 and body temperature is similar to that described for IL-6 and CRP although the time difference between changes in serum IL-6 concentration and axillary temperature is much shorter. Nevertheless, again the temporal relationship supports IL-6's putative role as an endogenous pyrogen, and is consistent with Le May et al's report that following injection of dogs with LPS, peak plasma IL-6 concentration preceded peak rectal temperature by 2-3 hours (138), and again the correlation between peak IL-6 levels and peak body temperature is rather weak although highly significant. Considerations similar to those in the case of CRP apply - namely that IL-6 may not act in a dose-dependent manner or that systemic IL-6 levels may poorly reflect those which exert the pathophysiological effect at the hypothalamus. However, Le May et al (137) have reported that intraperitoneal injection of fever inducing doses of lipopolysaccharide in rats produced significant elevations of IL-6 concentrations in CSF and plasma, both of which showed good correlation with changes in body temperature ($r = 0.77$ and $r = 0.84$ respectively) suggesting that IL-6 does behave in a dose-dependent manner and that physiologically relevant CSF levels do mirror systemic concentrations.

We have been unable to investigate whether the serum concentrations of IL-6 observed in our patients are capable of inducing fever when produced experimentally, so we cannot state with certainty that IL-6 acts as endogenous pyrogen. Nevertheless, the most likely explanation for our findings is that IL-6 is indeed an endogenous pyrogen but does not act in isolation. Interestingly, Le May et al observed that very much higher doses of recombinant IL-6 administered intracerebroventricularly to rats and dogs were required to produce fever than were

predicted by the CSF IL-6 concentrations which occurred following lipopolysaccharide administration (137,138). These findings support the hypothesis that the fever-inducing effect of IL-6 depends on or is magnified by the presence of other factors in the pathophysiological situation.

IL-6 AND HYPOFERRAEMIA AND HYPOZINCAEMIA

As we found no apparent relationship between reduction in iron/transferrin ratio and severity of surgical procedure, it is not surprising that there was no significant association between serum IL-6 response and reduction in serum iron/transferrin ratio, given that IL-6 has been shown to reflect the severity of surgical procedure. Moreover, the serum iron/transferrin ratio remained low although serum IL-6 levels were returning to normal. These findings make it unlikely that IL-6 plays an important role in inducing hypoferraemia and suggest that mechanisms distinct from those that induce acute phase protein synthesis are involved. Since IL-6 appears to be a major inducer of acute phase protein synthesis, one might reasonably have expected the correlation between hypoferraemia and IL-6 to be similar to that for CRP and IL-6 (although to date there is no direct evidence that IL-6 stimulates ferritin synthesis). Consequently, hepatic ferritin synthesis may not be the major mechanism by which hypoferraemia is achieved. Alternatively, the lack of association may be because IL-6 is not involved in ferritin synthesis. IL-1 and TNFα have both been shown to produce hypoferraemia in vivo (127,154-156), and TNFα and IL-1 have been shown to stimulate ferritin heavy chain synthesis in murine and human muscle cell cultures (240,241) and human fibroblasts (242). It is possible, then, that IL-1 or TNFα may stimulate hepatic synthesis of ferritin directly but unlikely given that there are no reports of hepatic ferritin synthesis stimulated by IL-1 or TNFα and that IL-6 has been shown to be a much more potent stimulus to hepatocytes than either TNFα or IL-1 (112). The likeliest explanation for the lack of association is that mentioned above, namely

that IL-6 is probably involved in ferritin synthesis but that this is not the major mechanism by which hypoferraemia is achieved.

Our findings, then, tend to support the lactoferrin hypothesis advocated by Van Snick (153) and backed by the findings of Goldblum et al who showed that endotoxin administration to rabbits and rats produced hypoferraemia which was neutrophil dependent and which could be reproduced by infusion of lactoferrin (154). However, it should be noted that Borish et al recently demonstrated in vitro that IL-6 could stimulate neutrophils to produce lactoferrin (243). Nevertheless the pathophysiological significance of this finding is unclear, and our results suggest that IL-6 is not a major factor in the production of the hypoferraemia of the acute phase response whatever the mechanism.

The significant correlation observed between serum IL-6 response and reduction in zinc/albumin ratio is compatible with a role for IL-6 in lowering plasma zinc concentrations by stimulating hepatic metallothionein synthesis. While a stronger correlation might be thought necessary to support (but not prove) a causal relationship between these variables, considerations similar to those in the case of CRP are applicable, namely that IL-6 probably does not act in isolation to induce metallothionein, or that serum IL-6 concentrations may not be an accurate reflection of tissue concentrations. Certainly the degree of association between IL-6 and reduction in zinc/albumin ratio is reasonably similar to that between IL-6 and CRP responses, indicating that the mechanism which produces hypozincaemia (unlike that which produces hypoferraemia) may be similar to the mechanism producing hepatic synthesis of CRP albeit on a different timescale. Peak IL-6 levels occurred approximately 9 hours post-incision in our 5 cholecystectomy patients, and lowest zinc/albumin ratios occurred at approximately the same time post-incision in the 9 cholecystectomy patients studied previously. The striking inverse relationship between changes in serum IL-6 and zinc/albumin ratio observed in these 2 groups of patients provides strong support for a role for IL-6 in

the production of hypozincaemia. Moreover, the relative time courses of the IL-6 and zinc responses are in keeping with the report that rat hepatocytes exhibit increased levels of metallothionein protein within 3 hours of stimulation with IL-6 (163). Interestingly, too, the hip group in which IL-6 levels did not peak until after 12 hours post-incision was the group in which zinc levels were still low at 48 hours post-incision.

Several in vivo studies have shown that IL-1 and TNF produce hypozincaemia when injected into animals (128,244,245). In particular, Cousins et al reported that administration of IL-1 to rats caused increased metallothionein gene expression resulting in a 14-fold increase in liver metallothionein and a transient depression of plasma zinc concomitant with zinc uptake by liver, thymus and bone marrow (246), while Karin et al found that IL-1 increased metallothionein gene expression in human hepatoma cells (247). However, Schroeder and Cousin reported that IL-6, but not IL-1, stimulated metallothionein in rat hepatocytes in the presence of glucocorticoids (163) although these findings have not as yet been repeated with human hepatocytes. Thus like other acute phase proteins (112), metallothionein gene expression appears to be dependent on IL-6 and glucocorticoids - results in keeping with previous observations that glucocorticoids stimulate metallothionein synthesis in vivo and in vitro (158,159). The reasons for the discrepancy between the in vitro results of Karin and those of Schroeder and Cousin are unclear but may involve differences in responsiveness among different cell lines. Nevertheless, it seems most probable that the in vivo actions of IL-1 reported by Cousins et al are mediated via IL-6. IL-1 and TNF cause release of IL-6 which in turn stimulates glucocorticoid secretion which in conjunction with IL-6 induces metallothionein expression causing hypozincaemia. Our findings are compatible with this hypothesis.

Clinical Value of IL-6

SURGICAL COMPLICATIONS

The 8 surgical patients who developed complications within 4 days of their operation had higher IL-6 levels 24 hours post-operation overall than those who did not develop complications. Furthermore, each of these 8 patients had higher 24 hour IL-6 levels than any of the uncomplicated patients in the same surgical group although 6 of these 8 patients had no clinical evidence of complications at that time. These findings suggest that IL-6 has potential as a predictor of early complications. In contrast, at 24 hours post-surgery, CRP discriminated poorly between patients who went on to develop clinical complications and those who did not. The two patients who developed complications on the sixth and seventh post-operative days had serum IL-6 concentrations 24 hours post-operation which were comparable to those in uncomplicated patients. This is not surprising since one would not expect a rise in serum IL-6 levels to precede the development of clinically apparent problems by more than 48 hours. Consequently, serum IL-6 on the first post-operative day would be of no value in selecting patients at risk of developing relatively late complications.

Given that the data from the uncomplicated surgical patients suggest that IL-6 is an early marker of tissue damage and that there are numerous reports of increased serum IL-6 levels in inflammatory, malignant and septic conditions, then the above results are to be expected. One would expect that any pathological process would result in continued or increased production of IL-6 since it appears to act as an inflammatory mediator. Very high levels of serum IL-6 have been reported in septic shock (168,180-182). In particular, a recent study of 40 critically ill surgical patients with documented sepsis found increased circulating levels of IL-6 in all patients, and reported a significant correlation between serum IL-6 and APACHE II score - a scoring system which assesses severity of illness ($r = 0.63$, p

<0.001). Moreover the mortality rate was higher (50% versus 21%) in patients whose IL-6 levels were greater than 1000 pg/ml suggesting that IL-6 may well have value as a predictor of mortality in septic patients (248). It is noteworthy that the patient in our group who developed septic shock and died (patient 6) had IL-6 levels which were far in excess of those of any other patient. Moreover, the only other patient who died (patient 5) had the second highest IL-6 concentration 24 hours post-operation.

The numbers here are small and consist of a heterogeneous group of patients. Nevertheless, there is a consistent pattern whereby serum IL-6 concentrations in patients who subsequently develop complications either fail to fall or increase post-operation producing high serum levels which generally precede the onset of clinical signs by at least 12 hours. For example, both patients who died had relatively straightforward operations and were clinically stable 24 hours post-operation when IL-6 levels were very high. Clinical deterioration did not become obvious in either case until after 36 hours post-operation. This lead time could be of value in a clinical setting and allow selected patients to be monitored more intensively and/or treated earlier. All reports of elevated circulating IL-6 in patients with sepsis, some of which suggest that IL-6 may have value as a prognostic indicator, have studied patients in whom sepsis is established. To my knowledge, no other workers have reported elevated IL-6 levels prior to the development of clinical sepsis or other surgical complications. It should be possible to study larger numbers of patients and establish ranges of serum IL-6 concentrations which are 'acceptable' following different types of operation. Values in excess of this would prompt more detailed review of the patient. It should be noted that 3 patients with high IL-6 levels 24 hours post-operation developed transient pyrexias of more than 38.5°C, which were unexplained and were associated with no adverse clinical consequences. It is possible then, that IL-6 might be too sensitive an indicator although this perhaps could be allowed for by setting cut-off points at an appropriate level. From our data, very high levels would suggest a serious

problem such as developing septic shock. Although we have no direct evidence that earlier prediction of complications would improve patient outcome, it is not unreasonable to suggest that it should favourably influence patient management and perhaps facilitate the optimal use of limited resources.

SUSPECTED MYOCARDIAL INFARCTION

Admission IL-6 levels in patients with chest pain provided complete discrimination between those who had unstable angina and those who had myocardial infarction. The poor discrimination with CRP is not surprising given the lack of time between onset of symptoms and admission. This factor may also explain the finding of 'normal' CK levels in 4 patients out of the 10 who had myocardial infarctions since CK levels do not peak until later than 24 hours post-infarction.

These results highlight the problems of using CK levels at presentation to diagnose myocardial infarction. A study of 639 patients presenting with acute chest pain found that the sensitivity of total CK on admission was only 38%, and that for CKMB on admission was even lower at 34% (although this rose to 57% if the sample was taken more than 12 hours after the onset of symptoms) (199). If serial measurements are used, the sensitivity is very good for total CK (98%) and near-perfect for CKMB (100%) (249). The sensitivity of 60% for total CK on admission observed here is probably roughly comparable with the figure of 38% given that our numbers are very small and constitute a more highly selected population. In contrast IL-6 had a sensitivity of 100% in diagnosing infarction in our patients. Because of its non-specificity, IL-6 could be used as a marker of myocardial damage only if strong clinical evidence existed that a patient's chest pain was cardiac in origin. If dubiety existed, then IL-6 would be less useful. In contrast, admission total CK activity and CKMB have been shown to be reasonably specific (80% and 88% respectively) (199).

Early diagnosis of myocardial infarction has two major advantages:- it selects patients who might benefit from thrombolytic therapy; and it facilitates the rational use of coronary care unit beds which are a limited resource. The first of these in particular has produced a demand for emergency biochemical tests to help diagnose myocardial infarction. It is possible that in some patients, IL-6 might be helpful to diagnose myocardial infarction within the limitations mentioned above. However in practice, the diagnosis of myocardial infarction and the decision to administer thrombolytic therapy on clinical and ECG grounds is not usually difficult. In fact there is some evidence to suggest that those patients in whom ECG findings are equivocal do not experience as much benefit from thrombolytic therapy. The ISIS-2 study found that administration of streptokinase alone or in conjunction with aspirin produced substantial benefit in patients whose ECG showed anterior ST elevation but little benefit in those whose ECG showed ST depression or was 'normal' (250). Thus it appears that the clinical value of admission IL-6 in the diagnosis of myocardial infarction may be limited, although it might be helpful in some difficult cases.

Depression of left ventricular function is known to be an important prognostic factor in coronary heart disease. Studies have demonstrated an association between LVEF measured with radionuclide angiography early after acute myocardial infarction and increased mortality during the subsequent year (251-253). In 1982, Sanz et al assessed 79 variables as predictors of late mortality in 259 men who survived acute myocardial infarction and were followed for a mean of 34 months. They found that LVEF (measured by left ventricular angiography) was the single best predictor of late survival (254). Mock et al studied more than 20,000 patients on the Coronary Artery Surgery Study Registry and found that LVEF (measured by radionuclide angiography) was a very important predictor of 4 year survival irrespective of the number of diseased coronary vessels (202). LVEF was measured in our patients by apical biplane cross-sectional echocardiography which has been found to produce similar results to both single-plane and biplane left

ventricular radionuclide angiography (236). Consequently, the finding of a good, negative correlation between admission IL-6 and LVEF is of interest and suggests that IL-6 is potentially useful as a predictor of residual myocardial function and therefore of survival once the diagnosis of unstable angina pectoris or myocardial infarction had been made. Admission CRP proved to be less closely associated with LVEF, while the correlation between admission CK and LVEF was poorer still, although it was better for peak CK. Serial CKMB estimations can be used to estimate infarct size ($r = 0.85$-0.89) (256), but this involves frequent sampling and the use of complex mathematical models. Determination of peak CK and peak CKMB also involves serial measurements but the correlation reported between peak CK and infarct size ($r = 0.68$) (256) is not good and is similar to that found here for peak CK with LVEF. Given these relatively poor correlations, it is not surprising that peak CK levels are reported to be poor predictors of mortality post-myocardial infarction (200). Since IL-6 was closely associated with LVEF, one may speculate that IL-6 could prove useful as a prognostic indicator. Anecdotal support for this is provided by the observation that the two patients with the highest IL-6 levels both died. These results are promising but a much larger prospective study is required to confirm that IL-6 is helpful in diagnosing myocardial infarction in the acute situation, and that it has prognostic value in patients with acute episodes of myocardial ischaemia.

ACUTE PANCREATITIS

The relative temporal relationship between IL-6 and CRP response in the pancreatitis patients was reasonably similar to that found in the elective surgical patients, although actual peak IL-6 and CRP levels occurred later in the pancreatitis patients, particularly those with severe attacks. This finding is probably a reflection of differences in the types of insult - the controlled tissue damage of an elective surgical procedure versus the ongoing inflammatory process of an attack of acute pancreatitis. More importantly, our results suggest that serum IL-6 concentration at

the time of admission discriminates well between severe and mild attacks of pancreatitis. Our finding that admission CRP levels did not discriminate confirms previous reports (206,257). These observations were to be expected given the time courses of IL-6 and CRP responses found following elective surgery. The delay between onset of symptoms and admission was variable in the pancreatitis patients but was usually less than 24 hours. Consequently, at admission one would have expected to find increased levels of IL-6 but not CRP.

The numbers involved in this study are small and larger numbers must be studied before definite conclusions can be drawn. However, admission IL-6, peak IL-6 and peak CRP concentrations all appear to have potential as severity indicators with roughly comparable efficiency, and were all more efficient at predicting severe attacks than the modified Glasgow score over which they have the added advantage of simplicity. The efficiency of peak CRP as a predictor of complicated attacks in this study is similar to that found by Wilson et al who studied 72 patients and found an efficiency of 85% using a cut-off concentration of 210 mg/l (206). Moreover, a recent study of 50 patients reported after this work was performed concluded that serum IL-6 concentration at days 1 and 2 post-admission reflected the severity of acute pancreatitis (258). These workers quoted a positive predictive value of 91% and negative predictive value of 82% for serum IL-6 concentration in predicting a severe disease course - results roughly comparable with those found here for admission IL-6. It should be emphasised that both IL-6 and CRP are non-specific and that levels may be used as severity indicators only once the diagnosis of pancreatitis has been made based on the clinical picture and serum amylase activity. (Our finding of no difference in admission serum amylase activity between the mild and severe groups confirms that serum amylase activity has no prognostic value).

The major advantage of admission IL-6 as a severity indicator is that it reduces the delay associated with using either CRP levels or complicated multifactorial scoring

systems. Such delay is unacceptable since a severely ill patient can deteriorate appreciably within 24 to 48 hours. Furthermore, at this stage, clinical assessment is better at predicting the severity of an attack (figures quoted for percentage correct prediction are 73% (204) and 83% (205)), and so the relative value of biochemical tests becomes smaller. Thus, the earlier a test is available the more valuable it is for patient management. An IL-6 result within hours of admission could help to select patients at risk of complications who might require more intensive monitoring, and/or who might benefit from more invasive or more expensive diagnostic procedures such as peritoneal aspiration or CT scanning.

Peritoneal aspiration is capable of predicting the severity of an attack of acute pancreatitis at presentation, but it is an invasive technique involving a risk of visceral perforation which has not been widely adopted into clinical practice. Contrast enhanced CT scanning has been reported to distinguish mild from severe pancreatitis with sensitivity and specificity of 71% and 77% respectively (259). However, these values compare poorly with those for both IL-6 and CRP and, moreover, CT scanning is very expensive, is not universally available and involves transferring acutely ill patients from the therapy area.

Two other potential early severity indicators in acute pancreatitis have been reported recently, both of which seem promising. These are trypsinogen activation peptides (TAP) and polymorphonuclear (PMN) elastase.

Free trypsinogen activation peptides are produced during pathological intrapancreatic trypsinogen activation when they are released into the peritoneal cavity and circulation. These peptides are then rapidly cleared by the kidney and excreted in the urine. Severe necrotising pancreatitis is thought to differ from the milder oedematous form by the presence of intrapancreatic trypsinogen activation. Consequently, measurement of released trypsinogen activation peptides could have predictive value in acute pancreatitis. Gudgeon et al measured urinary TAP concentrations in 55 patients with acute pancreatitis at the time of admission (260).

They found that this test predicted disease severity with a sensitivity of 80% and a specificity of 90% - diagnostic efficiency comparable with that which we have found for admission serum IL-6.

Polymorphonuclear leukocytes have a capacity to cause tissue damage which has been linked to the harmful potential of their lysosomal proteases, in particular elastase. The release of PMN elastase during the inflammatory response may be partly responsible for the tissue damage which occurs in many inflammatory pathologies, including acute pancreatitis, and this has prompted interest in the measurement of elastase as a severity indicator. A recent study of 182 patients with acute pancreatitis reported that admission serum elastase concentration predicted disease severity with a sensitivity of 93% and a specificity of 94% - diagnostic efficiency again comparable with admission IL-6 concentration (261).

Thus, although serum IL-6 on admission appears promising as a severity indicator once the diagnosis of acute pancreatitis has been made, two other promising indicators have been reported - PMN elastase and TAP. TAP may well have an advantage in that the other two are non-specific. Although the evidence to date suggests that the reliability of each test is comparable, the numbers studied are relatively small. Which of these is the single most reliable predictor of disease severity remains open to question, and a large prospective study in which all 3 of these parameters are measured and compared is necessary. The adoption of such tests into routine clinical practice is also likely to depend on available methodologies and sampling requirements. For example, the requirement of a urine sample for TAP assay may make this test less popular than one which requires serum or plasma.

RHEUMATOID ARTHRITIS

Other workers have reported elevated IL-6 in sera from patients with rheumatoid arthritis (219,223-224). Twenty-seven of our 33 patients had raised IL-6 concentrations, the highest being 414 units/ml. Houssiau et al reported detectable (raised) serum IL-6 levels in only one third of 54 patients when measured using the 7TDI bioassay (219). Moreover, all patients had concentrations less than 50 units/ml. The discrepancy between these results and ours may be explained by the different definition of a 'unit'. Houssiau et al define a unit as 'the dilution giving half-maximal proliferation of cells' which 'corresponds to approximately 5 pg of IL-6'. In contrast, the unitage in this work corresponds to approximately one pg of IL-6. Hovdenes et al measured IL-6 levels in 24 patients with rheumatoid arthritis and found detectable levels in about half (223). However, direct comparisons with our results are difficult because of methodological differences (these workers used a different cell line in their bioassay). None of these workers related serum IL-6 levels to clinical assessment of disease activity.

We found that serum IL-6 was weakly but significantly associated ($r = 0.35$) with clinical rheumatoid disease activity as assessed using the Ritchie Articular Index. In contrast, there was no correlation between either CRP or ESR and the Ritchie Articular Index. The presence of a significant correlation between serum IL-6 and Ritchie Articular Index is encouraging and not unexpected given that Miltenburg et al found a good correlation ($r = 0.72$, $p < 0.001$) between the IL-6 concentration in synovial fluid and clinical assessment of local disease activity (222). Furthermore, Leisten et al reported good correlation ($r = 0.88$, $p < 0.01$) between serum IL-6 levels and the degree of inflammation quantitated by measuring paw volume in arthritic rats (262).

The radiograph has evolved into the 'gold standard' of the rheumatoid patient's disease state because it is an objective method of assessing bone erosions (263).

However, significant technical and interpretative problems continue to exist with radiographic assessment. More importantly, it is a measure which is not sensitive to change, and which involves a considerable lag time between the onset of the disease process and the presence of radiographic change (which may not be present until the cartilage is irreversibly destroyed) (264,265). Magnetic resonance imaging has been reported to detect early synovial proliferation but this technique remains prohibitively expensive (266). In the absence of a true 'gold standard', clinical scoring systems such as the Ritchie Articular Index have been used as objective measures of disease activity. Using this index we found that serum IL-6 compared favourably with more conventional tests such as ESR and serum CRP as a marker of disease activity. These results are consistent with the findings from my study of surgical patients in which IL-6 was more closely associated with length of surgery than CRP. In the rheumatoid patients, however, there was poor correlation between IL-6 and no correlation between ESR and CRP and the Ritchie Articular Index. These results are surprising given the widespread measurement of ESR and CRP in patients with rheumatoid arthritis, although ESR is known to be affected by a variety of 'non-inflammatory' factors such as haematocrit. Other workers have reported conflicting results. Mallya et al found a significant correlation ($r = 0.48$) between serum CRP and the Ritchie Articular Index (226), whereas our findings are supported by Thompson et al who found no significant correlation between serum CRP and Ritchie Articular Index in 30 patients with rheumatoid arthritis (267). This lack of association may partly be due to the fact that the Ritchie Articular Index measures joint tenderness rather than overall inflammation (228). Nevertheless, joint tenderness is recognised as the most reliable measure of joint inflammation (268) and the discrepancy between our results and those of Mallya et al remains unexplained, although it is possible that inter-observer variation associated with scoring the Ritchie Articular Index is partly responsible. Certainly, in our patients it appears that IL-6 reflects the inflammatory process more accurately than does ESR or CRP.

Two other analytes have been reported to be significantly associated with clinical measures of disease activity. Hyaluronic acid is a glycosaminoglycan that is a major component of connective tissue and is present in synovial fluid and synovial tissues. Concentrations increase in the serum of rheumatoid patients on mild exercise, and this increase has been found to show good correlation ($r = 0.79$, $p < 0.001$) with the Ritchie Articular Index, suggesting that hyaluronic acid may prove useful as a disease marker (269) . It also has the advantage of being more specific than IL-6 or other acute phase reactants.

IL-1ß concentrations in serum have also been found to have a weak but significant association with disease activity ($r = 0.57$, $p < 0.001$) although considerable overlap existed between values for rheumatoid patients and normals (270).

The role of IL-6 in the pathogenesis of rheumatoid arthritis is not clear. IL-1 and TNFα cause arthritis in rabbits when injected intra-articularly (271). Both of these cytokines can stimulate prostaglandin E_2 and collagenase production by synovial fibroblasts and chondrocytes (272-275) implicating them in the mechanism by which pannus formation causes cartilage and bone destruction. This is not the case for IL-6. It is possible that IL-6 is a product of, and does not contribute to, the inflammatory process within the joint, and orchestrates the ensuing systemic inflammatory and immune responses. Certainly, we and other workers (219) have found comparable correlations between serum IL-6 and CRP in rheumatoid patients ($r = 0.65$ and $r = 0.69$ respectively). These are similar to the correlation found in the surgical patients and suggest that IL-6 has the same role in CRP synthesis both acutely and in chronic conditions. Also, synovial derived IL-6 stimulates immunoglobulin synthesis (49) and IL-6 levels have been found to show significant correlation with immunoglobulin concentrations in synovial fluid (221,276).

These results indicate that IL-6 compares favourably with CRP and ESR as a marker of disease activity in rheumatoid arthritis. However, both hyaluronic acid

and, to a lesser extent, IL-1ß look promising as such markers. Many more patients must be studied to determine which, if any, of these are reproducibly elevated in rheumatoid arthritis and which show good correlation with disease activity. Furthermore, longitudinal studies need to be performed to determine which of these parameters reflect changes in disease activity with the required sensitivity, and parallel the clinical course of the disease.

CONCLUSION

The findings from the study of 28 elective surgical patients provide support for the premise that IL-6 is a major mediator of such components of the acute phase response as hepatic CRP synthesis, increase in body temperature and hypozincaemia, but suggest that IL-6 is not involved in producing hypoferraemia.

The failure to detect TNFα or IL-1ß in the sera of surgical patients indicates that neither of these cytokines is likely to act systemically to produce these manifestations of the acute phase response, and that they probably exert their effects locally.

Of more importance clinically is that these results indicate that IL-6 is a marker of tissue damage which has potential advantages over CRP. Serum levels increased much earlier than CRP and peak concentrations were more closely associated with degree of tissue damage as reflected by duration of surgery.

The finding of higher serum IL-6 levels 24 hours post-surgery in patients who went on to develop post-operative complications indicates its potential value in predicting those surgical patients at risk of developing complications. Admission levels of IL-6 discriminated completely between patients with unstable angina and those having had a myocardial infarction, whereas CRP and CK discriminated poorly. In addition, admission IL-6 was closely associated with LVEF on the third hospital day, suggesting that it may well have prognostic value. IL-6 on admission discriminated well between those patients who had mild attacks and those patients who had severe attacks of pancreatitis. In patients suffering from rheumatoid arthritis, serum IL-6 levels were more closely associated than either ESR or CRP with a clinical index of disease activity.

These results are promising and support the concept that IL-6 is a better quantitative marker of inflammation than CRP and may well prove to be a valuable

marker of tissue damage in clinical practice. While IL-6 may be more useful than CRP (or ESR) in monitoring patients with chronic inflammatory conditions such as rheumatoid arthritis, it is likely to offer less benefit than in the acute situation. This is because the principal advantage of IL-6 over CRP in the acute situation is not that it provides a more accurate measure but that it provides an earlier measure of inflammation.

As is the case for CRP, one of the main limitations of IL-6 is likely to be its non-specificity. Nevertheless, provided clinicians are aware of this, there is no theoretical reason why IL-6 should not fulfil a similar role to CRP, with the advantage that it has a 24-hour lead time. However, the numbers of patients studied here are small and many more patients must be studied to confirm the clinical value of IL-6 as a marker of tissue damage.

The premise that IL-6 has potential as a severity indicator in clinical practice is dependent on results being available within a few hours of sample collection. The major drawback of the IL-6 bioassay used here is the very long turnaround time of five days. However, several commercial manufacturers have recently developed immunoassay kits for IL-6 which are now being marketed (Quantikine Human IL-6, British Bio-technology Ltd., Abingdon, Oxon; Biokine IL-6 Test Kit, T Cell Sciences, Cambridge; COALIZA IL-6, Kabi Diagnostica, Sweden; IL-6 EASIA, IL-6 IRMA, Medgenix Ltd, High Wycombe, Bucks), and which are claimed to have turnaround times of less than 6 hours. Consequently, the methodology is now available to produce IL-6 results within the time scale necessary for these to be of value clinically, and comparisons between immunoassays and bioassays require to be performed. The immunoassay kits are expensive - approximately £10-00 per test. Nevertheless, it is important that such immunoassays are used in any future studies, such as those outlined in the preceding discussion, which aim to confirm the value of IL-6 in given clinical situations, since it is assays of this type which would be used should measurement of IL-6 be incorporated into routine practice.

In conclusion, these results support the hypothesis that IL-6 is an important mediator of the systemic inflammatory response. The results reported in this thesis have shown that serum IL-6 is an early, quantitative, non-specific marker of tissue damage which offers potential advantages over CRP, particularly in acute clinical situations.

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