Inflammatory mechanisms in acute pancreatitis

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for Ishrat
I declare that:

1. this thesis has been composed by myself;
2. the research work is primarily my own;
3. while I do not hold the degree of MB ChB from the University of Edinburgh, all of the work contributing to this thesis was undertaken while employed as a lecturer by the University of Edinburgh, in the Lister Laboratories, Royal Infirmary of Edinburgh;
4. this thesis has not been submitted in candidature for any other degree, diploma or professional qualification.

Mr Andrew Charles de Beaux
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Abstract

Acute pancreatitis is an inflammatory condition. It is associated with a systemic inflammatory response, the degree of which appears to correlate with the severity of the illness. The role of circulating leucocytes and their production of cytokines in the development of severe acute pancreatitis is unknown. Monocytes are believed to be a major source of pro-inflammatory cytokines, but lymphocytes and endothelial cells also produce such cytokines. These cell types, in particular lymphocytes, also produce a variety of down-regulatory signals so that monocytes, lymphocytes and endothelial cells interact to produce a net systemic inflammatory signal, influenced further by the varying degree of lymphocyte sub-populations to undergo blastogenesis in response to inflammation. The focus of this thesis is on pro-inflammatory cytokines and their release in vitro from peripheral blood mononuclear cells (PBMCs) isolated from patients with acute pancreatitis.

On admission to hospital, patients with acute pancreatitis demonstrated increased interleukin-6 and interleukin-8 release but not tumour necrosis factor-α release from isolated PBMCs compared with healthy volunteers. The severity of the disease was not related to the level of cytokine release from a standard cell number. However, when allowance was made for the variation in PBMC numbers in the blood, the estimated IL-6 and IL-8 release per unit of blood was greater in those patients with severe disease compared with those with mild disease. Severe disease is also characterised by a more prolonged duration of increased pro-inflammatory cytokine release compared with patients with mild disease. Products of the cyclooxygenase pathway play a down-regulatory role in PBMCs in patients with acute pancreatitis as indomethacin (a cyclo-oxygenase inhibitor) had no significant effect.
on pro-inflammatory cytokine release by PBMCs isolated from healthy volunteers, but increased IL-6 and IL-8 release by PBMCs isolated from patients with both mild and severe disease. PBMC pro-inflammatory cytokine release remains sensitive to the down-regulatory action of the T-cell regulatory cytokines, interleukin-4 and interleukin-10. Lymphocyte proliferation (as measured by thymidine incorporation) is impaired in acute pancreatitis and correlates with the severity of the disease.

Following the successful isolation and culture of human umbilical vein endothelial cells, IL-4 and IL-10 (in contrast to their inhibitory action on PBMCs), produce a dose dependent increase in endothelial cell IL-6 and IL-8 release. TNFα is often undetectable in patients with acute pancreatitis on admission, even in severe disease. However, elevation in the serum concentration of soluble TNFα receptors would suggest significant TNFα-induced inflammation early in the course of the disease.

Glutamine is a conditionally essential amino acid in patients with severe acute pancreatitis and is important for immune function. A double blind, randomised controlled trial of glutamine supplemented versus conventional total parenteral nutrition in patients with severe acute pancreatitis demonstrated a trend towards improved lymphocyte proliferation in the glutamine supplemented group.

Furthermore, PBMC IL-8 release but not TNFα and IL-6 release was significantly reduced over the study period.

Severe acute pancreatitis is associated with prolonged PBMC pro-inflammatory cytokine release and impaired lymphocyte proliferation. However, these cells remain sensitive to the down-regulatory action of T-cell cytokines in vitro, but the exogenous administration of these cytokines may have an unpredictable clinical effect because of their different actions on various cell types. More general methods of immuno-modulation, such as the exogenous administration of glutamine may have therapeutic benefit in patients with severe acute pancreatitis.
Chapter 1

Introduction

Acute pancreatitis is an acute inflammatory process affecting the pancreas, with variable involvement of other regional tissues or remote organ systems. It is a common and potentially lethal disease. Although conservative treatment results in rapid recovery in the majority of cases, a proportion of patients develop extensive pancreatic inflammation and necrosis, a systemic inflammatory response, and subsequent multiple organ failure. This significant minority pose difficult management problems and they often have a protracted hospital stay, need intensive care support of their organ failure, and require major surgery to deal with the consequences of pancreatic necrosis (Carter, 1993; Fenton-Lee, 1993).

While acute pancreatitis remains a dangerous disease, mortality rates appear to be falling in recent analyses of national statistics. For example, in Finland the mortality fell from 5.9 % to 2.6 % in the period 1970 to 1989 (Jaakkola, 1993) while in Scotland it fell from 17.8 % in 1961 to 5.8 % in 1985 (Wilson, 1990). These trends however, may not be reflected in data from individual surgical units receiving a high proportion of patients with complicated and severe disease. In an audit of 279 patients with acute pancreatitis managed in the University Department of
Surgery, Royal Infirmary of Edinburgh, between 1989 and 1993 (de Beaux, 1995) there were only 4 deaths amongst 210 patients admitted directly to the department, a mortality rate of 1.9 %. This must be set against the overall mortality rate of 6.1 % and a mortality rate of 18.8 % (13 deaths) in the 69 patients transferred from other units for further management of local or systemic complications of the disease. The overall mortality in this series is similar to that of 7.6 % reported from Leeds for the years 1985 to 1987 (Larvin, 1989). Severe disease (as defined by the Atlanta Classification (Bradley, 1993): development of a local pancreatic complication and/or organ failure) was present in 130 (46.6 %) patients, 74 (35.0 %) in the direct and 56 (81.0 %) in the transfer group. Ninety (32.3 %) patients developed organ failure, (scoring 1 or more on the Goris organ failure score (Goris, 1985)), 55 (26.2 %) in the direct and 35 (50.7 %) in the transfer group. The number of patients surviving or dying in relation to their maximal Goris score is shown in Figure 1.1. It is of note that not one of the 189 patients with a Goris score of 0 (no organ failure), died. As the Goris score increased, so did the mortality, rising to a rate of 67 % in patients with acute pancreatitis with a Goris score of 5 or more. The prevention of organ failure would thus appear to be of central importance in reducing mortality in the disease, yet the pathophysiology of the disease is poorly understood. At the level of the pancreas itself, attempts to explain the varying aetiological agents of pancreatitis within a unified process has remained elusive. Furthermore, the sequence of events whereby local inflammation of the pancreas mediates a systemic inflammatory response, progressing to multiple organ dysfunction and death, is also unclear.

While progressive organ failure is the common pathway of death in patients with acute pancreatitis, the timing of onset of this organ failure defines a number of patient categories. In a number of patients, the onset of acute pancreatitis is followed by a rapid progression to multiple organ failure and often death within
Figure 1.1
Survival/non-survival in patients (n=90) with acute pancreatitis as a function of the worst Goris multiple organ failure score (Goris, 1985) during the illness. There were no deaths in 189 patients with a score of 0. Survivors (n=73;□) and non-survivors (n=17;■).
several days. In a second category, the initial presentation is less dramatic although the patient continues to be unwell on clinical grounds. Dynamic abdominal computer tomography scans typically reveal areas of pancreatic necrosis. Many of these patients will make a slow recovery on conservative treatment but a number will relapse with ensuing organ dysfunction. The trigger mechanism for this event are unclear, but may be related to bacterial infection of the once sterile pancreatic necrosis (Beger, 1986). While this dichotomy in the timing of events exists, it seems likely the mechanisms responsible for the development of organ failure are similar in the two groups, arising as a result of the initial pancreatic acinar cell injury or as a event subsequent to the infected pancreatic necrosis.

In this introduction, the evidence relating to the pathophysiology of acute pancreatitis is discussed, focusing predominantly on the potential mechanisms resulting in progression to severe disease and organ dysfunction. Pathological changes in the pancreas and distal organs are well reported and these are described briefly. The role of the pancreas as a digestive organ led to the hypothesis that the pathological changes observed in acute pancreatitis represented a state of autodigestion mediated by premature uncontrolled activation of pancreatic digestive enzymes and that derangement of structure and function in distant organs was a consequence of such activated enzymes spilling into the circulation. Attempts, however, to minimise such enzyme activity have enjoyed little clinical success. The potential role of oxygen-derived free radicals in mediating local and systemic effects was also recognised although the emphasis now appears to be more on free radical production from the parenchyma of the pancreas rather than from white blood cells. A variety of changes in the serum concentrations of a number of factors involved in the complement, kallikrein-kinin and coagulation cascades have been identified and point to the development of an acute phase response and a systemic inflammatory response. However, the demonstration that leucocyte depletion in experimental
acute pancreatitis attenuated a number of the systemic effects of the disease pointed to the key role of the leucocyte in mediating end organ damage. Whether leucocytes mediated such damage as a result of intra-vascular leucocyte activation directly or by opportunistic exploitation of the damaged endothelium by leucocytes was not clear from these studies. The evidence leading to the current hypotheses of the pathogenesis of acute pancreatitis are discussed in turn.

1.1 Pathology of acute pancreatitis

Amongst the earliest descriptions of the pathology associated with acute pancreatitis were those by Fitz (Fitz, 1889) and Opie (Opie, 1901) towards the end of the 19th century. They described a number of features associated with severe pancreatitis including fat necrosis, pancreatic haemorrhage and peri-pancreatic inflammation. Since then, changes in both the pancreas and other organs in experimental acute pancreatitis have been described (Becker, 1981). The earliest microscopic event to be observed appears to occur within the pancreatic acinar cell with the development of patchy vesiculation of the rough endoplasmic reticulum (Tardini, 1971; Watanabe, 1984). This progresses to show prominent vacuolation within the cell with an increased number and heterogeneity of the zymogen granules (Lombardi, 1975). Following these acinar cell changes, leucocyte congestion within the post capillary venules is observed proceeding to a marked inflammatory cell infiltrate into the gland (Aho, 1980). This emigration of leucocytes corresponds to changes in the micro-circulation including the initial increased fenestration of capillaries (Kelly, 1993b) progressing to more marked disruption of the endothelium with time. In-vivo microscopy techniques have demonstrated increased vascular permeability and reduced capillary blood flow with areas of blood stasis as a relatively early event (Kusterer, 1991). Micro-corrosion casts of the pancreatic
micro-vasculature in experimental acute pancreatitis (Kelly, 1993b; McEntree, 1989) show increasing irregularity of capillaries, loss of the typical smooth contour of vessels, increased tortuosity and abrupt termination of vessels with development of the local inflammation. Similar findings in the hepatic micro-vasculature (Andrzejewska, 1985; Delaney, 1990) are observed after a short lag phase behind the pancreatic changes. Some time later on, similar changes in the pulmonary micro-vasculature (Kelly, 1991) but not the renal circulation are observed over the study period (Kelly, 1993a). With increasing severity of the pancreatitis, patchy areas of acinar cell necrosis coalesce with concomitant haemorrhage, fat necrosis and oedema with such pathological changes being observed in regional tissues around the pancreas and in distant organs (Lankisch, 1983a).

1.2 Pancreatic digestive enzymes

1.2.1 Pancreatic enzyme synthesis and transport

Protein synthesis within the pancreas is greater than in any other organ. The two main enzyme series produced are the digestive enzymes and lysosomal hydrolases. The synthesis, transport and secretion of these enzymes are common to protein manufacture in other cell types (Caro, 1964). The protein sequences are synthesised from messenger RNA on ribosomes attached to the rough endoplasmic reticulum. The polypeptide chains produced are then transported to the golgi complex where the pathways of these two enzyme classes diverge: digestive enzyme proteins are packaged into zymogen granules and lysosomal hydrolases into lysosomes through an intermediate stage of the condensing vacuole (Steer, 1984). The mechanism by which the two protein classes are sorted from each other appears to involve post-translational modification of the lysosomal enzymes (Rosenfield, 1982). Following a secretory signal to the pancreatic acinar cell, for example, by
pancreozymin, zymogen granules fuse with the cell membrane at the apex of the cell and discharge their contents into the pancreatic duct, a process termed exocytosis.

1.2.2 Preservation of pancreas integrity

The integrity of the pancreas is protected from the potentially lethal effect of its enzymes by a number of mechanisms. The enzymes are present as inactive zymogens sequestered in zymogen granules (Rinderknecht, 1986; Steer, 1984). The zymogen granule is actively acidified precluding enzyme activity and favouring enzyme degradation rather than activation (Niederau, 1986). Low intra-granule calcium concentration promotes a similar environment (Rinderknecht, 1986).

Specific zymogen inhibitors are present in pancreatic secretions and zymogen granules (Watanabe, 1984). Activation of zymogens normally takes place in the duodenum, associated with the change in pH, calcium concentration and the presence of enterokinase, an enzyme released from enterocytes lining the duodenum that activates trypsin from trypsinogen. Trypsin then appears to be important in the activation cascade of the remaining pancreatic zymogens (Rinderknecht, 1986).

Whilst the separation, packaging and apical release of these potentially destructive enzymes would seem to be important for the preservation of the integrity of the pancreas, derangements in this system have been identified in experimental models of acute pancreatitis, although their relevance to the disease in man is controversial. Lombardi and co-workers (Lombardi, 1975) developed a murine model of acute pancreatitis, by feeding mice a choline-deficient, ethionine-enriched diet. Within hours of consumption of this diet, the number of zymogen granules present in the acinar cells rapidly increased suggesting either an accelerated production of these enzymes or an inhibitory block to granule exocytosis (Steer, 1987). Zymogen granules then appeared to fuse with the intracellular lysosomes, allowing pancreatic zymogens to come in contact with lysosomal hydrolases, an event that can mediate zymogen activation (Rao, 1980).
In another model of acute pancreatitis, rats were infused with caerulein, a pancreozymin analogue (Watanabe, 1984) mediating hyper-stimulation of the pancreas. Within 30 minutes of caerulein stimulation, large vacuoles appeared in the acinar cell, shown to contain both digestive enzymes and lysosomal hydrolases. This intra-vacuole mixing of enzymes is likely to occur as a result of abnormal condensing vacuole maturation which normally leads to the segregation of lysosomal hydrolases from digestive enzymes (Baniukiewicz, 1994; Steer, 1984). Another potentially damaging event, so called 'misdirected' exocytosis, has been observed in this hyper-stimulation model (Adler, 1982) and in a hyper-stimulation model of acute pancreatitis in dog (Tardini, 1971). Here the contents of zymogen granules are released on the basolateral surface of the acinar cell into the interstitium of the pancreas rather than on the apical surface into the pancreatic ductule.

1.2.3 Role of pancreatic enzymes in systemic organ damage

While pancreatic enzymes are likely to contribute to the local cell damage observed in acute pancreatitis, their role in the systemic effects of the disease remains not proven for a number of reasons. Pancreatic hyper-stimulation is not thought to be play a major role in the initiation of acute pancreatitis in man, except perhaps in more rare aetiologies of the disease such as following the bite of the Trinidad scorpion (Bartholomew, 1970) or the ingestion of insecticides with marked anti-cholinesterase activity (Dressel, 1979). Indeed, active trypsin is rarely found in more than trace amounts in acute pancreatitis (Mero, 1982) although trypsinogen is detectable in the serum for several days in patients with acute pancreatitis (Ventrucci, 1987). Furthermore, homogenates from both normal and inflamed pancreas have little in the way of proteolytic activity as a number of zymogen and enzyme inhibitors are released by the pancreas in significant amounts, even in disease states (Balldin, 1979; Rinderknecht, 1988). The micro-environment
generated by an inflammatory response tends to be low in calcium and pH, again reducing the chance of uncontrolled enzyme activation. Finally, therapeutic intervention to reduce pancreatic stimulation and further enzyme release with calcitonin (Goebell, 1979), glucagon (Condon, 1973), somatostatin (D'Amico, 1990; Usadel, 1980), atropine (Cameron, 1979), and nasogastric aspiration (Fuller, 1981) or by manoeuvres to neutralise activated pancreatic enzymes such as aprotinin (trasylol) (Imrie, 1978; Trapnell, 1974), gabexate mesilate (Büchler, 1993; Valderrama 1992), fresh frozen plasma (Goodman, 1986; Leese, 1991) or peritoneal lavage (Mayer, 1985) have shown no clinical benefit. The exact role of pancreatic enzymes in mediating distant organ damage is unclear. Infusion of high dose trypsin and phospholipase A2 in experimental models to reproduce a state of excess activation has resulted in changes in the blood coagulation system (Kwaan, 1971) lungs (Nevalainen, 1980; Tahamont, 1982) and brain (Nevalainen, 1980) typical of the disease. Whether such changes are mediated directly by a toxic effect of activated pancreatic enzymes in the circulation or by a more indirect mechanism is unclear. Furthermore, many of the systemic changes in acute pancreatitis are not specific to the disease, but are evident in other conditions such as those following major trauma, burns and sepsis where there is no evidence any significant release of pancreatic enzyme into the circulation.

1.3 Oxygen-derived free radicals

Another avenue of investigation in the pathophysiology of acute pancreatitis was the potential role of oxygen-derived free radicals, contributing to both pancreatic cell damage and spillage into the circulation thereby mediating distant organ damage. Oxygen-derived free radicals are highly charged, toxic intermediates normally produced in small amounts as a by-product of oxidative metabolism. Such
by-products are usually rapidly detoxified by endogenous enzymatic free radical scavengers such as superoxide dismutase, catalase and non-enzymatic free radical scavengers such as vitamins A, C and E (Reilly, 1991). However, in a number of pathological conditions, including acute pancreatitis (Scott, 1993), oxygen-derived free radical production may exceed the scavenging capacity, and tissue injury is produced largely through the peroxidation of structural lipids in the membranes of cells and organelles and disruption of membrane transport proteins. Injury of this nature in pancreatitis is of particular importance, as disruption of lysosomes and zymogen granules, with the subsequent intracellular release and activation of lysosomal and granule enzymes, could potentiate the free-radical induced cellular damage (Bulkley, 1983). It appears however, that pancreatic zymogens as well as trypsin inhibitors are protected from attack by oxygen-derived free radicals, resistance that may be conferred by the presence of a hydrogen peroxide-dependent enzyme in pancreatic juice (Guyan, 1986). Nevertheless, a number of studies using experimental models of acute pancreatitis have demonstrated a protective effect on the degree of pancreatic inflammation with the use of oxygen-derived free radical scavengers such as superoxide dismutase and catalase (Gough, 1990; Sanfey, 1984a), pre-treatment with allopurinol (a xanthine oxidase inhibitor) (Gough, 1990; Rutledge, 1987; Sanfey, 1985) or administration of a platelet activating factor antagonist (Dabrowski, 1989). The source of these oxygen-derived free radicals in acute pancreatitis is believed to be in the pancreas parenchyma, either the acinar cells or the vascular endothelial cells since leucocyte depletion prior to the induction of acute pancreatitis (thus removing the leucocyte as a potential source of oxygen-derived free radicals) had no effect on the degree of pancreatitis induced (Guice, 1989; Sarr, 1987). This finding is perhaps not surprising, as allopurinol was able to attenuate pancreatic damage in both leucocyte rich and depleted models of acute pancreatitis. Neutrophils contain little or no xanthine oxidase but generate oxygen-
derived free radicals via a membrane-associated NADH oxidase which is not inhibited by allopurinol (Baboir, 1978).

1.3.1 Xanthine oxidase system

Xanthine oxidase is translated in the form of xanthine dehydrogenase, an enzyme that is common to most cells and catalyses the terminal oxidation of purines to uric acid, coupled with the reduction of NAD+ to NADH. Conversion of xanthine dehydrogenase to xanthine oxidase is by proteolytic cleavage of a 26 kilo Dalton (kDa) peptide, producing an enzyme which is also able to catalyse the conversion of purines to uric acid, but now coupled with the reduction of molecular oxygen. When the extra electron is passed to the oxygen molecule, the highly reactive superoxide free radical anion is generated. The role of oxygen-derived free radicals produced by the pancreatic cells is not entirely clear, but they may well mediate the increased capillary permeability observed early in the course of acute pancreatitis (Parks, 1983a; Sanfey, 1984b). As well as an effect on capillary permeability, superoxide appears to catalyse the generation of a cascade of toxic oxidants (Reilly, 1991) that can signal integrin-mediated adhesion, trapping and activation of circulating neutrophils by the micro-vascular endothelium (Patel, 1991). It is evident that the recruitment of leucocytes to the tissue by oxygen-derived free radicals is not unique to acute pancreatitis. Reperfusion of an ischaemic tissue is associated with an inflammatory cell infiltrate into the previously ischaemic tissue and such leucocyte emigration is inhibited by allopurinol (Grace, 1994). It has been postulated that cell damage, produced by a noxious agent or hypoxia, is associated with the rapid conversion of xanthine dehydrogenase to xanthine oxidase, increased oxygen-derived free radical formation within the damaged cell, creating an effective mechanism of rapid onset to attract leucocytes to the vicinity of the cell damage, establishing an area of acute inflammation as part of the defence mechanism to limit spread of cellular injury to adjacent viable cells and
to aid in the repair and ultimate healing of the tissue (Bulkley, 1993; Dormandy, 1983). Any additional oxygen-derived free radical production by infiltrating leucocytes may then act as a second message to potentiate the immune response in the recruitment of further leucocytes to the area of pancreatic cell damage, perhaps accounting for the rapid inflammatory cell infiltrate into the gland observed in models of acute pancreatitis. From this proposal, it is evident that the formation of free radicals may not necessarily be deleterious to the host, perhaps accounting for the findings that reducing free radical formation may not alter the clinical outcome in terms of mortality in models of acute pancreatitis (Rutledge, 1987). However, to date, no trials of such therapies in patients with acute pancreatitis have been reported in the literature.

1.3.2 Role of oxygen-derived free radicals in systemic organ damage

Oxygen-derived free radicals are believed to mediate endothelial cell damage within the pancreas but can free radicals mediate distant organ damage? Infusion of intra-vascular hypoxanthine and xanthine oxidase, an oxygen radical generating system led to increased micro-vascular permeability in distant organs (Parks, 1983b). While oxygen-derived free radicals may account for some of the micro-vascular changes in distant organs seen in acute pancreatitis, whether this is a direct toxic effect (Johnson, 1981; Taylor, 1983) or mediated through the generation of additional neutrophil chemotactic factors (McCord, 1983), concomitant complement activation (Willemer, 1991) or intra-vascular leucocyte activation (Guice, 1989) is not clear. Neutrophils can also damage pulmonary endothelium through their own oxygen radical production (Guice, 1989), and there is evidence that interaction with pancreatic enzymes can enhance free radical formation by leucocytes (Tsuji, 1994). However, Rutledge and co-workers (Rutledge, 1987) found that while treatment with allopurinol in a model of acute pancreatitis reduced the degree of pancreatic inflammation, mortality was similar in the treated or untreated groups.
Nevertheless, Braganza (Braganza, 1995) and co-workers have reported evidence of oxidative stress that had pervaded the vascular compartment by the time of admission in patients with acute pancreatitis and the blood micro-nutrient antioxidant profile at this stage in these patients was consistent with compromised cellular capacity to withstand further oxidative assault.

### 1.4 Role of the inflammatory cell infiltrate

The normal function of the infiltrating leucocytes is to remove the offending material from the site of injury by engulfing it into a phagocytic vacuole where it is degraded. This process involves the stimulus-secretion coupling of leucocyte zymogen granules with the phagocytic vacuole (Weissmann, 1982). These granules contain a variety of proteases, including neutrophil elastase, phospholipase, DNAase, RNAase, many other lysosomal hydrolases, platelet activating factor, and also oxidases involved in the production of reactive oxygen species. In a self limiting disease, tissue damage is minimised and healing follows or is concurrent with the inflammation leading to recovery. However, circumstances may arise in which the inflammatory response develops in a direction more damaging to the host than the initial injury. This can occur when the quantity of material to be digested is overwhelming (Weissmann, 1971) or when the stimulation of neutrophils and macrophages is excessive and phagocytosis so vigorous that the granulocyte contents are spilled into the exterior of the phagocyte before the phagocytic vacuole has time to close off completely, so called overt secretion (Weissmann, 1982). Release of lysosomal enzymes, further reactive oxygen metabolites and other tissue toxic factors into the extra-vascular space will result in additional cell damage, increasing the intensity of the inflammatory response and initiating a vicious cycle of leucocyte recruitment, overt secretion, increased tissue injury and further
leucocyte recruitment. Evidence for this is now recognised in other inflammatory conditions such as gout and rheumatoid arthritis (Rae, 1982; Rinderknecht, 1988). It seems reasonable to postulate that in the case of severe initial injury to the pancreas, or prolonged activation by a noxious agent, the amount of cell debris and noxious agent to be phagocytosed may become overwhelming, provoking overt secretion of neutrophil granules into the interstitium of the pancreas. The normally protective apparatus of both leucocytes and acinar cells is unleashed on a course of local pancreas destruction. There is increasing indirect evidence that the magnitude of the inflammatory response may not simply relate to the extent of the initial pancreatic cell injury, but that the degree of activation of the inflammatory process may have a significant genetic component (Guillou, 1993; Rinderknecht, 1994). Thus, the extent or duration of the pancreatic injury coupled with the patient's idiosyncratic reaction to the presence of pancreatic inflammation that may determine the progression of the disease to involve distant organs.

1.5 Distant organ damage and the leucocyte

Spillage of a wide variety of products from activated leucocytes, endothelial cells and damaged parenchymal cells undoubtedly occurs, and many of these molecules are known to promote a pro-inflammatory effect on vascular beds distant from the area of initial inflammation. However, Barie and co-workers noted that the pulmonary sequelae of experimental acute pancreatitis in sheep (in particular the increased micro-vascular permeability) were prevented by leucocyte depletion prior to the induction of pancreatitis (Barie, 1982). Similarly, leucocyte depletion reduced acute lung injury in experimental acute pancreatitis in rats (Guice, 1989; Willemer, 1991). Furthermore, in other models of acute pancreatitis, pronounced margination of leucocytes was observed within the capillaries and venules of the
liver and lungs as an early event in the development of organ dysfunction and failure (Kelly, 1993b). These observations would suggest that the circulating leucocyte is an important mediator in the development of the systemic complications of acute pancreatitis, either interacting with an activated endothelium at the target organ, or as a consequence of intra-vascular leucocyte activation or as a combination of both distant endothelial injury and intra-vascular leucocyte activation. The evidence for these events in man is largely indirect through an interpretation of the changes in serum complement, kinin and coagulation cascades and the changes in serum markers of leucocyte activation in patients with acute pancreatitis.

1.6 Complement system

Acute pancreatitis is associated with reduced serum levels of complement (Foulis, 1982; Goldstein, 1978; Seelig, 1975). Furthermore, patients with severe disease manifest a greater depletion of serum complement (Goldstein, 1978; Lasson, 1985). This depletion of complement could result from reduced synthesis, but as complement factors are acute phase proteins (Alper, 1969) and acute pancreatitis is associated with a significant acute phase protein response, this explanation is unlikely. The more likely explanation for the depletion of complement is increased catabolism, either by activation of the classical or alternate complement pathways or perhaps by direct pancreatic enzyme digestion of the factors in the blood. It has been demonstrated that trypsin and other proteases can cleave C3 and C5 to yield biologically active fragments (Cochrane, 1968) which are chemotactic for leucocytes (Ward, 1971), increase capillary permeability, increase the adhesiveness of neutrophils (Craddock, 1977) and promote the release of lysosomal enzymes (Goldstein, 1973) from neutrophils. The possibility exists that inappropriate complement activation in patients with acute pancreatitis is sufficient to generate
humoral mediators of inflammation and tissue injury within the circulation. Indeed, there is evidence to support the role of complement activation in the development of acute lung injury (Craddock, 1977; Hammerschmidt, 1980). Hammerschmidt and co-workers demonstrated that serum C5a levels correlated with the development of acute lung injury (Hammerschmidt, 1980). Neutrophils aggregate in response to activated complement such as C5a (Craddock, 1977) and intra-vascular leucocyte aggregation, with the resultant formation of leucocyte emboli, has been postulated to mediate organ damage (Jacob, 1978). Indeed, C5a induced leuco-embolism has been observed by intra-vital microscopy of rat mesenteries (Hammerschidt, 1978).

1.7 Kallikrein-kinin system

There is depletion of prekallikrein and kininogen in both the serum and peritoneal fluid of patients with acute pancreatitis, and this depletion is most marked in patients with severe disease (Lasson, 1984a; Lasson, 1984b; Orlov, 1978). Conversely, kallikrein activation in blood and especially peritoneal fluid is enhanced in patients with acute pancreatitis while the ability of the patient’s serum to inhibit kallikrein activity is markedly reduced, being abolished in patients with severe disease (Lasson, 1984a). O’Brodovich and co-workers (O’Brodovich, 1981) demonstrated that pulmonary artery bradykinin levels rose in a sheep model of acute lung injury and suggested that bradykinin may contribute to the increased pulmonary endothelial permeability observed in their model.
1.8 Coagulation system

The initial change in the coagulation cascade in patients with acute pancreatitis is the development of a hyper-coagulable state (Goodhead, 1969; Murphy, 1977; Shinowara, 1963). Platelet count, fibrinogen, factor V and factor VIII concentrations rise as part of the acute phase response (Imrie, 1988). However, progression to a consumptive coagulopathy and disseminated intra-vascular coagulation in severe acute pancreatitis is manifest by decreased platelet count (Byrne, 1971), reduced serum levels of prothrombin, fibrinogen, clotting factors, anti-thrombin III (the main inhibitor of thrombin) (Innerfield, 1952; Lasson 1986) and evidence of increased fibrinolysis by reduced levels of fibrinogen and raised levels of fibrin degradation products (Agarwal, 1982; Ranson, 1977). Ranson and co-workers (Ranson, 1977) demonstrated that early changes in the serum of such coagulation factors correlated with respiratory, renal and hepatic dysfunction in patients with acute pancreatitis, and postulated that pancreatic enzyme mediated intra-vascular coagulation might be implicated in the pathogenesis of the systemic features of acute pancreatitis. Indeed, a consumptive coagulopathy similar to that seen in acute pancreatitis can be induced in dogs by the intravenous infusion of trypsin (Kwaan, 1971). Intra-vascular coagulation and fibrin micro-embolism involving the pulmonary micro-circulation has been implicated in the pathogenesis of acute lung injury (Blaisdell, 1970). Indeed, fibrinogen levels were elevated in 80% of patients with acute pancreatitis on admission and there was a negative correlation between these levels and the arterial partial pressure of oxygen (Berry, 1981). Saldeen (Saldeen, 1976) suggested that inhibition of normal fibrinolysis in the lungs might lead to pulmonary fibrin deposition. However the mechanism whereby fibrin deposition leads to pulmonary damage is not understood. It has been suggested that fibrin degradation may cause a local increase in capillary permeability (Belew, 1978). Peptides such as fibrinopeptide-A produced during the
conversion of fibrinogen to fibrin are also known to cause local pulmonary damage (Bayley, 1967). Thrombin, itself, has been demonstrated to enhance interleukin-1β and tumour necrosis factor α-induced polymorphonuclear leucocyte migration across endothelium (Drake, 1992).

Although changes in the serum complement, kallikrein-kinin and coagulation cascades described above are likely to play a role in the pathogenesis of end organ damage, perhaps through endothelial cell activation promoting leucocyte attraction, it is clear from the work by Barie (Barie, 1982), Guice (Guice, 1989) and Willemer (Willemer, 1991) that the presence of the leucocyte is imperative in the production of end organ damage, manifest clinically as organ failure. Furthermore, in a rat model of acid aspiration, leucocyte depletion showed a protective effect on multiple organ oedema normally observed in this model (Goldman, 1992). Similarly, in a guinea pig model of sepsis, granulocyte depletion prevented tumour necrosis factor mediated acute lung injury (Stephens, 1988). However, a key question that remains unanswered is do leucocytes merely exploit an activated or damaged endothelium allowing their migration into the tissues or is there associated intra-vascular leucocyte activation in patients with acute pancreatitis?

Rinderknecht (Rinderknecht, 1988) proposed that the systemic sequelae of acute pancreatitis arose following excessive leucocyte activation within the pancreas with the release of secondary mediators of inflammation such as the pro-inflammatory cytokines, tumour necrosis factor-α and interleukin-6 into the circulation which in turn, promoted intra-vascular leucocyte activation. Indirect evidence to support this hypothesis has come from the measurement of serum concentrations of neutrophil elastase in patients with acute pancreatitis and other inflammatory conditions. The capacity of neutrophils to cause tissue damage has been attributed to the effects of their lysosomal proteases, especially elastase, and to
the production by the phagocytic cell of oxygen-derived free radicals. Neutrophil elastase is released into the extra-cellular space during phagocytosis and is rapidly complexed in the circulation by α₁-proteinase inhibitor (Banks, 1991b). Levels of this complex are elevated in the sera of patients with a number of inflammatory conditions such as sepsis, rheumatoid arthritis and inflammatory bowel disease (Adewumi, 1985; Duswald, 1985) and are thought to be a crude marker of neutrophil activation. Circulating levels of neutrophil elastase-α₁-proteinase inhibitor complex are raised in patients with acute pancreatitis (Banks, 1991a; Domínguez-Muñoz, 1991; Gross, 1990) and the increase in the concentration of this complex has been shown to reflect the severity of the attack. Banks and co-workers (Banks, 1991a) found that on the first day of admission, there was no significant difference in the level of neutrophil elastase-α₁-proteinase inhibitor complex between patients with mild or severe disease. However, over the subsequent 4 days, levels of the complex remained high in the severe group but fell towards the normal range in the mild group. From day 5 onwards, once again, there was no significant difference between the two groups. Similar findings were observed in two further similar studies (Domínguez-Muñoz, 1991; Gross, 1990) suggesting that severe acute pancreatitis is associated with a prolonged period of neutrophil activation compared with mild disease.

A multitude of molecules with either pro-inflammatory actions or produced as a result of such pro-inflammatory stimulation are now recognised in acute pancreatitis including members of the cytokine family, platelet activating factor, acute phase proteins and endotoxin. Their appearance or increase in concentration within the circulation can be measured giving an indication of inflammatory events including leucocyte activation. The character of such compounds along with their possible role in pathophysiology of acute pancreatitis are discussed in the subsequent sections.


1.9 Cytokines

Cytokines, such as tumour necrosis factor, interleukin-6 and interleukin-8, are members of a large family of endogenous polypeptides or glycoproteins produced by leucocytes and in many cases, other cell types. They are capable of mediating a wide range of biological effects on diverse cell types, many of which are particularly essential to the hosts' metabolic, haemodynamic, immunologic and wound healing responses to injury and infection. The production and secretion of these cytokines is of limited duration and basal production is usually absent or occurs at low levels. Furthermore, most cytokines are not stored as preformed molecules and their production requires new gene transcription and translation in response to a stimulus (Lowry, 1993). The transcription of many cytokine genes results in an unstable messenger RNA providing a potential mechanism for tight regulation of their appearance.

All cytokines exert their action by binding to specific cell-surface receptors. As opposed to many other peptide hormones, cytokine activity is generally exerted over a short radius via binding to the cell of its origin (autocrine activity), or to a neighbouring target cell (paracrine activity). Some cytokines do exert an influence on distal target cells (endocrine activity) although in most cases the detection of such proteins in the circulation more likely reflects excessive activity of these mediators at the tissue level (Tracey, 1990). Once bound to the target cell, the post-receptor actions of cytokines are exerted through second messenger systems. Such events usually result in altered patterns of gene expression with new messenger RNA transcription. Many of these activities include some component of altered cell proliferation, differentiation or differentiated function and movement at nanomolar to picomolar concentrations.
Individually, cytokines are potent molecules. Redundant functions are demonstrated, at least in vitro, but it is clear that there are likely to be no circumstances in vivo in which cytokines are produced individually. Rather they are produced together with other cytokines in patterns characteristic of the particular stimulus or disease. Cytokine interactions are complex so that the ultimate cellular events elicited by the sequential or simultaneous presence of several cytokines is not always predictable. Thus, the potency of cytokines and their potential for amplification and damage which excessive cytokine production carries, has resulted in elaborate controls on cytokine production and action. The current view of cytokine biology is of a network of positive and negative cytokines, and cytokine inhibitors and inducers, which combine to give an overall biological or clinical response. While this complex interrelationship of cytokines (and a multitude of secondary mediators) stimulates inflammatory responses designed to protect the host, in some circumstances the deleterious effects of these factors may predominate.

1.9.1 Tumour Necrosis Factor-α

Tumour necrosis factor-α (TNFα) was first identified for its anti-cancer activity hence its name. The gene for TNFα exists on chromosome 6 within the major histocompatibility complex (Nedwin, 1985). The expression of TNFα messenger RNA encodes a precursor of 233 amino acids that is processed to a mature 157 amino acid protein of 17 300 Da. TNFα is normally secreted as a homotrimer; monomeric TNFα is not biologically active (Callard, 1994). While the major source of this cytokine is believed to be from macrophages, many other cell types including monocytes and lymphocytes produce TNFα. Challenge with endotoxin appears to be the classical induction agent for the release of TNFα. Other stimuli will release TNFα such as the binding of immune complexes, phagocytosis by mononuclear phagocytes, interferon gamma, interleukin-2 and viruses
Prostaglandins have demonstrated a suppressive effect on the release of macrophage derived TNFα production (Kunkel, 1988) and glucocorticoids suppress the transcription of TNFα (Remick, 1989).

TNFα receptors are present on nearly all cell types with few exceptions such as erythrocytes and resting T cells. Two receptors are described, Type I and Type II, both members of the nerve growth factor release superfamily found on chromosome 12 and chromosome 1 respectively. Soluble forms of both receptors are detectable in the serum and urine and are derived from the extra-cellular domain of each receptor. The two receptors have less than 25% homology and have no more homology to each other than to other members of the superfamily. Furthermore, there is no significant homology between the intracellular domains of the two TNFα receptors indicating different signalling mechanisms. Receptor cross-linking by the TNFα trimer is important for signal transduction although the exact signalling mechanism is not clear. However, it seems likely that the two TNFα receptors are coupled to distinct signal transduction pathways. Mice, for example, with type I receptor gene deletion are resistant to TNFα-mediated toxicity (Rothe, 1993).

TNFα has multiple biological activities (Semenzato, 1990). It serves as the most proximal cytokine mediator of the natural immune response to bacterial lipopolysaccharide and to activate inflammatory neutrophils and monocytes to affect microbial killing, stimulation of T- and B-cell function, and expression of class 1 major histocompatibility complex molecules. TNFα further stimulates the production of other pro-inflammatory cytokines, such as IL-6 and IL-8, and enhances endothelial cell adhesiveness for leucocytes, thereby contributing to the recruitment of these activated cells to sites of inflammation. In higher concentrations in the systemic circulation, TNFα can contribute to haemodynamic
collapse through reduced vascular smooth muscle tone, reduced myocardial contractility and activation of the coagulation cascade with associated intra-vascular thrombosis. The toxicity of TNFα, as well as other cytokines, is synergistically enhanced by other factors (Tracey, 1991). For example, interleukin-1 by itself, even when administered at high doses, is minimally toxic, yet when co-administered with normally non-toxic doses of TNFα, the combination becomes lethal. Endotoxin, along with platelet-activating factor and interleukin-6 also increase TNFα toxicity.

The central role of TNFα in orchestrating the early inflammatory responses in patients with severe sepsis is well established (Dinarello, 1993b). Elevated levels of TNFα have been documented in the sera of patients with sepsis (Damas, 1989; Debets, 1989; Dofferhoff, 1992) and following exposure to endotoxin (Cannon, Tompkins, 1990; Hesse, 1988; Michie, 1988b). However, TNFα is often undetectable in the serum of patients with acute pancreatitis, even in those with severe disease (Banks, 1991a; Domínguez-Muñoz, 1993; Exley, 1992). This may be due to the short serum half life of TNFα, binding of TNFα to other proteins, difficulties with the various methods of measuring TNFα in serum or breakdown of TNFα in the sample by neutrophil elastase (Van Kessel, 1991). Alternatively, it may be that TNFα is only transiently and repetitively produced and thus the peak concentration of TNFα could easily be missed by infrequent blood sampling, or only occasionally spills over into the circulation. In support of the role of TNFα in the pathogenesis of acute pancreatitis, messenger RNA for TNFα and TNFα itself have been demonstrated in the pancreatic acinar cell in an experimental model of acute pancreatitis (Formela, 1993). TNFα has also been demonstrated in pneumatocytes in the lung tissue of patients with acute pancreatitis dying from acute lung injury (Nash, 1991), including patients with acute pancreatitis. An additional factor in the interpretation of serum levels of TNFα relates to the soluble TNFα receptors. Shedding of TNFα receptors by the target cell may be a process whereby
the target cell down-regulates itself to a further TNFα related stimulus (Coyle, 1993; Tartaglia, 1992). However, these soluble TNFα receptors may bind free TNFα within the circulation or tissues, thereby reducing the bioavailability of TNFα, preventing TNFα related stimulation of more distant target cells. It is not inconceivable, also, that the TNFα/TNFα receptor complex has important biological activity of its own.

1.9.2 Interleukin-6

Interleukin-6 (IL-6) is the accepted term for hepatocyte stimulating factor, a substance found in the supernatant of leucocytes that could induce an acute phase protein response in hepatocyte cultures. The human IL-6 gene is located on the short arm of chromosome 7 (Heinrich, 1990). This gene translates into a 212 amino acid precursor protein with a molecular mass of 26 kDa. Following removal of a 28 amino acid signal peptide, the resulting protein is glycosylated and phosphorylated at varying sites. Although the unglycosylated IL-6 is biologically active, these post-translational modifications may play a role in tissue-specific functions. IL-6 is synthesised by a number of cells including monocytes/macrophages, fibroblasts, endothelial cells and lymphocytes. IL-6 production by these cells can be triggered by a number of stimuli. Monocytes/macrophages are preferentially triggered by bacterial lipopolysaccharide, while fibroblasts and endothelial cells respond better to endogenous cytokines such as TNFα. TNFα is also produced by monocytes/macrophages suggesting that these cells play a key role in the production of IL-6.

IL-6 has a multitude of actions. It is a major growth factor for the proliferation and differentiation of B-cell and T-cell lymphocytes. In contrast, IL-6 appears to be inhibitory on the growth of fibroblasts and endothelial cells, perhaps mediated in a negative autocrine feedback loop. IL-6 has been demonstrated to
increase endothelial permeability *in-vitro* (Maruo, 1992). However, the major target organ for IL-6 would seem to be the liver. Twenty minutes following an injection of radio-labelled, human recombinant IL-6 into rats, about 80 % had disappeared from the circulation and was found in the liver (Castell, 1989). Furthermore, it was exclusively localised on the surface of the liver parenchymal cells suggesting the existence of a membrane bound IL-6 receptor (IL-6R). This receptor is now characterised and is made up of 2 trans-membrane subunits (Callard, 1994). The IL-6R α chain subunit binds IL-6 but does not signal. The β subunit (gp130) does not itself bind IL-6 but interacts with the α chain/IL-6 complex and is responsible for signal transduction (Kishimoto, 1992). In the case of the hepatocyte, this signal transduction brings about the synthesis and release of the acute phase proteins. The nature and role of such proteins with regard to inflammation is described in section 1.12.

While many pro-inflammatory cytokines have significant cardiovascular effects (Reidy, 1993), the intravenous infusion of IL-6 into an animal has little in the way of haemodynamic change (Casey, 1993). However, IL-6 is now recognised as an endogenous pyrogen, and intravenous infusion of human recombinant IL-6 into rabbits leads to a rapid onset of fever (Heinrich, 1990).

In contrast to TNFα (section 1.9.1) IL-6 is commonly detected in the sera of patients with acute pancreatitis, particularly during the early course of the disease. Both the serum concentration of IL-6 on admission and the peak level of IL-6 correlate to a degree with the severity of the disease (Heath, 1993; Leser, 1991; Viedma, 1992; Windsor, 1993). IL-6 has also been shown in other conditions associated with an inflammatory response to correlate with the severity of the disease, including sepsis (Damas, 1992), thermal injury (Drost, 1993) and the development of organ failure in patients with intra-abdominal sepsis (Patel, 1994),
trauma (Hoch, 1993) and following cardio-pulmonary bypass in cardiac surgery (Casey, 1993).

1.9.3 Interleukin-8

Interleukin-8 (IL-8) belongs to a family of pro-inflammatory molecules called chemokines (Horuk, 1994). Prior to the introduction of interleukin nomenclature, a variety of descriptive terms were applied to molecules, often isolated from monocyte cultures, that could activate or were chemotactic for neutrophils (Callard, 1994). These molecules were subsequently found to be identical, i.e. IL-8. IL-8 is produced by multiple cell types, including monocytes, lymphocytes, granulocytes, fibroblasts, endothelial cells, bronchial epithelial cell and hepatocytes. Following stimulation of such cells, the production of IL-8 tends to lag behind that of TNFα or IL-6, although the production of IL-8 results from similar stimuli, for example, bacterial lipopolysaccharide in the case of monocytes and TNFα in the case of endothelial cells (Casey, 1993).

The gene for IL-8 is found on chromosome 4. It is generated as a 99 amino acid polypeptide. While IL-8 shows no significant sequence homology with other cytokines, it manifests considerable sequence homology with peptides secreted by platelets and macrophages that have similar activating and chemotactic effects on immune competent cells.

In common with other cytokines, IL-8 interacts with its target cell via surface bound receptors, and a number of these are described (Horuk, 1994; Rot, 1992). Two specific IL-8 receptors have been described, IL-8RA and IL-8RB (Callard, 1994). Both are seven transmembrane spanning, G-protein-linked receptors of the rhodopsin superfamily. Neutrophils, basophils and lymphocytes are the main cell types recognised to express these functional receptors. In contrast to
the narrow ligand specificity of IL-8RA and IL-8RB, receptors expressed by human erythrocytes have a broad specificity for chemokine attachment. This multi-specific receptor, designated the erythrocyte chemokine receptor has been identified as the Duffy blood group antigen, which is also the receptor for the human malarial parasite, *Plasmodium vivax* (Horuk, 1994). The role of this receptor is unclear but it is postulated that this receptor acts as a sink for IL-8 that may enter the circulation (Tilg, 1993). At least to date, binding of IL-8 to this receptor has not been shown to influence any of the physiological functions of erythrocytes.

IL-8 is thought to exert predominately pro-inflammatory activity and is present in the sera of patients with sepsis (van Zee, 1991) as well as those with acute pancreatitis (Galloway, 1994; Gross, 1992). The serum IL-8 concentration also tends to correlate with the severity of disease in acute pancreatitis. Furthermore, the concentration of IL-8 in bronchoalveolar lavage fluid, obtained on the first day of admission from patients with acute pancreatitis was shown to be greater in those patients who subsequently developed acute lung injury compared with those who did not (Donnelly, 1993). However, the role of IL-8 in inflammatory conditions is still not clear.

### 1.10 Platelet-activating factor

Platelet-activating factor (PAF) (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is an ether phospholipid molecule that is stored in precursor form within cell membranes of a number of cell types including endothelial cells, leucocytes and pancreatic acinar cells. PAF is produced by *de novo* synthesis or by remodelling with the consecutive enzymatic action of phospholipase A2 and acetyl transferase on 1-O-alkyl-2-acyl-sn-glycero-3-phosphocholine. (In the remodelling
pathway, phospholipase A₂ is the rate-limiting enzyme. This enzyme is known to
be elevated in patients with acute pancreatitis and the highest serum levels tend to be
found in those with severe disease (Bird, 1989; Nevalainen, 1993; Schroder, 1980). A multitude of stimuli for the production of PAF exist and include vasoactive
agonists such as thrombin, histamine and bradykinin as well as cytokines such as
TNFα and interleukin-1β, and there is evidence that PAF itself, may induce PAF
synthesis (Heller, 1992).

PAF exerts its action via a cell surface receptor and is believed to play a role
in a large number of cell signalling mechanisms involved with inflammation (Chao,
1993). These transmembrane spanning receptors, functionally linked to G-
proteins are found on a variety of cell types including most leucocytes, platelets,
macrophages, including Kupffer cells and alveolar macrophages, and endothelial
cells throughout the body. The regulatory factors on PAF receptors is unclear,
although PAF itself has been shown to down-regulate its own receptor.

The term platelet-activating factor is somewhat of a misnomer, as platelet
activation is just one of its many functions. PAF is able to evoke neutrophil
activation causing chemotaxis, aggregation, superoxide release and degranulation.
PAF also increases neutrophil adhesiveness to endothelial cells (Kubes, 1993) and
increases both endothelial permeability and leucocyte migration across the
endothelium (Heller, 1992; Zimmerman, 1992). An additional, important action of
PAF in inflammation is in arachidonic acid metabolism. Various cell types release
arachidonic acid when PAF is synthesised as well as in response to PAF stimulation
(Chao, 1993). The production of leukotrienes, thromboxanes and prostaglandins
from arachidonic acid may amplify and/or mediate the actions of PAF.
Emanuelli and co-workers (Emanuelli, 1989) demonstrated that the injection of PAF into the superior pancreatico-duodenal artery of rabbits increased serum amylase and induced the morphological changes typical of acute pancreatitis. Since then a number of groups have demonstrated a protective effect on pancreatic cell damage by pre-treatment with PAF receptor-antagonists in animal models of acute pancreatitis (Dabrowski, 1989; Fujimura, 1992; Hirano, 1992; Jancar, 1988; Konturek, 1992; Zhou, 1993). These PAF receptor-antagonists are chemically similar to PAF but do not initiate a biological response following binding with the PAF receptor. Treatment with such PAF receptor-antagonists after the initiation of acute pancreatitis appears to be less successful (Leonhardt, 1992). Nevertheless, pre-treatment with PAF receptor-antagonists in experimental acute pancreatitis has also been demonstrated to attenuate a number of systemic effects typical of the disease such as haemodynamic upset (Ais, 1992) and acute lung injury (Zhou, 1992). Indeed, a PAF receptor-antagonist is currently undergoing clinical trials in patients with acute pancreatitis.

1.11 Acute phase proteins: C reactive protein

The acute phase proteins are a group of proteins synthesised by the liver in response to a variety of stimuli such as tissue trauma, ischaemia, inflammation, infection and malignancy. They include C-reactive protein, serum amyloid A, haptoglobin, transferrin, ceruloplasmin, $\alpha_1$-anti-trypsin, $\alpha_1$-anti-chymotrypsin, fibrinogen (see section 1.4) and a number of the complement proteins (see section 1.6). The concentration of these acute phase proteins in the serum can rise 20 to 100 fold following hepatocyte stimulation (Stahl, 1987). The chief messenger in this respect is considered to be IL-6, exerting its effect at the level of transcription of the acute phase protein genes (Marinkovic, 1989; Morrone, 1988). Interleukin-$1\beta$ and
TNFα also influence the acute phase protein response, perhaps indirectly by stimulating IL-6 production, and directly at the level of acute phase protein gene translation (Mazlam, 1994). Glucocorticoids also have a positive feedback on this response (Castell, 1989). In addition, unknown control mechanisms must regulate acute phase proteins individually to allow the varying acute phase protein profiles seen in differing diseases.

The acute phase proteins possess wide ranging functions including modulation of the host response at the site of inflammation and the binding of toxins such as bacterial products and proteolytic enzymes released from the sites of inflammation. C-reactive protein (CRP) was discovered in 1930 as a substance which precipitated pneumococcal C-polysaccharide (Tillett, 1930), hence its name. CRP acts as an opsonin, recognising and binding in a calcium dependent manner to molecular groups found on a wide variety of bacteria and fungi. This process can activate complement and enhance phagocytosis. Haptoglobin's main action is to bind free haemoglobin in the plasma. It also binds free iron and by reducing the free iron concentration in the plasma or tissues may act as a bacteriostat as most bacteria require a level of free iron in the tissues in order to proliferate (Eaton, 1982). Transferrin is also an important iron carrying molecule and may contribute to the iron control system attempting to minimise bacterial proliferation. An additional function of transferrin is in host cell proliferation. Many human cells express receptors to transferrin on the cell surface before undergoing cell division. The availability of sufficient transferrin may be a requirement for the proliferation of many cell types, including T-cell lymphocytes (Tormey, 1972). The primary function of ceruloplasmin is as a copper transport protein. However, its action as a circulating antioxidant has been demonstrated (Goldstein, 1982). α1-anti-trypsin and α1-anti-chymotrypsin, as their names suggest, are important anti-proteases, complexing out proteolytic enzymes released from damaged parenchymal cells and
leucocytes at sites of inflammation. The acute phase protein response is clearly important to the host but in some cases will contribute significantly to the hypermetabolism associated with a systemic inflammatory response which is believed to drive the development of organ failure in critical illness (Cerra, 1987).

CRP is the most rapidly detectable of the acute phase proteins and has proved a useful marker of disease activity in a variety of inflammatory conditions. Serum CRP concentrations are elevated in patients with acute pancreatitis and both overall and peak CRP level have been shown to distinguish mild from severe disease (Mayer, 1984; Puolakkainen, 1987; Wilson, 1989). The rise in serum CRP lags behind the peak in serum IL-6 concentration by some 24 to 36 hours (Colley, 1983; Windsor, 1993).

1.12 Endotoxin

The terms endotoxin and bacterial lipopolysaccharide are often used synonymously, although more precisely, endotoxin refers to the impure extract of lipopolysaccharide found in combination with different proteins. Endotoxin is derived from the cell wall of Gram-negative and some Gram-positive bacteria following bacterial lysis. The bacterial cell wall is made up of a mucopolysaccharide-peptidoglycan layer, a phospholipid layer and an outermost lipopolysaccharide layer. Lipopolysaccharide serves as a selective permeability barrier and controls the transport of molecules into and out of the cell and it aids in the interaction of the bacterium with its environment (Watson, 1994). The lipopolysaccharide molecule consists of a polysaccharide chain that extrudes into the environment and shows marked heterogeneity among different bacterial strains. The inner part of the polysaccharide chain, called the core region is more conserved
and anti-core antibodies may therefore recognise endotoxin derived from different bacteria. The best conserved part of the molecule is the lipid moiety, termed lipid A. This is bound covalently to the polysaccharide chain and serves to anchor the lipopolysaccharide molecule in the bacterial membrane (Kimmings, 1994). It is the lipid A component that contains virtually all the biological activity of endotoxin.

Endotoxaemia is a feature of acute pancreatitis (Exley, 1992; Foulis, 1982; Kivilaakso, 1984). Foulis and co-workers (Foulis, 1982) detected endotoxin in the serum of 50% (13/26) of patients with acute pancreatitis. More recently, Windsor and co-workers (Windsor, 1993) have shown a significant fall in the serum levels of endogenous anti-endotoxin core antibodies in 28 of 33 cases, implying exposure to endotoxin. Furthermore, severe disease was associated with a greater depletion of antibody. The source of endotoxin may be from an infective focus but is more likely to come from the gut, as a result of increasing intestinal permeability in response to the stress of the disease (Roumen, 1993; van Deventer, 1988). Exposure to bacterial lipopolysaccharide (LPS) results in monocyte and tissue macrophage activation, and indeed, LPS is used as a standard stimulus of such cells in vitro. While monocytes are able to recognise LPS directly (Dentener, 1993), activation is potentiated when LPS form a complex with serum proteins such as LPS-binding protein (a glycoprotein synthesised by the liver as an acute phase protein) (Mathison 1992) which then interact with the CD14 receptor on the surface of the monocyte (Wright, 1990). Such cellular activation includes the increased synthesis and release of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6 and IL-8 (Andersson, 1992). Monocytes in turn, down-regulate their responsiveness by shedding their surface CD14 receptor (Bazil, 1991).

Endotoxaemia is observed in a number of conditions and it is unlikely to be the primary event in the pathogenesis of acute pancreatitis. Nevertheless, its
presence could contribute to intra-vascular monocyte activation and the subsequent development of the systemic inflammatory response syndrome associated with the disease. Endotoxin is recognised to activate the complement system and may be responsible for changes in the complement system described above (Foulis, 1982; Goldstein, 1978). Endotoxin is also able to induce endothelial cell activation, possibly directly or mediated via LPS interaction with the soluble CD14 receptor (Arditi, 1993; Issekutz, 1993). Exposure to LPS has also been implicated in mediating increased neutrophil adhesiveness by activation of the CR3 receptor (Wright, 1991). Furthermore, endotoxin appears not only to stimulate the production of pro-inflammatory cytokines, but may also potentiate their action at the target cell.

An extension to the endotoxin story is the presence of pancreatic sepsis complicating the acute inflammation. The presence of proven clinical pancreatic sepsis is recognised as a risk factor for increased morbidity and mortality in acute pancreatitis (Beger, 1986; Widdison, 1993). The method of infection is not fully established but may be haematogenous or may represent bacterial translocation from the gut. Such a phenomenon has been demonstrated in a rat (Tarpila, 1993) and a feline (Widdison, 1994b) model of acute pancreatitis but its relevance as a mechanism of pancreatic infection in human disease is unclear. Furthermore, a randomised multi-centre clinical trial of antibiotic prophylaxis in patients with acute pancreatitis while reducing septic complications had no effect on the incidence of multiple organ failure or mortality (Pederzoli, 1993). Nevertheless, it is now clear that during critical illness, including acute pancreatitis, structural and functional changes occur throughout the intestinal tract that serve to reduce the integrity of the gut (Lacey, 1990). The mechanisms for such alteration in gut integrity include ischaemia, hypoxia and malnutrition (Saadia, 1990), and indeed may be exacerbated by the presence of pancreatic enzymes within the gut lumen (Bounous, 1977). The
state of nutrition at the level of the gut during illness is of current interest and research. The amino-acid glutamine, is now recognised to be an important energy substrate for the gut, and indeed, most rapidly dividing cells such as the immune system. (Glutamine and its role in gut and immune function is discussed more fully in Chapter 9). However, this non-essential amino-acid in health, becomes conditionally essential during illness as its requirement appears to exceed synthesis at such times manifest by a fall in glutamine concentration in both plasma and the intracellular free amino acid pool (Lacey 1990). There is increasing evidence to suggest that the exogenous administration of glutamine, either by the enteral or parenteral route, may have beneficial effects on gut and immune cell function (van der Hulst, 1993; O'Riordain, 1994).

The findings of scientific research presented in this introduction so far have indicated that severe acute pancreatitis is associated with increased levels of serum markers of a systemic inflammatory response compared with patients with mild disease. However, the serum markers measured may have been derived from the tissues, endothelial cells or leucocytes but the source of such mediators cannot be identified from these studies. Nevertheless, the findings reflect the host systemic response to pancreatic inflammation and suggest an up-regulated inflammatory response contributing to, if not mediating, systemic organ dysfunction and eventually failure. The next section, continues to explore the role of such inflammatory mediators in the pathogenesis of acute pancreatitis, both at the level of the pancreas itself and in particular, the development of distant organ injury.
1.13 Potential biological effects of serum pro-inflammatory molecules in acute pancreatitis

Acute pancreatitis, by definition, involves acute inflammation. Acute inflammation is a process that occurs when living tissue is damaged whereby the surrounding areas undergo a series of changes which result in phagocytic cells and elements of circulating plasma entering the damaged area. The aim is to limit further tissue injury and at the same time is a mechanism of repair and healing. The presence of pro-inflammatory cytokines, platelet-activating factor, acute phase proteins and other vasoactive substances within the region of pancreatic inflammation during acute pancreatitis is generally considered beneficial, acting as messengers or signals to recruit and activate immune competent cells to the site of pancreatic injury. And thus, in the majority of patients with acute pancreatitis, it is a self-limiting condition and unlikely to reoccur if the precipitating factor is identified and removed. However, a proportion of patients, as already discussed, can develop a lethal, fulminant systemic illness often associated with the development of multiorgan failure. This has been attributed to a reaction to infection of pancreatic necrosis but these life threatening events can also occur in the absence of any identifiable focus of infection (Allardyce, 1987). While the patients may appear septic, they do not all have an underlying infection and dead or injured pancreatic tissue can replace bacteria as the stimulus for the septic or systemic inflammatory response (Rinderknecht, 1988).

It is now realised that in the development of a systemic inflammatory response, the host is not an innocent bystander whose tissues are being directly ravaged by products of injured tissue, but instead is an active participant in this destructive process (Deitch, 1992). A number of hypotheses have been put forward to explain these events which contain overlapping elements but the basic element is
an uncontrolled or persistent immuno-inflammatory response (Border, 1988). Clearly, an initiating clinical event is required, in this case acute pancreatitis, which effects multiple normal homeostatic mechanisms. These altered, normally well controlled, homeostatic systems interact to amplify or modulate each other. For example, tissue hypoxia within the pancreatic inflammation, activates resident macrophages and circulating monocytes and neutrophils and multiple humoral plasma protein cascades. Products of these activated leucocytes and protein cascades may in turn further impair oxygen delivery by their effects on the microcirculation. Further leucocyte recruitment to the site of inflammation occurs and this vicious cycle continues.

1.13.1 Macrophage hypothesis

In the macrophage hypothesis, excessive or prolonged activation or stimulation of macrophages ultimately results in excessive production, surface expression and liberation of cytokines and other products, which exert initially local and then systemic deleterious effects. In other words, the inflammatory response can escape the local environment and induce a generalised systemic response, resulting in the activation of multiple inflammatory effector cells such as macrophages, neutrophils and lymphocytes as well as the intra-vascular activation of humoral protein cascades. Through this uncontrolled intra-vascular response, the vascular endothelium may be damaged, thereby further potentiating distant organ injury. Ultimately, systemic inflammation may become self-perpetuating because of both the continued leak or spill-over of locally or systemically produced inflammatory mediators into the circulation and inadequate regulation of the inflammatory response by the host. Thus, while inflammation aids the host at the tissue level, systemic activation can represent a major potential host liability. This hypothesis, that distant organ injury is related to an uncontrolled or persistent inflammatory state is consistent with the post-mortem study of Nuytinck and co-
workers (Nuytinck, 1986) who found an association between the presence of acute lung injury and histologic evidence of organ inflammation in patients with major trauma.

Support for this hypothesis is based on the recognition that macrophage activation and cytokine release can produce a systemic inflammatory response, indistinguishable from severe infection. Elevation of serum TNFα, IL-6 and IL-8 levels in patients with acute pancreatitis have been discussed in section 1.9. Furthermore, the injection of TNFα into human volunteers produces many of the systemic and immunologic signs associated with the systemic inflammatory response (Michie, 1988a). While TNFα may mediate some of its damaging effects directly, evidence is pointing towards other mediators, downstream of TNFα, as key mediators. One of these mediators could be IL-8.

IL-8 is a potent chemo-attractant for circulating leucocytes to sites of acute inflammation (Baggiolini, 1989; Kuijpers, 1992; Nourshargh, 1992). Endothelial surface-bound IL-8 is known to promote the CD11-CD18 integrin-mediated adhesion between neutrophils and endothelial cells (Huber, 1991; Lo, 1989; Tanaka, 1993). In addition, IL-8 induces neutrophil shape change and the shedding of L-selectin from the surface of neutrophils adhered to endothelial cells, events that are essential for neutrophil emigration (Burns, 1994; Smith, 1991). However, several groups (Gimbrone, 1989; Luscinskas, 1992; Moser, 1993) have shown that while certain inflammatory stimuli render cultured human endothelial cells hyper-adhesive for neutrophils, this state is transient and reversible. The mechanism for the attenuation of endothelial cell-neutrophil interaction was attributed in part to the secretion by activated endothelial cells of a soluble leucocyte adhesion inhibitor, identified as IL-877, the extended amino-terminal IL-8 variant (Gimbrone, 1989). Furthermore, recombinant human IL-8 inhibited neutrophil adhesion to IL-1-
activated endothelial monolayers, protecting the monolayers from neutrophil-mediated damage (Gimbrone, 1989). One explanation for the observations reported by Gimbrone and others is that IL-8, like all chemo-attractants, is an ineffective promoter of neutrophil invasion if the stimulus is distributed equally between apical and basal compartments thereby destroying the concentration gradient. Under normal conditions of blood flow through a vessel, the IL-8 released by endothelial cells from the lumenal surface may be bound to antibodies and red cells and removed (Horuk, 1994; Tilg, 1993). However, if neutrophils come into contact with soluble blood-borne IL-8, they may undergo shape change and shedding of the L-selectin receptor before their initial contact with endothelial cells, thus losing their ability to effectively adhere to activated endothelium and migrate (Moser, 1993; Rot, 1992). Support for this hypothesis is demonstrated in a mouse model where neutrophils activated in vitro did not home in to inflammatory sites in vivo (Jutila, 1989). These prematurely intra-vascular activated neutrophils, because of their shape change or via complement induced aggregation, lose their deformability and thus cannot pass through capillary beds. They are thus carried in the circulation to sequester in the next micro-circulation they encounter (Hechtmann, 1991). This sequence of events such as this may account for the findings described earlier of prominent leucocyte margination in the liver and then the lungs in models of acute pancreatitis (Kelly, 1991).

1.13.2 Micro-circulatory hypothesis

In essence, the micro-circulatory hypothesis of multiple organ failure proposes that organ injury is related to ischaemia or vascular endothelial cell injury. Inadequate oxygen delivery (Cain, 1991), ischaemia-reperfusion injury (Grace, 1994; Metinko, 1992) and tissue injury due to endothelial-leucocyte interactions (Osborn, 1990) contribute to the local and systemic tissue destruction. However, the micro-circulatory and macrophage hypotheses overlap as systemic inflammation
adversely affects the micro-circulation whereas ischaemia can exaggerate the host's inflammatory response to subsequent stimuli by activating neutrophils and priming macrophages (Pober, 1990).

Endothelial cells are active participants in the regulation of blood flow, coagulation and inflammation. TNFα (and to a lesser extent other cytokines) are able to induce a change in endothelial phenotype from a non-inflammatory to a pro-inflammatory, pro-coagulant phenotype (Pober, 1990). The endothelial cells express tissue factor, can bind factor VII a and thus activate the extrinsic clotting pathway. In addition, these endothelial cells express surface receptors that promote leucocyte adherence and secrete leucocyte-activating factors such as PAF and IL-8. This shift in endothelial phenotype ultimately results in focal micro-vascular thrombosis and leucocyte-mediated endothelial cell injury. If widespread, this phenomenon can progress to tissue ischaemia and ultimately organ failure.

1.13.3 Gut hypothesis

In this hypothesis, intestinally derived bacteria or endotoxin serve as triggers to initiate or perpetuate or exacerbate the septic state. Bacteria and endotoxin from the gut efficiently induce cytokine secretion by resident tissue macrophages, promote a pro-inflammatory endothelial cell phenotype, stimulate neutrophil protease and oxidant production and activate multiple humoral protein cascades. In acute pancreatitis, the gut hypothesis is unlikely to be the primary initiating event of the systemic inflammatory response observed in patients with severe disease. However, this response is associated with impaired gut perfusion which will increase intestinal permeability, potentiate endotoxin translocation and feedback in a positive manner to the systemic inflammatory response state (Saadia, 1990). Once this cycle is initiated, it can become self-sustaining with progression of the systemic
inflammatory response, perhaps independent at this stage from the initial pancreatic cell injury.

The finding of elevated serum levels of pro-inflammatory cytokines and other inflammatory molecules from activated leucocytes does not in itself, confirm their role as mediators of the systemic inflammatory responses. More compelling evidence comes from interventional animal studies, and in particular, those employing antibodies to such cytokines. An antibody to endotoxin in conjunction with the antibiotic gentamycin decreased TNFα production which correlated with survival in a mouse model of Gram-negative sepsis (Mayoral, 1990). Anti-TNFα monoclonal antibodies have been shown to reduce lethality from endotoxaemia in rats (Sheppard, 1989) and baboons (Hinshaw, 1990; Tracey, 1987) although this is not a universal finding (Eskandari, 1992). Anti-TNFα antibodies reduced the pulmonary micro-vascular permeability but not pulmonary neutrophil sequestration following intestinal ischaemia-reperfusion in a rat model (Caty, 1989). In a porcine model of endotoxaemia, anti-TNFα antibodies attenuated the pulmonary microvascular leak as well as pulmonary neutrophil sequestration (Windsor, 1994). Anti-IL-6 monoclonal antibodies also protect against lethal *Escherichia coli* infection and lethal TNFα challenge in mice (Starnes, 1990). An anti-TNFα antibody in a rat model of acute pancreatitis attenuated the serum changes in amylase, calcium and glucose concentration as well as reducing the volume of ascites and fall in haematocrit (Grewal, 1994). While pancreatic inflammation as assessed by histological scores was unchanged, the lung scores were improved in the antibody treated group compared with the untreated group.
1.14 Leucocyte function in acute pancreatitis

A large body of evidence to support the presence of a systemic inflammatory response in patients with acute pancreatitis has been presented above. Furthermore, the greater the response (as determined by the serum concentration of a variety of inflammatory mediators including pro-inflammatory cytokines) the more likely the patients will progress to severe diseases characterised by pancreatic necrosis and organ dysfunction. While such serum changes would point to a state of leucocyte activation, there is little direct evidence to support the presence of generalised intra-vascular leucocyte activation in patients with acute pancreatitis. Larvin and co-workers (Larvin, 1993) measured the clearance of trypsin from the plasma as an index of phagocytic function in patients with acute pancreatitis. Those patients with severe disease had significantly prolonged clearance of trypsin compared with patients with mild disease and healthy volunteers suggestive of impaired phagocytic function in such patients. In the same study, monocytes and polymorphonuclear leucocytes were isolated from peripheral blood and the \textit{in vitro} zymosan-stimulated phagocytic index measured. There was a step wise reduction in median monocyte phagocytosis between healthy volunteers, patients with mild disease and those with severe disease. In contrast, there was no significant difference in polymorphonuclear leucocyte phagocytosis between the three groups. In another study (Widdison, 1994c), \textit{in vitro} neutrophil chemotaxis in patients with either mild or severe acute pancreatitis was no different from controls while random motility (reflecting increased metabolic activity) was reduced in patients with severe disease but not mild disease compared with controls. Antibody independent opsonisation was similar for the three groups while chemiluminescence (an indirect measure of bactericidal oxidative metabolism) was increased in patients with severe disease but not mild disease compared with controls.
1.12.1 CD4-positive T helper lymphocyte dysfunction in acute pancreatitis

T helper cells are important lymphocytes which control and modulate the development of immune responses. They express a 62 kDa glycoprotein, the CD (cluster designation) 4 antibody, in contrast to the 76 kDa glycoprotein on CD8 positive T cells that defines the T cytotoxic/suppresser cell population. T helper cells produce a number of cytokines, in particular interleukin-4 and interleukin-10. These cytokines have major down-regulatory effects on other lymphocytes as well as macrophage cytokine synthesis and effector function (Sher, 1992). Reduced T helper cell numbers and T cell proliferation occur in patients with burns and trauma and correlate with the incidence of sepsis and outcome (Curley, 1993). It is possible that the presence of T helper cell suppression, in conditions manifesting a systemic inflammatory response (and associated elevated serum levels of pro-inflammatory cytokines) such as burns, trauma and acute pancreatitis, may contribute to excessive macrophage activation, and pro-inflammatory cytokine production.

Curley and co-workers (Curley, 1993) have demonstrated a depletion of CD4-positive T helper lymphocytes in patients with acute pancreatitis with the greatest depletion occurring in those with severe disease. At the same time, it was observed that these patients with severe disease had significantly higher serum IL-6 levels compared with those patients with mild disease. Following resolution of the pancreatitis and recovery of the patient, the CD4-helper population returned towards normal values. In this study, CD8-positive suppresser lymphocytes numbers were not measured to allow determination of the CD4/CD8 ratio. It is not clear whether the CD4/CD8 ratio or the absolute numbers of CD4 positive cells is more important in determining a more useful index of lymphocyte imbalance. Nevertheless, similar findings have been reported for CD4-positive T helper cell depletion following thermal injury (O-Mahony, 1985) and trauma (Faist, 1987). It is postulated that this
depletion of CD4-positive T helper cells causes an imbalance in the regulatory control of the immune response early in inflammatory conditions such as acute pancreatitis, effectively diminishing the brake on leucocyte activation and allowing deregulated leucocyte activation (Casey, 1993). In the case of monocytes and macrophages, this would allow up-regulation of pro-inflammatory cytokine production which could mediate progression of the disease to multiple organ failure as discussed above.

### 1.15 Study hypothesis

In view of the reported changes in inflammatory mediators in the serum of patients with acute pancreatitis, in particular IL-6 but also TNFα, IL-8 and other pro-inflammatory molecules, severe acute pancreatitis appears to be associated with a greater systemic inflammatory response compared with patients with mild disease. Local leucocyte as well as endothelial cell activation within the pancreas in response to the pancreatic cell injury are likely to be the main source of these inflammatory mediators. It is hypothesised, that in some cases of the disease, perhaps if the pancreatic injury is large, or if there is an idiosyncratic reaction to the pancreatic injury, spill over of these inflammatory mediators into the portal and systemic circulation can induce and propagate a generalised state of leucocyte and endothelial cell activation. This generalised inflammatory cell activation is responsible not only for the degree of pancreatic damage but also mediates distant organ dysfunction, potentiating further systemic inflammatory responses which in turn correlate with the severity of the disease.

The working hypothesis of this thesis is that the release of pro-inflammatory cytokines from peripheral blood mononuclear cells (isolated from patients with
acute pancreatitis) is increased in patients with acute pancreatitis early in the course of the disease and that this increased cytokine release contributes to the severity of the disease. Peripheral blood mononuclear cells are a mixed cell population comprising monocytes and lymphocytes. Both cell populations are recognised to produce pro-inflammatory cytokines such as TNFα, IL-6 and IL-8. PBMCs are an important cell population involved early in acute inflammation in the recognition of cellular damage and the presence of foreign antigenic material such as endotoxin. These cell types participate in inter-cellular signalling which can significantly modify each others cell function when incubated as co-cultures in comparison with cell function when incubated as monocultures (Mattern, 1994; Oka, 1993). Thus co-culture of mononuclear cells is important as this may more accurately reflect the net effect of their respective actions in vivo.

1.16 Study aims

The aim of this thesis was to examine a number of inflammatory-related events occurring in the systemic circulation in patients with acute pancreatitis and to relate these events to the severity of the disease. The pilot study was to compare in vitro pro-inflammatory cytokine release from peripheral blood mononuclear cells isolated from healthy volunteers and patients with acute pancreatitis shortly after their admission to hospital. Leading on from this, pro-inflammatory cytokine release over the early course of the disease along with the influence of known peripheral blood mononuclear cell regulatory factors (such as the T helper cell cytokines, interleukin-4 and interleukin-10, and prostaglandins) was investigated in vitro. T-cell function, in particular, blastogenesis was also studied over a similar time period.
Endothelial cells are also responsible for pro-inflammatory cytokine production. The potential regulatory role of T helper cell cytokines on endothelial cytokine release is investigated. TNFα is often undetectable in the sera of patients with acute pancreatitis. An additional aim of this study was to investigate serum levels of soluble TNFα receptors in patients with acute pancreatitis and to relate these findings to the severity of the disease. The final aim was to investigate in a double blind randomised controlled manner, the effect of glutamine supplemented total parenteral nutrition compared to conventional nutrition on peripheral blood mononuclear cell pro-inflammatory cytokine release and T-cell blastogenesis in patients with severe acute pancreatitis.
Chapter 2

Materials and Methods

2.1 Patients

2.1.1 Patients with acute pancreatitis

The diagnosis of acute pancreatitis was based on the presence of appropriate clinical or radiographic (abdominal ultrasound or computed tomography) evidence accompanied by a serum amylase concentration greater than 1 000 units/l (Phadebas; Pharmacia Daignostics, Uppsala, Sweden; normal range 70-300 units/l). All such patients were admitted and cared for by named consultant surgeons in the Royal Infirmary of Edinburgh.

2.1.2 Healthy volunteers

Control subjects were healthy volunteers, on no medication and with no known pancreatic or inflammatory disease.
2.2 Serum collection

Blood was collected for the separation of serum as follows: 10 mls of blood was removed from a peripheral vein under aseptic conditions into plain tubes (Sarstedt, Nümbrecht, Germany) through a 21 G needle (Sarstedt, Nümbrecht, Germany). The blood was transported on ice before being centrifuged (1 400 x g for 10 minutes) and the serum aliquoted into 1 ml apex tubes (Alpha Laboratories Ltd, Eastleigh, U.K.). Samples were stored within 1 hour of collection at -70 °C until subsequent batch analysis.

2.3 Isolation and culture of peripheral blood mononuclear cells

2.3.1 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood (20 mls) was collected from patients and volunteers under aseptic conditions into non-pyrogenic lithium heparin tubes (Sarstedt, Nümbrecht, Germany). The blood was layered onto an equal volume of Histopaque® (polysucrose/sodium diatrizoate)(Sigma Chemicals, Poole, U.K.) in a 50 ml polypropylene tube (Costar, Cambridge, U.S.A.) and centrifuged at 600 x g for 30 minutes. The cells at the interface were removed and washed 3 times by centrifugation in RPMI 1640 (ICN Flow Laboratories, Irvine, U.K.). The cell pellet was resuspended in RPMI 1640 medium supplemented with penicillin (50 IU/ml)(ICN Flow Laboratories, Irvine, U.K.), streptomycin (50 μg/ml)(ICN Flow Laboratories, Irvine, U.K.) and 2 mM glutamine (ICN Flow Laboratories, Irvine, U.K.) (complete medium) and either 5 % heat inactivated (56 °C: 30 min) fetal calf serum (FCS)(ICN Flow Laboratories, Irvine, U.K.) or 5 % autologous serum (AS) according to the experimental protocol to be followed. The cells were counted
using a haemocytometer and resuspended at 2 x 10^6 cells/ml. Viability was assessed by trypan blue exclusion and was 95-98%.

### 2.3.2 PBMC culture

PBMCs were cultured in triplicate at 2 x 10^5 cells per well in 200 µl of complete medium containing 5% FCS or 5% AS in 96-well flat-bottomed tissue culture plates (Costar, Cambridge, U.S.A.) and in the presence or absence of endotoxin (LPS)(bacterial lipopolysaccharide - *Escherichia coli* 0127:B9, Sigma Chemicals, Poole, U.K.) at a final concentration of 5 µg/ml. The cells were incubated at 37 °C in 5% carbon dioxide, 95% humidified air for 24 hours. The supernatants were then removed and stored in aliquots at -70 °C for subsequent batch cytokine analysis.

### 2.4 PBMC lymphoproliferation assay

PBMCs were isolated as described above (Section 2.3.1) and cultured in triplicate in the presence of phytohaemagglutinin (PHA)(Wellcome Diagnostics, Dartford, U.K.) at final concentrations of 10 µg/ml and 100 µg/ml. Control wells were cultured in the absence of PHA. After culture for 72 hours, 1 µCi [³H]-methyl-thymidine (³HTdr)(Amersham, Aylesbury, U.K.) was added to each well. After 4 hours the plates were removed and stored at -70 °C.

To determine ³HTdr uptake, the plate contents were thawed and the cells harvested onto glass microfibre filter papers (Whatman, Maidstone, U.K.) using an Automash cell harvester (Dynatech, Billinghurst, U.K.). The discs when dry were counted in 4 ml of scintillation fluid (Toluene Scintillator, Packard, Groningen, Netherlands) in an Isocap liquid scintillation counter (Searle, Uithoorn,
Netherlands) for 10 minutes with the $^3$HTdr uptake expressed as counts per minute (cpm).

2.5 Isolation and culture of human umbilical vein endothelial cells

Unlike the other methods described in this chapter, the isolation and culture of endothelial cells had not previously been undertaken in the laboratory, although there was expertise in the laboratory with regard to the isolation and culture of other primary cells, in particular, hepatocytes. A good deal of trial and error was required to obtain endothelial cell cultures which are somewhat fastidious cells to isolate, culture and passage successfully. The method described below was the outcome of this preparatory work and in my hands became for the most part, a reliable method.

2.5.1 Isolation of human umbilical vein endothelial cell cultures

Human umbilical vein endothelial cells (HUVECs) were obtained by a modification of the method of Jaffe (Jaffe, 1973). Human umbilical cords were obtained fresh and transported in ice cold Hanks balanced salt solution (HBSS)(Sigma Chemicals, Poole, U.K.) containing penicillin (100 IU/ml) and streptomycin (100 µg/ml)(HBSS). Both ends of the umbilical vein were cannulated with a 14 G intra-venous cannula (Viggo-Spectramed, Helsingborg, Sweden), secured with a nylon ligature (Ethicon, Edinburgh, U.K.) and the vein flushed with HBSS to remove any retained blood. The vein was then infused with 15 ml of 0.05 % collagenase H (Boehringer Mannheim, Lewes, U.K.) in HBSS containing 180 mM CaCl$_2$ and incubated for 15 minutes at 37 °C. Following this period the vein was flushed with RPMI 1640, the perfusate collected in 50 ml polypropylene tubes (Falcon, U.K.) and cells pelleted by centrifugation at 500 x g for 10 minutes. The
cells were washed by centrifugation in endothelial-serum free (E-SFM) growth medium (Gibco BRL, Paisley, U.K.) before being resuspended in E-SFM plating media (Gibco BRL, Paisley, U.K.) supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml), hydrocortisone (1 μg/ml) (Sigma Chemicals, Poole, U.K.), endothelial cell growth factor (50 μg/ml) (ECGF) (Boehringer Mannheim, Lewes, U.K.) and recombinant epidermal growth factor (10 ng/ml) (rEGF) (Gibco BRL, Paisley, U.K.) (complete plating medium).

2.5.2 HUVEC culture

HUVECs were cultured in 75 cm³ plastic flasks (Costar, High Wycombe, U.K.) in complete plating medium. Subsequent media change was with E-SFM growth media supplemented with the same additives as the complete plating medium (Section 2.5.1). Passage of HUVEC was as follows: primary cultures were detached by incubation with trypsin-EDTA (0.25 % trypsin, 0.02 % EDTA in phosphate buffered saline; pH 7.4), washed serially by centrifugation with RPMI 1640 supplemented with 10% FCS, then E-SFM growth media before being resuspended in complete plating medium. For the purpose of experimentation, HUVECs were cultured in triplicate and grown to confluence in 96-well flat-bottomed tissue culture plates at 37 °C in 5% carbon dioxide, 95% humidified air. All HUVEC cell lines were characterised by the expression of von Willebrand factor (Section 2.5.3) and were used for experiments between the second and fourth passage. Confluent HUVECs were incubated in the presence LPS at a final concentration of 10 ng/ml with 5% human AB serum (Sigma Chemicals, Poole, U.K.) for 5 hours. Following this period, the cells were washed with E-SFM growth media and incubated in fresh E-SFM growth media for 12 hours. The supernatant were then removed and stored in aliquots at -70 °C for subsequent batch cytokine assay.
2.5.3 von Willebrand factor characterisation of HUVECs

Cytospin slide preparations of HUVECs were made and allowed to air dry before being fixed in cold methanol for 5 minutes. The slides were washed in TBS (20 mM Tris-HCl, 150 mM NaCl: pH 7.4) and then blocked with 20 % rabbit serum (Scottish Antibody Production Unit, Carluke, U.K.) diluted in TBS for 20 minutes. The slides were washed in TBS and the primary antibody, sheep anti-factor VIII (vWF) (Scottish Antibody Production Unit, Carluke, U.K.) or the control antibody, sheep serum (Scottish Antibody Production Unit, Carluke, U.K.) diluted 1/100, 1/1000 or 1/5000 in TBS added and incubated for 30 minutes. The slides were washed in TBS and incubated with the secondary antibody, peroxidase conjugated anti-goat IgG (Sigma Immunochemicals, Poole, U.K.) diluted 1:1000 for 30 minutes. The slides were washed in TBS and the substrate, 3,3'-diaminobenzidine tetrahydrochloride (Sigma Immunochemicals, Poole, U.K.) (1 mg/ml in 0.2 M Tris-HCl, 0.01 M imidazole; pH 7.6 containing 0.15 μl of 30 % H2O2 per ml) added observing for a brown colour change. The slides were washed in tap water, counterstained for 10 minutes in 25 % Harris haematoxylin and further washed in tap water. The slides were dehydrated serially in 64 % ethanol, 74 % ethanol, absolute ethanol and xylene at 1 minute per stage. The preparation was mounted in DePex (BDH Ltd, Poole, U.K.) and examined by light microscopy.

2.6 Cytokine and C-reactive protein enzyme-linked immunosorbant assays

2.6.1 Tumour necrosis factor-α ELISA

Ninety-six well ELISA plates (Costar, Cambridge, U.S.A.) were coated with mouse monoclonal anti-human-TNFα clone 199 antibody (Boehringer Mannheim, Lewes, U.K.) diluted in coating buffer (50 mM carbonate-bicarbonate; pH
9.6)(Sigma Chemicals, Poole, U.K.), according to the manufacturers instructions, at 100 μl per well. After a 4 hour incubation at room temperature, the antibody was removed and the wells blocked with coating buffer containing 1 % bovine serum albumin (BSA)(Sigma Chemicals, Poole, U.K.) for 20 minutes at room temperature. The plates were then washed five times in TBS containing 0.1 % Tween-20 (Sigma Chemicals, Poole, U.K.). Sera diluted 1:5, or PBMC supernatants diluted 1:7 and 1:28 in TBS containing 10 mM EDTA (Sigma Chemicals, Poole, U.K.) and 1 % BSA were added to wells in duplicate. Standards of recombinant human TNFα (R & D Systems, Oxon, U.K.) in doubling dilution from 400 pg/ml to 3.12 pg/ml were added to wells in duplicate. Additionally, two wells were filled with diluent only and two with an international standard TNFα (National Institute for Biological Standard and Control, Potters Bar, U.K.) at 50 pg/ml. After overnight incubation at 4 °C, the plates were washed again and 100 μl of peroxidase-conjugated mouse monoclonal anti-human-TNFα, clone 196, Fab fragments (Boehringer Mannheim, Lewes, U.K.) diluted according to the manufacturers instructions was added for 2 hr at room temperature. The plates were washed as described before. The substrate solution, 0.1 mg/ml tetramethylbenzidine (Boehringer Mannheim, Lewes, U.K.) in 100 mM sodium citrate-acetate buffer; pH 4.9 (Sigma Chemicals, Poole, U.K.) was added at 100 μl per well. The reaction was stopped with 100 μl per well 1 M H₂SO₄ (BDH Ltd, Poole, U.K.) and the plates read at 450 nm using a MR5000 ELISA plate reader (Dynatech, Billinghurst, U.K.). Concentrations in the samples were calculated with "AssayZap" computer software (Biosoft, Cambridge, U.K.). The intra-assay co-efficient of variation was 3.3 % and the inter-assay co-efficient of variation was 3.2 % calculated by the method described by Tijssen (Tijssen, 1985).
2.6.2 Interleukin-6 ELISA

IL-6 was assayed in serum diluted 1:5 and 1:30, PBMC supernatants diluted 1:7 and 1:28 and HUVEC supernatants diluted 1:5 and 1:30. The method was essentially as described for the TNFα ELISA (Section 2.6.1). The coating antibody was mouse monoclonal anti-human-IL-6 antibody (Boehringer Mannheim, Lewes, U.K.) diluted to 3 μg/ml. The standard curve was recombinant human IL-6 (R & D Systems, Oxon, U.K.) in doubling dilution from 4000 pg/ml to 7.8 pg/ml with an international standard IL-6 (National Institute for Biological Standard and Control, Potters Bar, U.K.) at 500 pg/ml. The second antibody was goat polyclonal (non-enzyme-labelled) anti-IL-6 (R & D Systems, Oxon, U.K.) diluted to 3 μg/ml. This was detected by incubation with polyclonal sheep anti-goat-IgG conjugate with horseradish peroxidase (Sigma Immunochemicals, Poole, U.K.) diluted 1:10 000 for 2 hours at room temperature. The substrate was added as described above (Section 2.6.1). The intra-assay co-efficient of variation was 3.6 % and the inter-assay co-efficient of variation was 4.4 %.

2.6.3 Interleukin-8 ELISA

IL-8 was assayed in serum diluted 1:5 and 1:30, PBMC supernatants diluted 1:28 and 1:98 and HUVEC supernatants diluted 1:5 and 1:30. The method was essentially as described for the TNFα ELISA (Section 2.6.1). The coating antibody was rabbit polyclonal anti-human-IL-8, (AMS Biotechnology UK Ltd, Witney, U.K.) diluted 1:2 000. The standard curve was recombinant human IL-8 (Genzyme, West Malling, U.K.) in doubling dilution from 10 000 pg/ml to 20 pg/ml with an international standard IL-6 (National Institute for Biological Standard and Control, Potters Bar, U.K.) at 500 pg/ml. The second antibody was goat polyclonal (non-enzyme-labelled) anti-IL-8 (R & D Systems, Oxon, U.K.) diluted to 3 μg/ml. This was detected by incubation with polyclonal sheep anti-goat-IgG conjugate with horseradish peroxidase (Sigma Immunochemicals, Poole, U.K.) diluted 1:10 000 for
2 hours at room temperature. The substrate was added as described above (Section 2.6.1). The intra-assay co-efficient of variation was 1.3 % and the inter-assay co-efficient of variation was 1.3 %.

2.6.4 Soluble TNFα receptor (55 and 75 kDa) ELISAs

Soluble TNFα receptors (sTNFR55 and sTNFR75) were assayed in serum samples diluted 1:5 and 1:30. The antibodies were kindly donated by Dr. W. Buurman, Maastricht, Netherlands. The method was essentially as described for the TNFα ELISA (Section 2.6.1). The coating antibodies were mouse anti-sTNFR55 or sTNFR75 diluted 10 μg/ml and 5 μg/ml respectively. Purified sTNFR55 and sTNFR75 were used to construct standard curves from 2 500 pg/ml to 20 pg/ml. The second antibody was biotinylated rabbit anti-sTNFR55 or sTNFR75, diluted 1:6 000 and 1:4 000 respectively, and detected by incubation with streptavidin ABC complex/HRP (DAKO, High Wycombe, U.K.) diluted 1:200 in TBS for 1 hour at room temperature. The substrate was added as described above (Section 2.6.1). The intra-assay co-efficient of variation for sTNFR55 and sTNFR75 was 2.4 and 1.9 % and the inter-assay co-efficient of variation was 2.4 and 1.9 % respectively.

2.6.5 C-Reactive protein ELISA

CRP was assayed in serum samples diluted 1:7 000 and 1:100 000. The method was essentially as described for the TNFα ELISA (Section 2.6.1). The coating antibodies was rabbit anti-human CRP (DAKO, High Wycombe, U.K.) diluted 1:800. A human serum CRP calibrator (DAKO, High Wycombe, U.K.) was used to construct standard curves from 100 μg/ml to 0.39 μg/ml. The second antibody was peroxidase-conjugated rabbit anti-human CRP (DAKO, High Wycombe, U.K.) diluted 1:3 000. The substrate was added as described above (Section 2.6.1). The intra-assay co-efficient of variation was 3.3 % and the inter-assay co-efficient of variation was 3.2 %.
2.7 Preparation of PBMCs for fluorescence activated cell sorter (FACS) analysis

PBMCs were isolated from whole blood by density centrifugation as described in Section 2.3.1. Following counting using a haemocytometer, the cells were pelleted and washed by centrifugation in PBS/1 % BSA with azide. The cells were resuspended in 20 % rabbit serum (Scottish Antibody Production Unit, Carluke, U.K.) in PBS/1 % BSA with azide at 1 x 10^5 to 10^6 cells per 50 μl and left at room temperature for 20 minutes in order to block any non-specific Fc receptor binding which might occur. 50 μl of individual antibody (neat supernatant) and 50 μl of the cell suspension were added to LP3 tubes, mixed by agitation and left at room temperature for 30 minutes. The control antibody was anti-phycoerythrin antibody, an algal protein of mixed isotypes which should have no reactivity with human tissues (obtained from Dr von Heyningen, MRC Human Genetics Unit, Edinburgh, U.K.) The test antibodies were against the T cell CD3 antigen, the helper T cell CD4 antigen, the cytotoxic/suppressor T cell CD8 antigen (obtained from ECACC, Porton Down, U.K.) and the monocyte CD14 antigen (obtained from Professor Zola, University of Melbourne, Australia). The cells were washed by centrifugation in PBS/1 % BSA with 0.01 % azide before 50 μl of 1/100 sheep anti-mouse FITC F(ab)_2 added to the cell pellet, mixed by agitation and left at room temperature for 30 minutes. The cells were washed by centrifugation in PBS/1% BSA with azide and finally resuspended in 1 ml FACS fix (1 % formaldehyde in PBS-BSA-azole). Flow cytometry analysis was performed using a Coulter XL flow cytometer (Coulter, U.K.).
2.8 Conclusion

Every attempt was made to ensure the use of laboratory grade standard products throughout the study. However, variation in the standard concentration or biological activity of reagents, particularly those obtained from animal origin such as LPS, FCS, collagenase and cytokine standards, is well recognised. While products with the same lot number were used for similar experiments, it is inevitable that variation in final concentration or final biological activity did vary throughout the experimental work. This is reflected in variation of baseline values throughout the thesis, making comparison or pooling of results performed several months apart less reliable.
Chapter 3

Pro-inflammatory cytokine release by peripheral blood mononuclear cells from patients with acute pancreatitis

3.1 Introduction

This chapter investigates pro-inflammatory cytokine release by peripheral blood mononuclear cells (PBMCs). PBMCs are a mixed cell population comprising monocytes and lymphocytes. Both cell populations are recognised to produce pro-inflammatory cytokines such as TNFα, IL-6 and IL-8. PBMCs are an important cell population involved early in acute inflammation in the recognition of cellular damage and the presence of foreign antigenic material such as endotoxin.

Monocytes arise from progenitor cells in the bone marrow. They differentiate into blood monocytes and represent a circulating pool of phagocytes that are able to migrate into various organs and tissues to become macrophages. It is a relatively large cell (10-18 μm in diameter) containing a well developed Golgi complex and many intra-cytoplasmic lysosomes. These lysosomes contain a number of acid hydrolases and peroxidase which is important in intracellular killing.
of micro-organisms. Monocytes will adhere strongly to glass and plastic surfaces. Adherence and ingestion of cellular matter including micro-organisms is promoted when the cells bind to the micro-organism through specialised cell surface receptors for certain carbohydrates, immunoglobulin and complement. A number of these receptors are found in intracytoplasmic vesicles and are rapidly expressed following activation. Following activation, monocytes produce and release a number of inflammatory active molecules termed cytokines. Class II major histocompatibility antigens are expressed on some monocytes and are important in presentation of antigens to T-cells. In return, the functions of monocytes can be enhanced or inhibited by factors released from T-cells.

Lymphocytes are produced in the thymus and bone marrow. In peripheral blood, they are heterogeneous in both size (6-10 μm in diameter) and morphology. They are subdivided into T-cells and B-cells. B-cells are involved in the humoral immune response, producing antibodies in response to exposure to specific antigens. They have little role in acute pancreatitis and are not discussed further. In contrast, T-cells are involved in the cellular immune response. When specific antigen is encountered in the tissues, the T-cells programmed to recognise that particular antigen transform into lymphoblasts. Lymphoblasts divide by mitosis to produce activated T-cells. These activated T-cells produce a variety of substances, collectively called lymphokines which attract and activate local and blood-borne monocytes/macrophages. Activated macrophages possess greatly enhanced phagocytic activity which is directed towards the destruction and elimination of antigen. Other activated T-cells called cytotoxic T-cells promote direct destruction of cells. The CD3 complex is a human leucocyte antigen that is specific for T-cells. These cells can be further subdivided by cell surface expression of human leucocyte antigens into CD4 positive (T-helper cells) and CD8 positive (T-
cytotoxic/suppressor cells). It is the CD4 positive T-cells that appear to be most important in the regulation of monocytes.

As alluded to already, monocytes and lymphocytes participate in intercellular signalling which can significantly modify each others cell function when incubated as co-cultures in comparison with cell function when incubated as monocultures (Mattern, 1994; Oka, 1993). Thus co-culture of mononuclear cells is important as this may more accurately reflect the net effect of their respective actions in vivo. In order to examine aspects of intra-vascular leucocyte activation directly, this chapter investigates the spontaneous and endotoxin-stimulated release of pro-inflammatory cytokines from PBMCs isolated from patients with acute pancreatitis. (Endotoxaemia, measured directly or indirectly by a depletion of endogenous anti-endotoxin core antibody has been implicated in the development of multiple organ failure in patients with acute pancreatitis (Windsor, 1993). Secondly, endotoxin is a recognised standard stimulus for testing stimulated cytokine release.) Measuring such stimulated cytokine release gives important additional information compared with spontaneous release alone, indicating if increased spontaneous release is actually maximal cytokine release or if further cell stimulation can lead to further increases in cytokine release (Andersson, 1992). The multitude of inflammatory molecules found in the serum of patients with acute pancreatitis has been outlined in Chapter 1. In order to investigate the influence of serum factors on cytokine release, PBMCs were incubated in both autologous serum and fetal calf serum. Changes in cytokine release in vitro were related to the clinical course of the patients and to the serum concentration of the corresponding cytokine.
3.2 Patients and methods

Initial dose response curves were performed on PBMCs isolated from healthy volunteers to identify the appropriate working concentrations of serum in the culture medium and the dose of endotoxin for stimulation with respect to pro-inflammatory cytokine release. Time course experiments on pro-inflammatory cytokine release from PBMCs were also performed. Furthermore, cytokine release from PBMCs was compared with cytokine release from the adherent and non-adherent PBMC sub-populations (Bauer, 1988).

3.2.1 Patients

Sixteen consecutive patients with a diagnosis of acute pancreatitis (as defined in Section 2.1.1) were admitted to the University Department of Surgery, Royal Infirmary of Edinburgh. Peripheral blood was taken from the patients on the first day of admission for the isolation of peripheral blood mononuclear cells, acquisition of serum and white cell counting. The progress of the patient was evaluated with regard to the development of pancreatic complications as defined by the 1992 Atlanta Convention (Bradley, 1993) or the development of multiple organ failure as defined by the Goris score (Goris, 1985). No patients were on steroid, non-steroidal anti-inflammatory or any other immunosuppressive medication on admission. Six control subjects were healthy volunteers, (Section 2.1.2). The patients characteristics are given in Table 3.1.

3.2.2 PBMC study protocols

PBMCs were isolated and cultured as described in Sections 2.3.1 and 2.3.2.
Table 3.1

Details of patients studied. Pancreatic complications defined by the Atlanta criteria (Bradley, 1993) and organ failure by the modified Goris score (Goris, 1985). The mononuclear cell count was measured by the Department of Haematology, Royal Infirmary of Edinburgh on peripheral venous blood taken following admission to hospital.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Aetiology</th>
<th>Pancreatic complication</th>
<th>Organ failure score</th>
<th>Mononuclear cell count (x10⁹ cell/l)</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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Volunteers

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<th>Aetiology</th>
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<tr>
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<td>F</td>
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</tr>
</tbody>
</table>
3.2.2.1 Effect of fetal calf serum concentration on PBMC IL-6 and IL-8 release

PBMCs (isolated from 2 volunteers) were incubated at 2 x 10^5 cells per well in complete media (Section 2.3.2) supplemented with FCS at 0, 1, 2.5, 5 and 10 % final concentration in the presence or absence of LPS at 5 μg/ml final concentration. Following 24 hr incubation, the supernatants were removed and stored in aliquots at -70 °C for subsequent IL-6 and IL-8 assay.

3.2.2.2 Effect of lipopolysaccharide concentration on PBMC IL-6 release

PBMCs (isolated from 3 volunteers) were incubated at 2 x 10^5 cells per well in complete media (Section 2.3.2) supplemented with 5 % FCS and LPS at a concentration of 0, 0.02, 0.08, 0.31, 1.25, 5 and 20 μg/ml final concentration. Following 24 hr incubation, the supernatants were removed and stored in aliquots at -70 °C for subsequent IL-6 assay.

3.2.2.3 Effect of incubation period on PBMC TNFα, IL-6 and IL-8 release

PBMCs (isolated from 2 volunteers) were incubated at 2 x 10^5 cells per well in complete media (Section 2.3.2) supplemented with 5 % FCS in the presence or absence of LPS at 5 μg/ml final concentration. Following incubation for 3, 6, 9, 12, 18 or 24 hr, the supernatants were removed and stored in aliquots at -70 °C for subsequent TNFα, IL-6 and IL-8 assay.

3.2.2.4 PBMC IL-6 release from adherent cell, non-adherent cell and adherent cell/non-adherent cell co-cultures

PBMCs (isolated from 2 volunteers) at 2 x 10^5 cells per well were incubated in 200 μl of complete media (Section 2.3.2) supplemented with 5 % FCS for 2 hours
to allow cell adherence. Following this time period, media in the adherent cell/non-adherent cell co-culture wells was carefully changed to fresh complete media with 5 % FCS. The wells designated for adherent cell were washed 3 times with fresh media to remove the non-adherent cells, the washings collected and the wells refilled with 200 µl of complete media with 5 % FCS. The washing were centrifuged at 500 x g for 10 minutes and the cell pellet resuspended in 200 µl of complete media per well washed and replated into clean wells. Following 24 hr incubation, the supernatants were removed and stored in aliquots at -70 °C for subsequent IL-6 assay.

3.2.2.5 FACS analysis of PBMC subsets in whole blood and following purification by density centrifugation

PBMCs were isolated from two volunteers by density centrifugation as described in Section 2.3.1 and prepared for flow cytometry as described in Section 2.7. Cells were also prepared for whole blood FACS analysis. 2 ml of peripheral blood was mixed with 45 ml of lysis buffer (8.29 g NH4Cl, 1 g KHCO3, 3.7 mg EDTA in 1 l distilled water at pH 7.2). Following 10 minutes at room temperature, the cells were washed by centrifugation at 4 °C and then resuspended in PBS/0.1 % BSA at a cell concentration around 5 x 10⁶/ml. The cell suspension was then prepared for flow cytometry as described in Section 2.7. The antibodies used were antiphycoerythrin (APE) as the control along with CD3, CD4, CD8 and CD14.

3.2.2.6 PBMC TNFα, IL-6 and IL-8 release from patients and volunteers

PBMCs (isolated from 16 patients and 6 volunteers) were incubated at 2 x 10⁵ cells per well in complete media (as described in Section 2.3.2) supplemented with 5 % FCS or AS in the presence or absence of LPS at 5 µg/ml final
concentration. Following incubation for 24 hr, the supernatants were removed and stored in aliquots at -70 °C for subsequent TNFα, IL-6 and IL-8 assay.

### 3.2.3 TNFα IL-6 and IL-8 ELISAs

Supernatants and serum were assayed for TNFα, IL-6 and IL-8 by ELISAs as described in Sections 2.6.1, 2.6.2 and 2.6.3 respectively.

### 3.2.4 White cell counting

The total white cell count and the neutrophil, monocyte and lymphocyte population count in patients with acute pancreatitis was performed by the Department of Haematology, Royal Infirmary of Edinburgh.

### 3.2.5 Statistical analysis

Comparison between patient and control groups or between patients with mild and severe disease was made with the Mann-Whitney U test while comparison within patient or control groups was made with the paired Student’s t test with significance taken at a p value < 0.05.

### 3.3 Results

There was no significant difference in the mean age (55.8 years, range 23-96 v 47.5 years, range 26-73, p = 0.41) between the acute pancreatitis group and the control groups with a 1:1 sex ratio in both groups (Table 3.1). Five of the 16 patients were considered to have severe disease in that they developed complications related to their disease. Two patients had pancreatic necrosis demonstrated by contrast-enhanced CT scanning during the first week of admission and one of these patients later developed a pancreatic pseudocyst requiring surgical
drainage. Three patients had renal impairment on admission, scoring 1 on the Goris score for the renal system (serum creatinine greater than 170 μmol/l). Renal function in these three patients returned to normal limits with conservative therapy.

3.3.1 Effect of fetal calf serum concentration on PBMC IL-6 and IL-8 release

The concentration of FCS in the culture medium produces a dose-dependent increase on both spontaneous and LPS-stimulated IL-6 and IL-8 release from PBMCs. The results of a representative experiment are shown in Figure 3.1. A concentration of 5 % FCS was chosen for further PBMC culture.

3.3.2 Effect of lipopolysaccharide concentration on PBMC IL-6 release

LPS stimulation resulted in an increased release of IL-6 from PBMCs which did not appear to be dose-dependent over the dose range studied. The results of a representative experiment are shown in Figure 3.2. The final concentration of LPS chosen was 5 μg/ml. While the results would suggest that a lower concentration of LPS could have been used without reducing the degree of PBMC IL-6 release, previous PBMC experimentation in the same laboratory had employed LPS at a final concentration of 5 μg/ml and this concentration was continued to allow more direct comparison of results.

3.3.3 Effect of incubation period on PBMC TNFα, IL-6 and IL-8 release

Both spontaneous and LPS-stimulated release of TNFα, IL-6 and IL-8 from PBMCs into the culture supernatant increased with time, reaching a maximum for TNFα at 12 hr, IL-6 at 18 hr and IL-8 at 24 hr. The results of a representative experiment are shown in Figure 3.3.
Figure 3.1

Spontaneous (—) and lipopolysaccharide-stimulated (final concentration 5 μg/ml) (— -) IL-6 (○) and IL-8 (□) concentration in the culture supernatant from peripheral blood mononuclear cells isolated from healthy volunteers as a function of fetal calf serum concentration in the culture medium. 2 x 10^5 cells per well were incubated in triplicate under standard conditions in the presence of fetal calf serum (0-10 %) for 24 hr. IL-6 and IL-8 were measured by ELISA. Results of a representative experiment.
Figure 3.2

IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from healthy volunteers as a function of lipopolysaccharide concentration in the culture medium. 2 x 10⁵ cells per well were incubated in triplicate under standard conditions in the presence of lipopolysaccharide (0-20 µg/ml) for 24 hr. IL-6 was measured by ELISA. Results of a representative experiment.
Figure 3.3

Spontaneous (—) and lipopolysaccharide-stimulated (final concentration 5 μg/ml)(- -) TNFα (Δ), IL-6 (○) and IL-8 (□) concentration in the culture supernatant from peripheral blood mononuclear cells isolated from healthy volunteers as a function of the period of incubation. 2 x 10⁵ cells per well were incubated in triplicate under standard conditions for a period of time up to 24 hr. TNFα, IL-6 and IL-8 were measured by ELISA. Results of a representative experiment.
3.3.4 PBMC IL-6 release from adherent cell, non-adherent cell and adherent cell/non-adherent cell CO-culture

Spontaneous release of IL-6 from either adherent or non-adherent PBMC cultures was undetectable. In contrast, IL-6 was detectable in both adherent and non-adherent LPS-stimulated PBMC cultures. Spontaneous and LPS-stimulated release of IL-8 was detectable from either adherent or non-adherent PBMC cultures. However, both spontaneous and LPS-stimulated IL-6 and IL-8 release from adherent and non-adherent PBMC co-cultures was greater than the sum of cytokine released from the separate culture of either adherent or non-adherent PBMCs under similar culture conditions. The results of a representative experiment are shown in Table 3.2. These findings support the concept of studying cytokine release from PBMC rather than the cytokine release from isolated monocytes and lymphocyte monocultures.

3.3.5 FACS analysis of PBMC subsets in whole blood and following purification by density centrifugation

The percentage recovery of T lymphocytes including their subsets and of monocytes by density centrifugation is similar to the relative proportions found on whole blood analysis (Table 3.3). This finding alleviates the concern that density centrifugation alters the relative proportion of T-cells and monocytes making extrapolation of the in vitro results back to the in vivo situation less valid.

3.3.6 PBMC TNFα, IL-6 and IL-8 release from patients and volunteers

The mean spontaneous TNFα release from PBMCs of patients with acute pancreatitis, whether incubated in FCS or AS, was similar to that of the control group (Table 3.4). Similarly, the mean release of TNFα from LPS-stimulated PBMCs was not significantly different between the patient and the control group (Table 3.4). In contrast, the mean 24 hour, spontaneous IL-6 release from PBMCs
Table 3.2
Spontaneous and LPS-stimulated (final LPS concentration 5 μg/ml) IL-6 and IL-8 concentration in the supernatant from adherent PBMCs, non-adherent PBMCs and adherent/non-adherent PBMC co-culture (2 x 10^5 cells per well in triplicate) following 24 hour incubation. Results of a representative experiment in a healthy volunteer.

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous</th>
<th>LPS-stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-6 (pg/ml)</td>
<td>IL-8 (ng/ml)</td>
</tr>
<tr>
<td>Adherent</td>
<td>&lt;200</td>
<td>0.67</td>
</tr>
<tr>
<td>Non-adherent</td>
<td>&lt;200</td>
<td>6.34</td>
</tr>
<tr>
<td>Co-culture</td>
<td>1560</td>
<td>8.78</td>
</tr>
</tbody>
</table>
Table 3.3

FACS analysis of PBMC subsets in whole blood and following purification by density centrifugation (Histopaque® 1077). Cell surface antigens examined were CD3 (T lymphocyte), CD4 and CD8 (T lymphocyte sub-populations) and CD14 (monocyte). A minimum of 5000 cells were evaluated for each antigen. Results of a representative experiment in a healthy volunteer.

<table>
<thead>
<tr>
<th></th>
<th>Density Separation</th>
<th>Whole blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD3 positive</td>
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<td>29.3</td>
</tr>
<tr>
<td>% CD4 positive</td>
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</tr>
<tr>
<td>% CD8 positive</td>
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</tr>
<tr>
<td>% CD14 positive</td>
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</tr>
<tr>
<td>Ratio CD3/CD14</td>
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<td>3.57</td>
</tr>
<tr>
<td>Ratio CD4/CD8</td>
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<td>% CD3 and CD14 positive</td>
<td>96.3</td>
<td>37.5</td>
</tr>
</tbody>
</table>
Table 3.4

Spontaneous and lipopolysaccharide-stimulated (final LPS concentration 5 μg/ml) TNFα, IL-6 and IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers and during the first day of admission from 16 patients with acute pancreatitis. 2 x 10⁵ cells per well were incubated in triplicate in the presence of 5% fetal calf (FCS) or 5% autologous serum (AS) for 24 hr under standard conditions. * 0.81 > p > 0.18 (not significant). † 0.037 > p > 0.001. Mann-Whitney U test comparing the acute pancreatitis group with the control group in each culture condition.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>TNFα (pg/ml) Mean (SEM)</th>
<th>IL-6 (ng/ml) Mean (SEM)</th>
<th>IL-8 (ng/ml) Mean (SEM)</th>
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<tbody>
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<tr>
<td>Control</td>
<td>1356 (376) *</td>
<td>6.9 (1.5) *</td>
<td>103 (17) †</td>
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<tr>
<td>Pancreatitis</td>
<td>1339 (374)</td>
<td>10.6 (1.3)</td>
<td>238 (26)</td>
</tr>
<tr>
<td>Spontaneous in AS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2053 (741) *</td>
<td>9.3 (1.7) †</td>
<td>128 (22) †</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>2425 (441)</td>
<td>20.7 (4.6)</td>
<td>283 (40)</td>
</tr>
<tr>
<td>LPS-stimulation in FCS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2596 (839) *</td>
<td>11.5 (0.9) †</td>
<td>192 (20) †</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>3554 (825)</td>
<td>24.4 (2.5)</td>
<td>427 (35)</td>
</tr>
<tr>
<td>LPS-stimulation in AS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3994 (1199) *</td>
<td>17.7 (2.6) †</td>
<td>135 (16) †</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>4799 (708)</td>
<td>35.8 (5.5)</td>
<td>419 (56)</td>
</tr>
</tbody>
</table>
was significantly increased in the pancreatitis group compared with the control group when cultured in AS but this effect was not seen when the cells were cultured in FCS (Table 3.4). The mean 24 hour, LPS-stimulated IL-6 release from PBMCs was significantly increased in the patient group compared with the control group when cultured with either FCS or AS (Table 3.4). The mean 24 hour, spontaneous and LPS-stimulated release of IL-8 from PBMCs was significantly increased in the pancreatitis group compared with the control group, with the effect seen when the cells were incubated with either FCS or AS (Table 3.4).

Release of TNFα, IL-6 and IL-8 from PBMCs isolated from patients with acute pancreatitis and healthy volunteers was significantly increased when the cells were incubated in the presence of LPS in comparison with spontaneous cytokine release (Table 3.4). Although the mean ratio of LPS-stimulated TNFα and IL-6 release over spontaneous cytokine release appeared greater in the pancreatitis group compared with the control group, 3.5 (2.4-4.9) mean (95% confidence limit) v 2.3 (1.4-3.3) for TNFα and 2.8 (1.7-4.0) v 1.7 (1.2-2.5) for IL-6 respectively, this did not achieve statistical significance (p > 0.24).

Incubation of PBMCs with AS in comparison with FCS resulted in a greater spontaneous TNFα and IL-6 release in both the pancreatitis and control groups. The spontaneous release of TNFα and IL-6 was significantly increased in the pancreatitis group compared with the control group 3.4 (2.1-5.0) mean (95% confidence limit) v 1.7 (1.6-1.9); p = 0.01 and 2.0 (1.6-2.8) v 1.6 (1.4-1.9); p = 0.04 respectively. IL-8 release was not significantly enhanced in either the pancreatitis or the control group from PBMCs cultured in media supplemented with AS when compared with PBMCs cultured in media supplemented with FCS 1.4 (0.9-1.8) v 1.2 (0.8-1.7); p = 0.36.
TNFα and IL-6 were detectable on the first day of admission in the serum of 7 patients, 5 of whom developed severe disease as described above (Figure 3.4). However, there was no correlation between the serum TNFα and IL-6 concentration and the spontaneous PBMC cytokine release (r = 0.10; p = 0.71 and r = 0.22; p = 0.42 for TNFα and IL-6 respectively). IL-8 was detected in the sera of only 2 patients. TNFα, IL-6 and IL-8 were undetectable in the sera of the volunteers.

The spontaneous release of TNFα, IL-6 and IL-8 from PBMCs isolated from patients with acute pancreatitis on the first day of admission, and cultured at a standard cell concentration of $10^6$/ml are depicted in Figure 3.5, Figure 3.6 and Figure 3.7 respectively. There was no significant difference between the 11 patients with mild and 5 patients with severe pancreatitis in PBMC release of TNFα, IL-6 or IL-8. To allow an estimation of cytokine production per unit volume of blood in order to take into account the variation in white cell count between patients, cytokine production from the standard cell concentration was multiplied by the mononuclear cell count (Table 3.1)(monocyte count plus lymphocyte count) measured on the first day of admission. The results for TNFα, IL-6 and IL-8 are depicted in Figure 3.5, Figure 3.6 and Figure 3.7 respectively. PBMC IL-6 and IL-8 release per unit volume of blood in patients with severe disease was significantly increased compared with mild disease (Figure 3.6 and Figure 3.7). However, there was no significant difference in PBMC TNFα release between the two groups (Figure 3.5).
Figure 3.4

Serum TNFα and IL-6 concentration in 16 patients with acute pancreatitis measured on the first day of admission. Patients with severe disease (n=5; •) and mild disease (n=11; ○)(Atlanta classification (Bradley, 1993)). TNFα and IL-6 were measured by ELISA with the minimum level of detection 15 pg/ml and 40 pg/ml respectively, represented by (—).
Figure 3.5

Spontaneous TNFα concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 16 patients with acute pancreatitis on the first day of admission (O), stratified for mild (n=11) and severe (n=5) disease (Atlanta classification (Bradley, 1993)). 2 x 10⁵ cells per well incubated in triplicate under standard conditions for 24 hr. Spontaneous TNFα concentration in the culture supernatant from peripheral blood mononuclear cells isolated from the same 16 patients multiplied by the peripheral blood mononuclear cell count (●) and stratified for disease severity as before. TNFα was measured by ELISA. * p > 0.13 (N.S.). Mann Whitney-U test comparing patients with mild and severe disease.
Figure 3.6

Spontaneous IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 16 patients with acute pancreatitis on the first day of admission (O), stratified for mild (n=11) and severe (n=5) disease (Atlanta classification (Bradley, 1993)). 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. Spontaneous IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from the same patients multiplied by the peripheral blood mononuclear cell count (●) and stratified for disease severity as before. IL-6 was measured by ELISA. * p = 0.31 (N.S). † p = 0.04. Mann Whitney-U test comparing patients with mild and severe disease.
Figure 3.7

Spontaneous IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 16 patients with acute pancreatitis on the first day of admission (O), stratified for mild (n=11) and severe (n=5) disease (Atlanta classification (Bradley, 1993)). 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. Spontaneous IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from the same patients multiplied by the peripheral blood mononuclear cell count (●) and stratified for disease severity as before. IL-8 was measured by ELISA. * p = 0.16 (N.S.). † p = 0.02. Mann Whitney-U test comparing patients with mild and severe disease.
3.4 Discussion

The findings that both adherent and non-adherent PBMC sub-populations release the pro-inflammatory cytokines measured and the synergistic effect on such cytokine release when both sub-populations are co-cultured re-inforce the value of studying mixed cell cultures when seeking to understand events that may be occurring in vivo (Table 3.2). Furthermore, the procedure of density centrifugation appears to be an appropriate method of isolation of PBMCs, achieving similar mononuclear cell populations in vitro to that in vivo as per the manufacturers instructions (Table 3.3). Clearly, the cell numbers are preserved but what is less certain is the effect of density centrifugation on cell function.

The studies described in this chapter demonstrate an increased release of IL-6 and IL-8 from PBMCs in the blood of patients with acute pancreatitis compared with healthy volunteers, (Table 3.4). While there was no significant difference in pro-inflammatory cytokine release per cell when comparing patients with mild or severe disease (Figure 3.5, Figure 3.6 and Figure 3.7), making allowance for the white cell count revealed that IL-6 and IL-8 release per unit volume of blood was significantly greater in patients with severe disease (Figure 3.6 and Figure 3.7). This effect was not observed for PBMC TNFα release (Figure 3.5) despite the observation that TNFα plays an important early role in the cascade of inflammatory events (Lowry, 1993). Although blood was withdrawn from patients as soon after admission as feasible, it was evident that the disease process, as manifest by patient symptoms, had been present for many hours and in some cases, a day or two prior to admission. It is thus likely that significant release of TNFα from PBMCs had already occurred, as indicated by elevated serum TNFα levels in a number of patients (Figure 3.4). It is perhaps of greater significance, with regard to the pathogenesis of acute pancreatitis and its complications, that PBMCs in patients
were still able to mount a similar TNFα response to healthy volunteers despite the elevated serum TNFα concentration without the appearance of tolerance to the initial stimulus (Mathieson, 1990).

Monocyte activation generally follows exposure to bacterial LPS. While monocytes are able to recognise LPS directly (Dentener, 1993), activation is potentiated when LPS forms a complex with serum proteins such as LPS-binding protein which then interact with the CD14 receptor on the surface of the monocyte (Wright, 1990). LPS-binding protein is present in human serum but being an acute phase protein, its serum concentration is elevated in patients with inflammatory conditions such as acute pancreatitis. The concentration of LPS-binding protein in fetal calf serum is not known. However, the presence of a LPS-binding protein appears to be a consistent finding in other animals (Schumann, 1990) and its presence in fetal calf serum is likely. In the present study, incubation with endotoxin led to an increased release of TNFα, IL-6 and IL-8 from PBMCs in both the patient and control groups, although the mean stimulation index (comparing LPS-stimulated cytokine release with spontaneous cytokine release) was not significantly different between the patient and control groups. While endotoxaemia is unlikely to be the primary event in the pathogenesis of acute pancreatitis, its presence could contribute to the state of monocyte activation and the subsequent development of the systemic inflammatory response syndrome associated with the disease.

Culture of isolated PBMCs in either autologous or fetal calf serum allows comparison between the behaviour of cells under the same conditions (FCS) or in the conditions prevailing in vivo (AS). Incubation with AS increased both spontaneous and LPS-stimulated TNFα and IL-6 release in both patients and controls, but this effect was not seen with regard to IL-8 release (Table 3.4).
Furthermore, spontaneous TNFα and IL-6 PBMC release in patients with acute pancreatitis in comparison with the control group, showed a greater cytokine release in response to incubation with AS compared with FCS. This finding suggests that circulating factors in the sera of patients with acute pancreatitis contribute to the state of PBMC activation with regard to TNFα and IL-6 secretion. Damaged acinar cells and the leucocyte infiltrate associated with the pancreatitis are known to secrete a wide variety of pro-inflammatory agents, including complement factors (Lasson, 1984a), oxygen derived free radicals (Sanfey, 1985), cytokines, platelet activating factor (Fujimura, 1992) and products of the lipo-oxygenase pathway such as leukotriene B₄ (Rinderknecht, 1988). All these agents are known to affect endothelial cells and promote circulating leucocyte activation. Differences in endotoxin concentration between FCS and AS are unlikely to account for this observation as the lipopolysaccharide stimulation concentration used in this study protocol had been shown to induce maximal cytokine release (Figure 3.2) with no evidence of cell cytotoxicity at higher LPS stimulation doses.

Elevation of serum IL-6 levels early in the course of acute pancreatitis is well documented (Heath, 1993; Leser, 1991; Viedma, 1992). While a number of immunocompetent cells secrete IL-6 in vivo, the increased secretion of IL-6 by PBMCs observed in patients in the present study suggests that this source may contribute significantly to elevated serum IL-6 concentrations. Studies have reported that both the concentration on admission and the peak concentration of serum IL-6 correlate with disease severity (Leser, 1991; Viedma, 1992). This correlation is also described in other conditions where inflammation plays a key role. For example, the risk of sepsis correlates with serum IL-6 concentration in patients following major burns (Drost, 1993) and in post-operative patients in an intensive care unit (Damas, 1992). Furthermore, Faist and co-workers (Faist, 1992) have shown that monocyte IL-6 secretion is increased two-fold in patients by sepsis.
In an intervention study using a mouse burns-sepsis model, IL-1β and indomethacin administration reduced the production of TNFα, IL-1β and IL-6 by cultured splenocytes towards normal control levels with a corresponding improvement in survival (O'Riordain, 1992). These results suggest that in inflammatory conditions, such as acute pancreatitis, excessive production of IL-6 from PBMCs may be altered by therapeutic intervention and could conceivably influence outcome. Alternatively, IL-6 may simply be a marker of more important pro-inflammatory mediators.

Although there is evidence that serum IL-8 levels are elevated in acute pancreatitis (Gross, 1992), IL-8 was detected in the serum on the first day of admission in only two patients in the present study. However, the IL-8 ELISA did not allow accurate measurement of serum IL-8 concentrations below 200 pg/ml. Furthermore, blood has a large capacity for inactivating IL-8, principally through irreversible binding with red blood cells (Horuk, 1994; Tilg, 1993), and thus serum IL-8 measurement early in the course of an acute inflammatory disease might not reflect cellular or PBMC IL-8 production. Nevertheless, the increase in PBMC IL-8 release in vitro observed in the present study may help explain the elevation of serum IL-8 in acute pancreatitis. Similar up-regulation of IL-8 release from monocytes has been observed in a study simulating ischaemia-reperfusion (Metinko, 1992). However, Faist (Faist, 1992), who observed up-regulation of IL-6 release from monocytes in patients with sepsis or multiple trauma, found that monocyte IL-8 release was suppressed in the same patients.

The results of the present study reveal increased LPS-stimulated IL-6 and IL-8 release and spontaneous IL-8 release from PBMCs in patients with acute pancreatitis early in the course of their disease. Increased de novo synthesis of these cytokines could possibly account for these findings although this can not be
determined from the present study. Increased synthesis is likely as it is recognised that messenger RNA can be detected for these cytokines within minutes of stimulation (Zhong, 1993). However, the link between disease progression and resolution and such pro-inflammatory cytokine release is not known. Furthermore, whether this up-regulation results from an imbalance in the production of inflammatory mediators or a loss of sensitivity to down-regulatory signals is not known.
Chapter 4

Changes in peripheral blood mononuclear cell pro-inflammatory cytokine release from patients with acute pancreatitis and correlation with disease progression

4.1 Introduction

The previous chapter demonstrated that IL-6 and IL-8 release from PBMCs isolated on the first day of admission from patients with acute pancreatitis was increased compared with healthy volunteers. However, the intensity of the pro-inflammatory cytokine release under standard conditions did not appear to be associated with subsequent disease severity. Only when allowance was made for the white cell count prevailing at the same time in patients with the disease, total IL-6 and IL-8 release (per unit volume of blood) from PBMCs appeared to be greater in those who subsequently developed severe disease. However, the changes in such PBMC cytokine release with time during the course of the illness is not known.

The aim of this study was to compare PBMC IL-6 and IL-8 release on the first and fifth day of admission in patients confirmed to have mild or severe disease.
Day 5 was chosen for several reasons. On clinical grounds, the differentiation as to the severity category of the patient is usually clear and the study of patients with mild disease beyond this time point can be difficult as the patient is fit for discharge from hospital. Furthermore, studies looking at indirect evidence of leucocyte activation such as neutrophil elastase or IL-6 in the serum report a difference in the median between the two groups of patients up to this time (Banks, 1991a; Heath, 1993). As in chapter 3, both spontaneous and LPS-stimulated cytokine release was examined.

### 4.2 Patients and methods

#### 4.2.1 Patients

The study group comprised 14 patients with acute pancreatitis as defined in Section 2.1.1. Peripheral blood was taken from patients on the first and fifth day of admission for the isolation and culture of PBMCs (as described in Section 2.3). The progress of the patient was evaluated with regard to the development of pancreatic complications as defined by the 1992 Atlanta Convention (Bradley, 1993) or the development of multiple organ failure as defined by the Goris score (Goris, 1985). The patient characteristics are given in Table 4.1. Six healthy volunteers were also studied to provide an indication of 'normal' cytokine release.

#### 4.2.2 PBMC study protocol

PBMCs were incubated at $2 \times 10^5$ cells per well in the presence or absence of LPS (final concentration 5 μg/ml). Following 24 hr incubation, the supernatants were removed and stored in aliquots at -70 °C for subsequent batch cytokine assay.
Table 4.1

Details of patients studied. Pancreatic complications defined by the Atlanta criteria (Bradley, 1993) and organ failure by the modified Goris score (Goris, 1985).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Aetiology</th>
<th>Pancreatic complication</th>
<th>Organ failure score</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>90</td>
<td>F</td>
<td>gallstones</td>
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<td>0</td>
</tr>
<tr>
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<td>gallstones</td>
<td>no</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
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<td>no</td>
<td>0</td>
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<tr>
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<td>61</td>
<td>F</td>
<td>gallstones</td>
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</tr>
<tr>
<td>5</td>
<td>79</td>
<td>M</td>
<td>idiopathic</td>
<td>no</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>69</td>
<td>M</td>
<td>gallstones</td>
<td>necrosis</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>47</td>
<td>F</td>
<td>alcohol</td>
<td>necrosis</td>
<td>5 (Died)</td>
</tr>
<tr>
<td>8</td>
<td>54</td>
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<td>gallstones</td>
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<td>0</td>
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<tr>
<td>9</td>
<td>31</td>
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<tr>
<td>14</td>
<td>53</td>
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<td>gallstones</td>
<td>necrosis</td>
<td>7</td>
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<td>Volunteers</td>
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<td></td>
</tr>
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<tr>
<td>5</td>
<td>48</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>M</td>
<td></td>
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</table>
4.2.3 IL-6 and IL-8 ELISAs

Supernatants and serum were assayed for IL-6 and IL-8 by ELISAs as described in Sections 2.6.2 and 2.6.3 respectively.

4.2.4 Statistical analysis

Initial comparison between patients with mild disease, severe disease and volunteers was performed using the Kruskal-Wallis test. Thereafter, Mann-Whitney U tests were applied to compare differences between any two groups while comparison within the acute pancreatitis group was made with the paired Student’s t test with significance taken at a p value < 0.05.

4.3 Results

While the volunteer group were younger than the patient group (40 years, range 28-49 v 63 years, range 31-90 respectively, p = 0.70), there was no significant difference in the mean age between patients classified as mild or severe disease (65 years, range 42-90 v 61 years, range 31-84 respectively, p = 0.70). Six of the 14 patients were considered to have severe disease in that they developed complications related to their disease. Three of these patients had pancreatic necrosis demonstrated by contrast-enhanced CT scanning during the first week of admission. Five of the six patients with severe disease scored 1 or more on the modified Goris score, one of whom died of multiple organ failure.

The mean spontaneous release of IL-6 in patients with either mild or severe acute pancreatitis on the first day of admission was significantly increased compared with the volunteer group (mild 2855 pg/ml or severe 3488 pg/ml v volunteer 791 pg/ml; p<0.02, Table 4.2). There was no significant difference in the mean
Table 4.2

Mean (standard error of the mean) spontaneous IL-6 and IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers and from 14 patients with acute pancreatitis on day 1 and day 5 of admission, stratified for mild (n=8) and severe (n=6) disease (Atlanta classification (Bradley, 1993)). 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 and IL-8 were measured by ELISA.

<table>
<thead>
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<th>Cytokine</th>
<th>Volunteer</th>
<th>Day 1</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild</td>
<td>Severe</td>
<td>Mild</td>
</tr>
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<td>IL-6 (pg/ml)</td>
<td>791</td>
<td>2855</td>
<td>3488</td>
</tr>
<tr>
<td></td>
<td>(168)</td>
<td>(615)</td>
<td>(980)</td>
</tr>
<tr>
<td>IL-8 (ng/ml)</td>
<td>35.0</td>
<td>146</td>
<td>174</td>
</tr>
<tr>
<td></td>
<td>(16.5)</td>
<td>(45.9)</td>
<td>(49.7)</td>
</tr>
</tbody>
</table>
spontaneous release of IL-6 in patients with mild disease compared with severe disease on the first day of admission (2855 and 3488 pg/ml respectively; p=0.15, Table 4.2). In contrast, on the fifth day of admission in patients with acute pancreatitis, mean spontaneous release of IL-6 remained significantly elevated compared with the volunteer group (6724 pg/ml; p=0.01, Table 4.2) while the mean spontaneous release of IL-6 in the mild pancreatitis group was not significantly different compared with the volunteer group (1538 pg/ml; p=0.30, Table 4.2). The individual data points for PBMC IL-6 release for the 14 patients with acute pancreatitis are shown in Figure 4.1. All the patients with severe disease demonstrated on day 5 an IL-6 release increased or within 90 % of the day 1 value. In contrast, PBMC IL-6 release in the mild disease group on day 5 fell by more than 10 % in all patients except one.

The mean spontaneous release of IL-8 in patients with either mild or severe acute pancreatitis on the first day of admission was significantly increased compared with the volunteer group (mild 85.5 ng/ml or severe 174 ng/ml v volunteer 35.0 ng/ml; p<0.03, Table 4.2). There was no significant difference in the mean spontaneous release of IL-8 in patients with mild disease compared with severe disease on the first day of admission (146 and 174 ng/ml respectively; p=0.07, Table 4.2). In contrast, on the fifth day of admission in patients with acute pancreatitis, mean spontaneous release of IL-8 remained significantly elevated compared with the volunteer group (131 ng/ml; p=0.02, Table 4.2) while the mean spontaneous release of IL-8 in the mild pancreatitis group was not significantly different compared with the volunteer group (53.8 pg/ml; p=1, Table 4.2). Furthermore, on day 5 IL-8 release in the severe group was elevated compared with the mild group (p=0.04). The individual data points for PBMC IL-8 release for the 14 patients with acute pancreatitis are shown in Figure 4.2. All the patients except two with severe
Figure 4.1

Spontaneous IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers and from 14 patients with acute pancreatitis on day 1 and day 5 of admission, stratified for mild (n=8) and severe (n=6) disease (Atlanta classification (Bradley, 1993)). $2 \times 10^5$ cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 was measured by ELISA.
Figure 4.2

Spontaneous IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers and from 14 patients with acute pancreatitis on day 1 and day 5 of admission, stratified for mild (n=8) and severe (n=6) disease (Atlanta classification (Bradley, 1993)). 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-8 was measured by ELISA.
disease demonstrated on day 5 a fall in IL-8 release of greater than 10% of the day 1 value.

LPS-stimulation resulted in an increase in both IL-6 and IL-8 in all the study groups compared with spontaneous release. The mean stimulation index for each study group is shown in Table 4.3. While the mean stimulation index was higher in the pancreatitis groups compared with the volunteer group, this was not significant on statistical analysis for either IL-6 (p=0.66) or IL-8 (p=0.43).

4.4 Discussion

The present study again demonstrates (in keeping with the findings of Chapter 3) an increased release of IL-6 and IL-8 from PBMCs isolated from the blood of patients with acute pancreatitis compared with those isolated from healthy volunteers, (Table 4.2, Figures 4.1 and 4.2). Again, there was no significant difference in pro-inflammatory cytokine release per cell when comparing patients with mild or severe disease on the day of admission. However, by day 5 of admission, PBMC IL-6 and IL-8 release from patients subsequently shown to have mild disease have returned to levels approaching that for healthy volunteers. In contrast, patients with severe disease had persisting significantly increased IL-6 and IL-8 release from PBMCs. Thus severe disease is characterised more by the duration of increased pro-inflammatory cytokine release rather than the intensity of the release when measured on admission to hospital. What is not clear from this study, is whether this prolonged duration of increased IL-6 and IL-8 release is the cause or the result of developing complications of severe disease.
Table 4.3

Mean (standard error of the mean) lipopolysaccharide-stimulation index for IL-6 and IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers and from 14 patients with acute pancreatitis on day 1 and day 5 of admission, stratified for mild (n=8) and severe (n=6) disease (Atlanta classification (Bradley, 1993)). 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 and IL-8 were measured by ELISA. There was no significant difference in the mean stimulation index in any of the acute pancreatitis groups compared with the mean volunteer stimulation index for either IL-6 (p=0.66) or IL-8 (p=0.43), Mann-Whitney U test.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Volunteer</th>
<th>Mild</th>
<th>Severe</th>
<th>Mild</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>8.70</td>
<td>11.58</td>
<td>12.47</td>
<td>12.08</td>
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<td></td>
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<td>(2.21)</td>
<td>(1.44)</td>
<td>(2.99)</td>
<td>(2.74)</td>
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<tr>
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<td>3.46</td>
<td>6.70</td>
<td>8.05</td>
<td>9.51</td>
<td>5.15</td>
</tr>
<tr>
<td></td>
<td>(0.65)</td>
<td>(1.71)</td>
<td>(4.40)</td>
<td>(2.37)</td>
<td>(1.61)</td>
</tr>
</tbody>
</table>
The two patients in the severe group who demonstrated a further marked increase in IL-6 release between day 1 and day 5 represent patients 7 and 14 detailed in Table 4.1, both of whom developed multiple organ failure, one of whom succumbed on day 9 from the disease. In accordance with the findings of the present study, Faist and co-workers reported that monocyte IL-6 secretion is increased two-fold in patients with sepsis compared with controls but no data on the time course of this increase is described (Faist, 1992). In the same report, patients undergoing major elective surgery also demonstrated increased monocyte IL-6 release in the week following surgery. However, PBMC IL-6 release in this group was also greater pre-operatively compared with the control group and it is of note that the surgery group was composed of patients predominately with malignancy. Chronically increased TNFα and IL-6 release has been reported from PBMCs isolated from patients with pancreatic cancer (Falconer, 1994). It is interesting to note that PBMC IL-6 release in patients following major trauma (thought to have a similar common pathogenesis as acute pancreatitis with the development of a systemic inflammatory response and the potential for multiple organ failure) is suppressed on day 1 of the injury compared with controls and returns to levels comparable with the controls by day 7. The explanation for this conflicting finding is not clear. Major trauma is associated with a marked monocytosis (Faist, 1988) and it is possible that this rapid monocyte recruitment is of a population of functionally immature cells which behave quite differently from monocytes released from a pool of marginating cells under less severe conditions.

The pattern of IL-8 release from PBMCs in patient with acute pancreatitis was similar to that of IL-6. A variation to this pattern is noted on day 5, where IL-8 release even in the severe group is generally returning towards levels found in healthy volunteers. Two patients in the severe group did manifest a further increase in IL-8 release between day 1 and day 5 but only one of these (compared with both
patients with rising IL-6 release) progressed to multiple organ failure. It is interesting to note that in a study where PBMCs were exposed *in vitro* to anoxia followed by normal conditions of incubation induced less of an IL-8 response compared with a similar anoxic stress followed by a period of hyperoxia (Metinko, 1992). These experimental conditions may well be mimicked *in vivo* following therapeutic intervention in patients with ischaemia-reperfusion type injury, including those with acute pancreatitis. In contrast, Faist and co-workers (Faist, 1992) who observed up-regulation of IL-6 release from monocytes in patients with sepsis, found that monocyte IL-8 release was suppressed in the same patients. This suppression of IL-8 release had reverted to normal by the seventh day of admission.

The trigger for enhanced IL-6 and IL-8 release is not clear from this study. The current understanding of the role of endotoxin in acute pancreatitis has already been discussed in Chapter 1, Section 1.11 and in Chapter 3, Section 3.4. The present study again demonstrates (in keeping with the findings of Chapter 3) that incubation with lipopolysaccharide results in an increased release of IL-6 and IL-8 from PBMCs in both the patient and volunteer groups (Table 4.3), although the mean stimulation index (comparing LPS-stimulated cytokine release with spontaneous cytokine release) was not significantly different between the patient and control groups. The stimulation indices in the experimental findings reported in this chapter are several fold higher than those found in Chapter 3. The reason for this is not clear but is likely to be related to variation in culture reagents, and in particular, the use of a new batch of stock lipopolysaccharide. Variations in the lipopolysaccharide-stimulation index have also been noted in the laboratory with changes in the batch of fetal calf serum and may relate to the concentration of LPS-binding protein present in different batches. Nevertheless, the further increase in IL-6 and IL-8 release by LPS-stimulation is important as it implies that PBMCs that already appear to have been activated in patients with acute pancreatitis are able to
respond to further stimulation. Thus, while endotoxaemia (Exley, 1992; Windsor, 1993) is unlikely to be the primary event in the pathogenesis of acute pancreatitis, its presence could exacerbate mononuclear cell activation and the subsequent development of the systemic inflammatory response syndrome associated with the disease. Furthermore, it implies that mononuclear cells possess the ability to produce a graded response to a stimulus, suggesting that control mechanisms exist to regulate the response such as the degree of pro-inflammatory cytokine release.
Chapter 5

Regulatory control by indomethacin and Type II cytokines of pro-inflammatory cytokine release by peripheral blood mononuclear cells isolated from patients with acute pancreatitis

5.1 Introduction

A number of control mechanisms exist for the regulation of pro-inflammatory cytokines (Type I cytokines) from PBMCs. Intracellular control mechanisms are recognised, principally through products of the cyclo-oxygenase pathway such as prostaglandins and intercellular control mechanisms such as the Type II cytokines (that include interleukin-4 and interleukin-10), secreted principally from subsets of CD4 positive T-helper cells.

Among the prostaglandin series of compounds, attention has focused on the role of prostaglandin E$_2$ as a modulator of monocyte, lymphocyte and monocyte/lymphocyte interaction functions (Goldyne, 1980; Goodwin, 1983). PGE$_2$ is produced endogenously by a number of immune competent cells including monocytes (Ellner, 1979; Miller-Graziano, 1988) and macrophages (Molloy, 1993;
Roland, 1994). In addition to the effects on lymphocyte function including impaired blastogenesis, lymphokine production and cytotoxicity (Ellner, 1979; Goodwin, 1983), endogenous PGE2 appears to exert an autocrine feedback loop on monocytes themselves (Callery, 1991). The plateau of TNFα release from LPS-stimulated murine peritoneal macrophages was concomitant with an accelerated increase in PGE2 production (Kunkel, 1988). Furthermore, the addition of PGE2 demonstrated a dose-dependent effect in LPS-induced activity as well as a significant reduction in TNFα messenger RNA accumulation. A number of groups have confirmed the co-production of TNFα and PGE2 and the regulatory feedback by PGE2 on monocyte and macrophage TNFα production (Callery, 1991; Molloy, 1993; Spengler, 1989). In accordance with this, the administration in vitro and in vivo of indomethacin (a cyclo-oxygenase inhibitor) appears to suppress endogenous PGE2 production (Callery, 1990; Roland, 1994), while increasing the release and synthesis of a number of cytokines including TNFα, IL-1β, IL-2, IL-6 and IL-8 (Callery, 1990; Chouaib, 1984; Endres, 1989; Faist, 1987; Roland, 1994; Spengler, 1989; Standiford, 1992). This is not a universal finding, as lipopolysaccharide and PGE2 synergistically induced IL-6 release from intestinal epithelial cells (Meyer, 1994). Nevertheless, increased production of PGE2 can be detected in the sera of patients following major burns (Ninnemann, 1984).

The human immune response to inflammation is thought to be regulated in part by the balance between so-called Type I and Type II cytokines (Modlin, 1993; Sher, 1992). These latter cytokines are generally considered to be inhibitory or anti-inflammatory (Colquhoun, 1993; Howard, 1992). Not only can they regulate the number of monocytes and neutrophils released from bone marrow progenitor cells (Snoeck, 1993), but IL-4 and IL-10 are known to down-regulate pro-inflammatory cytokine production from a wide-variety of immune cells including mononuclear (de Wall Malefyt, 1993; Donnelly, 1990; Essner, 1989; Fiorentino, 1991; Lee,
and polymorphonuclear (Cassatella, 1993) cells isolated from normal subjects. These regulatory cytokines are produced mainly by specific sub-populations of CD4-positive T helper cells but also, in the case of IL-10, by cells of the monocyte lineage (Howard, 1992). Curley and co-workers (Curley, 1993) have demonstrated a depletion of CD4-positive T helper lymphocytes in patients with acute pancreatitis with the greatest depletion occurring in those with severe disease who had significantly higher serum IL-6 levels. Following resolution of the pancreatitis and recovery of the patient, the CD4-helper population returned towards normal values. Similar findings have been reported for CD4-positive T helper cell depletion following thermal injury (O-Mahony, 1985) and trauma (Faist, 1987). It has been proposed that this depletion of CD4-positive T helper cells causes an imbalance in the regulatory control of the immune response early in acute pancreatitis, diminishing the down-regulatory control on leucocyte activation and allowing up-regulation of pro-inflammatory cytokine release with subsequent progression to multiple organ failure (Casey, 1993).

The aims of this chapter were to examine the effects of indomethacin, PGE₂, IL-4 and IL-10 on the spontaneous release of IL-6 and IL-8 from PBMCs isolated from patients with acute pancreatitis compared with healthy volunteers.

5.2 Patients and methods

5.2.1 Patients

The study group comprised a total of 20 patients with acute pancreatitis as defined in Section 2.1.1. For the IL-4/10 experiments, peripheral blood was taken from patients on the first day of admission for the isolation and culture of PBMCs (as described in Section 2.3). For the indomethacin/PGE₂ experiments, peripheral
blood was taken from patients on the first and fifth day of admission for the isolation and culture of PBMCs (as described in Section 2.3). The progress of the patient was evaluated with regard to the development of pancreatic complications as defined by the 1992 Atlanta Convention (Bradley, 1993) or the development of multiple organ failure as defined by the Goris score (Goris, 1985). The patient characteristics are given in Table 3.1, Chapter 3 (page 61) and represent patients numbered 6 to 11 for the IL-4/10 experiments and Table 4.1, Chapter 4 (Page 86) for the indomethacin/PGE₂ experiments. Six healthy volunteers were also studied to provide an indication of 'normal' cytokine release; the volunteers detailed in Table 3.1, Chapter 3 (page 61) and Table 4.1, Chapter 4 (Page 86) were used for the IL-4/10 and indomethacin/PGE₂ experiments respectively.

5.2.2 PBMC study protocol

5.2.2.1 IL-4/10 protocol

PBMCs from both patients and volunteers were incubated at 2 x 10⁵ cells per well in the presence or absence of LPS (final concentration 5 μg/ml) and in the presence of IL-4 or IL-10 (final concentration 0 - 50 ng/ml)(Genzyme, West Malling, U.K.) for 24 hours. PBMCs from 4 volunteers were incubated at 2 x 10⁵ cells per well in the presence or absence of LPS (final concentration 5 μg/ml) for 24 hours and in the presence of IL-4 or IL-10 (final concentration 5 ng/ml) with the addition of IL-4 and IL-10 to the culture medium taking place at fixed time points between 0 and 24 hours incubation. Supernatants were removed and stored in aliquots at -70 °C for subsequent batch cytokine assay.

5.2.2.2 Indomethacin/PGE₂ protocol

PBMCs from both patients and volunteers were incubated at 2 x 10⁵ cells per well in the presence or absence of indomethacin (final concentration 10⁻⁶ M)(Sigma Chemicals, Poole, U.K.) and in the presence of indomethacin and PGE₂ (final concentration 10⁻⁶ M and 10⁻⁶ to 10⁻⁹ M to respectively)(Sigma Chemicals,
Supernatants were removed and stored in aliquots at -70 °C for subsequent batch cytokine assay.

5.2.3 IL-6 and IL-8 ELISAs

Supernatants and serum were assayed for IL-6 and IL-8 by ELISAs as described in Sections 2.6.2 and 2.6.3 respectively.

5.2.4 Statistical analysis

5.2.4.1 IL-4/10 protocol

Comparison of patient and control groups was made with the Mann-Whitney U test while comparison of the dose response to IL-4 or IL-10 was made with analysis of covariance of the log10 transformed data with significance taken at a P value < 0.05 (Altman, 1991; Altman, 1989).

5.2.4.2 Indomethacin/PGE2 protocol

Comparison of patient and control groups was made with the Mann-Whitney U test while comparison within each acute pancreatitis group was made with the paired Student's t test with significance taken at a P value < 0.05.

5.3 Results

5.3.1 IL-4/10 protocol

There was no significant difference in the mean age between the patient and control groups (55.4 years, range 34-82 v 47.5 years, range 26-73, respectively, p=0.40) with an equal sex ratio in both groups. Three of the 6 patients were considered to have severe disease.
The mean spontaneous 24 hr PBMC IL-6 release was not significantly different in the pancreatitis group compared with the control group (13.7±3.0 v 9.7±2.7 ng/ml; Mean±SEM, p=0.61). However, mean 24 hr LPS-stimulated IL-6 PBMC release was significantly increased in the patient group compared with the control group (31.5±5.2 v 14.1±2.1 ng/ml, p=0.02). Mean 24 hr spontaneous and LPS-stimulated IL-8 PBMC release was significantly increased in the pancreatitis group compared with the control group (337±48 v 106±10 ng/ml, p=0.002 and 553±49 v 112±13 ng/ml, p=0.002 respectively).

Both IL-4 and IL-10 inhibited spontaneous and LPS-stimulated PBMC IL-6 release in a dose dependent manner, and there was no significant difference in the percentage inhibition between the patient and control groups, p>0.1 (Figures 5.1 and 5.2). In a similar manner, both IL-4 and IL-10 inhibited spontaneous and LPS-stimulated PBMC IL-8 release in a dose dependent manner, and there was no significant difference in the percentage inhibition between the patient and control groups, p>0.1 (Figures 5.3 and 5.4).

The degree of inhibition of both spontaneous and LPS-stimulated PBMC IL-6 release by IL-4 or IL-10 was preserved even if the addition of IL-4 or IL-10 was delayed up to 2 hours after plating the cells, (Figure 5.5). However, delaying the addition of IL-4 or IL-10 by 4 hours after the plating of PBMCs, reduced the degree of inhibition, (Figure 5.5) in PBMCs isolated from healthy volunteers.

5.3.2 Indomethacin/PGE2 protocol

While the volunteer group were younger than the patient group (40 years, range 28-49 v 63 years, range 31-90 respectively, p=0.70), there was no significant difference in the mean age (65.0 years, range 42-90 v 60.5 years, range 31-84, p=0.70) between patients classified as mild or severe disease. Six of the 14
Figure 5.1

Inhibition of the mean spontaneous IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers (- -) and from 6 patients with acute pancreatitis on the first day of admission (---) by IL-4 (□) or IL-10 (○)(dose range 0-50 ng/ml). Error bars represent the standard error of the mean. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 was measured by ELISA. P > 0.1 comparing the patient with the corresponding volunteer group (analysis of covariance, log_{10} transformed data).
**Figure 5.2**

Inhibition of the mean lipopolysaccharide-stimulated (final concentration 5 μg/ml) IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers (----) and from 6 patients with acute pancreatitis on the first day of admission (-----) by IL-4 (□) or IL-10 (○)(dose range 0-50 ng/ml). Error bars represent the standard error of the mean. 2 x 10⁵ cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 was measured by ELISA. P > 0.1 comparing the patient with the corresponding volunteer group (analysis of covariance, log₁₀ transformed data).
Figure 5.3

Inhibition of the mean spontaneous IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers (---) and from 6 patients with acute pancreatitis on the first day of admission (---) by IL-4 (□) or IL-10 (○)(dose range 0-50 ng/ml). Error bars represent the standard error of the mean. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-8 was measured by ELISA. P > 0.1 comparing the patient with the corresponding volunteer group (analysis of covariance, log_{10} transformed data).
Figure 5.4

Inhibition of the mean lipopolysaccharide-stimulated (final concentration 5 μg/ml) IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers (---) and from 6 patients with acute pancreatitis on the first day of admission (—) by IL-4 (□) or IL-10 (○)(dose range 0-50 ng/ml). Error bars represent the standard error of the mean. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-8 was measured by ELISA. P > 0.1 comparing the patient with the corresponding volunteer group (analysis of covariance, log_{10} transformed data).
Figure 5.5
Mean spontaneous (- -) and lipopolysaccharide-stimulated (—)(final concentration 5 μg/ml) IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 4 healthy volunteers. IL-4 (□) or IL-10 (○)(both 5 ng/ml final concentration) were added at time points from the start of cell incubation. Results are expressed as the mean percentage of IL-6 released when IL-4 or IL-10 was added at the end of the 24 hour incubation period and the error bars represent the standard error of the mean. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 was measured by ELISA.
patients were considered to have severe disease in that they developed complications related to their disease. Three of these patients had pancreatic necrosis demonstrated by contrast-enhanced CT scanning during the first week of admission. Five of the six patients with severe disease scored 1 or more on the modified Goris score, one of whom died of multiple organ failure.

Indomethacin in the culture medium had no significant effect on spontaneous IL-6 or IL-8 release from PBMCs isolated from volunteers, as shown in Table 5.1. In contrast, indomethacin increased IL-6 release from PBMCs isolated from patients with acute pancreatitis with both mild and severe disease on day 1 and also on day 5 of admission (Table 5.1). Furthermore, indomethacin increased IL-8 release from PBMCs isolated from patients with acute pancreatitis with both mild and severe disease on day 1 and also on day 5 of admission, (Table 5.1). (The mean PBMC IL-6 and IL-8 release from both the patient group and the healthy volunteer group varied between the IL-4 or IL-10 and the indomethacin experiments. This was most marked for IL-6 release with a 10 fold variation observed in the mean IL-6 release for the IL-4 or IL-10 and the indomethacin experiments. The reason for this is not clear. The two sets of experiments were performed a number of months apart during which time, many of the reagents had changed including the IL-6 standard for the ELISA. It is likely that I made a dilution error in the stock IL-6 standard)

While indomethacin had no significant effect on PBMC IL-6 release in the volunteer group, indomethacin in combination with PGE2 (both at 10^-6 M) resulted in a significant increase in IL-6 release as shown in Figure 5.6. Reducing the concentration of PGE2 led to a reduced response and indeed there appeared to be an inhibition of IL-6 release at lower PGE2 concentrations compared with indomethacin alone (Figure 5.6). Similar findings were observed for patients with
Table 5.1

Effect of indomethacin (final concentration $10^{-6}$ M) on the mean spontaneous IL-6 and IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers and from 14 patients with acute pancreatitis on day 1 and day 5 of admission, stratified for mild (n=8) and severe (n=6) disease (Atlanta classification (Bradley, 1993)). 2 x $10^5$ cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 and IL-8 were measured by ELISA. Results are expressed as the mean (SEM). The paired t test compares cytokine release in the presence or absence of indomethacin for each group.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 5</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Volunteer</td>
<td>Mild</td>
</tr>
<tr>
<td><strong>IL-6 (pg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No indomethacin</td>
<td>791</td>
<td>2855</td>
</tr>
<tr>
<td>(168)</td>
<td>(615)</td>
<td>(980)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>941</td>
<td>7669</td>
</tr>
<tr>
<td>$(10^{-6}$ M)</td>
<td>(212)</td>
<td>(1438)</td>
</tr>
<tr>
<td>paired t test</td>
<td>p&gt;0.29</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td><strong>IL-8 (ng/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No indomethacin</td>
<td>35.0</td>
<td>146</td>
</tr>
<tr>
<td>(16.5)</td>
<td>(45.9)</td>
<td>(49.7)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>37.6</td>
<td>246</td>
</tr>
<tr>
<td>$(10^{-6}$ M)</td>
<td>(20.0)</td>
<td>(53.7)</td>
</tr>
<tr>
<td>paired t test</td>
<td>p&gt;0.59</td>
<td>p&lt;0.02</td>
</tr>
</tbody>
</table>
Figure 5.6

Mean spontaneous IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers (▲) and from 14 patients with acute pancreatitis on day 1 (●, ■) and day 5 (○, □) of admission, stratified for mild (n=8; ○) and severe (n=6; □) disease (Atlanta classification (Bradley, 1993)). Results are expressed as the ratio of IL-6 release when co-cultured in indomethacin (10^{-6} M) and PGE2 (dose range 10^{-6} M to 10^{-9} M) over IL-6 release when cultured in indomethacin alone (10^{-6} M) against the PGE2 concentration. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 was measured by ELISA.
Figure 5.7

Mean spontaneous IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from 6 healthy volunteers (▲) and from 14 patients with acute pancreatitis on day 1 (●,■) and day 5 (○,□) of admission, stratified for mild (n=8; ○) and severe (n=6; □) disease (Atlanta classification (Bradley, 1993)). Results are expressed as the ratio of IL-8 release when co-cultured in indomethacin (10^{-6} M) and PGE_{2} (dose range 10^{-6} M to 10^{-9} M) over IL-8 release when cultured in indomethacin alone (10^{-6} M) against the PGE_{2} concentration. 2 \times 10^{5} cells per well incubated in triplicate under standard conditions for 24 hr. IL-8 was measured by ELISA.
both mild and severe acute pancreatitis on days 1 and 5 of admission (Figure 5.6). The findings for PBMC IL-8 release in the presence of indomethacin alone and indomethacin with PGE₂ are similar as shown in Figure 5.7.

5.4 Discussion

The findings of this chapter demonstrate that indomethacin increases IL-6 and IL-8 release from PBMCs isolated from patients with acute pancreatitis but not healthy volunteers while PGE₂ produces a dose-dependent increase or decrease in such cytokine release in both healthy volunteers and patients with acute pancreatitis. The dose dependent inhibition of IL-6 and IL-8 release from PBMCs by IL-4 or IL-10 seen in healthy volunteers is preserved in patients with acute pancreatitis.

Under normal conditions, spontaneous cytokine release does not appear to be under the control of products of the cyclo-oxygenase pathway (Callery, 1990; Goodwin, 1983) and the findings of the present study are in agreement with this. However, LPS-stimulated PBMCs from healthy volunteers appear to be under such control (Endres, 1989). In contrast, in patients with both mild and severe acute pancreatitis on admission, a product of the cyclo-oxygenase pathway appears to be active in an attempt to minimise the increased release of IL-6 and IL-8 from PBMCs. Furthermore, this control mechanism appears to still be active on day 5 of admission in such patients. Of note, while IL-6 and IL-8 release on day 5 in patients with mild disease have returned to levels comparable with healthy volunteers, the presence of indomethacin in the culture medium significantly increases such cytokine release to levels comparable with patients with severe disease (Table 5.1). It is postulated that the use of indomethacin and perhaps other cyclo-oxygenase inhibitors may adversely influence pro-inflammatory cytokine
release in patients with acute pancreatitis. Whereas Lankisch and co-workers (in Hollender, 1983) noted a life-prolonging effect of indomethacin in a rat model of acute pancreatitis, another group observed an aggravation of the disease following the administration of indomethacin or aspirin in a rat model (Olazabal, 1980). There are no reports in the literature to my knowledge that indicate that patients taking aspirin or cyclo-oxygenase inhibitors do better or worse on such medication. However, an association between the use of such agents and complicated diverticular disease has been demonstrated (Campbell, 1991); the postulated mechanism being an impairment in the immune response to the diverticulitis to limit the inflammatory/infective process.

The dose response of PGE2 on IL-6 and IL-8 release from indomethacin treated PBMCs isolated from both healthy volunteers and patients with acute pancreatitis was the reverse of that expected (Molloy, 1993). If the effect of indomethacin was to inhibit endogenous PGE2 production and thus block the regulatory effect of PGE2, then the administration of exogenous PGE2 would conceivably restore the control mechanism. But despite healthy volunteers showing no significant change in cytokine release in the presence of indomethacin, PGE2 increased IL-6 and IL-8 release in a dose-dependent manner. In patients with acute pancreatitis, PGE2 appeared to increase IL-6 and IL-8 release at 10^{-6} M concentration while being inhibitory at 10^{-9} M concentration. Furthermore, the dose response curve for both IL-6 and IL-8 release tended to regress towards the curve for healthy volunteers with improvement in the patients condition (Figures 5.6 and 5.7). A possible explanation for this finding is that it is not the PGE2 concentration alone but the ratio of PGE2 to other prostaglandins or products of the cyclo-oxygenase pathway that is the important in the control of cytokine release (Goodwin, 1983). Indeed, PGE2 and lipopolysaccharide synergistically induced IL-6 release from intestinal epithelial cells (Meyer, 1994) and treatment with
indomethacin in a murine burn-sepsis model reduced the overproduction of TNFα and IL-6 from splenocytes normally observed in this model (O'Riordain, 1992). Another possible explanation is that indomethacin exerts effects through pathways in addition to cyclo-oxygenase inhibition (Mathieu, 1994). It has been reported that indomethacin can act directly on normal purified T lymphocytes to increase IL-2 production independent of arachidonic metabolism but related to a rise in calcium influx and to an increase in T cell membrane fluidity (Flescher, 1991). In retrospect, an important 'control' arm to these experiments was omitted, namely the dose response of PGE2 on PBMCs in the absence of indomethacin. However, the use of PGE2 in experimental acute pancreatitis has not been of significant benefit (Lankisch, 1983b; Martin, 1981).

The dose dependent inhibition of IL-6 and IL-8 release from PBMCs by IL-4 or IL-10 is preserved in patients with acute pancreatitis in a similar manner to healthy volunteers despite the increased release of IL-6 and IL-8 in patients with acute pancreatitis compared with healthy volunteers. It is evident that IL-4 and IL-10 have a potent immunoregulatory function and this is reflected in their ability to suppress cellular responses in vitro (Cassatella, 1993; Fiorentino, 1991; Lee, 1990). IL-4 and IL-10 have also been used as immunotherapy in a variety of situations. In both experimental arthritis (Allen, 1993) and in cancer patients receiving immunotherapy (Wong, 1993), the production of the pro-inflammatory cytokine IL-1 is reduced by the administration of IL-4 although there is a related toxicity at higher dosages (Gilleece, 1992). In addition, IL-10 protects mice against Staphylococcus enterotoxin B-induced lethal shock (Bean, 1993) and IL-4 and IL-10 exhibit synergy in the inhibition of cell-mediated immunity in a mouse model of leishmaniasis (Powrie, 1993). However, it is evident that not all cell types respond uniformly to the regulatory effect of the Type II cytokines. IL-4 induced a selective production of IL-6 from normal human B lymphocytes (Smeland, 1989). IL-4 also
appears to up-regulate IL-6 release from endothelial cells (Howells, 1991) and work in the present thesis shows that both IL-4 and IL-10 increase IL-6 and IL-8 release from LPS-stimulated endothelial cells (see Chapter 7). These regulatory events on endothelium, if they pertain in vivo, have the potential to exacerbate the systemic inflammatory response.
Chapter 6

Studies on PBMC proliferation and cell sub-types in patients with acute pancreatitis

6.1 Introduction

The findings of Chapter 5 demonstrated the dose dependent inhibition of IL-6 and IL-8 release from PBMCs by exogenous IL-4 or IL-10 is preserved in patients with acute pancreatitis in a similar manner to healthy volunteers despite the increased release of IL-6 and IL-8 in patients with acute pancreatitis compared with healthy volunteers. While sensitivity to the signal is retained, perhaps the production of the signal is reduced in patients with acute pancreatitis to allow increased pro-inflammatory cytokine release. These regulatory cytokines are produced mainly by specific sub-populations of CD4-positive T helper cells. The findings of Curley and co-workers (Curley, 1993) have already been discussed but to recap, they demonstrated a depletion of CD4-positive T helper lymphocytes in patients with acute pancreatitis with the greatest depletion occurring in those with severe disease who had significantly higher serum IL-6 levels. Following resolution of the pancreatitis and recovery of the patient, the CD4-helper population returned towards normal values. One flaw in this study was that CD8-positive suppressor
lymphocytes numbers were not also measured to allow determination of the CD4/CD8 ratio which may be the more useful index of lymphocyte imbalance. Nevertheless, similar findings have been reported for CD4-positive T helper cell depletion following thermal injury (O-Mahony, 1985), trauma (Faist, 1987) and septic patients with multiple organ failure (Nishijima, 1986) although the CD4/CD8 ratio was only significantly altered compared with controls in the trauma study.

In addition to the changes in the phenotype of PBMC sub-populations, lymphocyte function may also be deranged. T cell DNA synthesis as determined by thymidine incorporation in vitro has been shown to correlate well with host resistance to infection in vivo (Moss, 1988). PBMCs isolated from patients following trauma demonstrated significant impairment in both phytohaemagglutinin (Faist, 1987) and pokeweed mitogen (McRitchie, 1990) induced blastogenesis compared with controls. A similar result was observed in septic patients with multiple organ failure (Nishijima, 1986).

The aims of this chapter were to determine the phenotype of PBMC populations and to assess lymphocyte function by the incorporation of thymidine into unstimulated and phytohaemagglutinin-stimulated PBMCs isolated from patients with acute pancreatitis compared with those isolated from healthy volunteers.

6.2 Patients and methods

6.2.1 Patients

The study group comprised 14 patients with acute pancreatitis as defined in Section 2.1.1. These patients have been described in Table 4.1, Chapter 4, (Page
Peripheral blood was taken from patients on the first and fifth day of admission for the isolation and culture of PBMCs (as described in Section 2.3). The progress of the patient was evaluated with regard to the development of pancreatic complications as defined by the 1992 Atlanta Convention (Bradley, 1993) or the development of multiple organ failure as defined by the Goris score (Goris, 1985). Six healthy volunteers were also studied and these are similarly described in Chapter 4, Table 4.1, (Page 86).

6.2.2 PBMC phenotype

PBMCs were obtained by density centrifugation from 10 patients with acute pancreatitis and 6 healthy volunteers and were prepared for flow cytometry as described in Section 2.7, Chapter 2. The surface markers examined were CD3, CD4, CD8 and C14.

6.2.3 Phytohaemagglutinin-stimulated PBMC protocol

PBMCs from 14 patients with acute pancreatitis and 6 healthy volunteers were incubated at $2 \times 10^5$ cells per well in the presence or absence of phytohaemagglutinin (final concentration 0, 10 or 100 $\mu$g/ml). Subsequent incubation, pulse with tritiated thymidine, storage and cell harvest are described in Section 2.4, Chapter 2.

4.2.4 Statistical analysis

Initial comparison in tritiated thymidine uptake between patients with mild disease, severe disease and volunteers was performed using the Kruskal-Wallis test. Thereafter, Mann-Whitney U tests were applied to compare differences between any two groups.
6.3 Results

While the volunteer group were younger than the patient group (40 years, range 28-49 v 63 years, range 31-90 respectively, p = 0.70), there was no significant difference in the mean age between patients classified as mild or severe disease (65 years, range 42-90 v 61 years, range 31-84 respectively, p = 0.70). Six of the 14 patients were considered to have severe disease in that they developed complications related to their disease. Three of these patients had pancreatic necrosis demonstrated by contrast-enhanced CT scanning during the first week of admission. Five of the six patients with severe disease scored 1 or more on the modified Goris score, one of whom died of multiple organ failure.

6.3.1 PBMC phenotype

The percentage of cells expressing the cell surface antigens, CD3, CD4, CD8 and CD14 for each volunteer and patient are shown in Table 6.1. The mean percentage of cells expressing CD3 fell in the patient groups compared with the volunteer group, but this was only significant in the severe groups on day 1 and day 5: volunteer, 58.5 % (2.8) Mean (SEM); mild pancreatitis day 1, 44.4 % (7.2); mild pancreatitis day 5, 52.3 % (6.4); severe pancreatitis day 1, 22.4 % (6.9) p<0.01 and severe pancreatitis day 5, 24.3 % (6.8) p<0.01. Similarly, the mean percentage of cells expressing CD4 fell in the patient groups compared with the volunteer group, but this was only significant in the severe groups on day 1 and day 5: volunteer, 39.8 % (2.2) Mean (SEM); mild pancreatitis day 1, 33.1 % (5.1); mild pancreatitis day 5, 38.1 % (3.9); severe pancreatitis day 1, 17.8 % (4.4) p<0.01 and severe pancreatitis day 5, 17.0 % (6.8) p=0.04. There was no significant difference in the mean percentage of cells expressing CD8 in the patient groups compared with the volunteer group, (p>0.31): volunteer, 28.7 % (2.1) Mean (SEM); mild pancreatitis day 1, 20.0 % (4.6); mild pancreatitis day 5, 22.8 % (3.1); severe pancreatitis day 1,
Table 6.1
Percentage of PBMCs isolated from 6 healthy volunteers and 10 patients with acute pancreatitis on day 1 and day 5 of admission (stratified for mild (n=5) and severe (n=5) disease (Atlanta classification (Bradley, 1993)) expressing the cell surface antigens examined by flow cytometry. Minimum of 5 000 cells examined per episode for each subject.

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22.5 % (7.5) and severe pancreatitis day 5, 15.0 % (4.7). However, the ratio of CD4 positive cells to CD8 positive cells in the study groups was found to be similar as depicted in Figure 6.1. The increase in the mean percentage of cells expressing CD14 in the patient groups compared with the volunteer group just failed to reach statistical significance, (p=0.0528): volunteer, 12.8 % (0.9) Mean (SEM); mild pancreatitis day 1, 28.5 % (3.6); mild pancreatitis day 5, 25.8 % (7.0); severe pancreatitis day 1, 25.1 % (3.8) and severe pancreatitis day 5, 21.7 % (6.1). However, the ratio of the sum of the percentage of CD4 positive cells and CD8 positive cells over the percentage of CD14 positive cells fell significantly in the patient groups compared with the volunteer group as depicted in Figure 6.2.

During FACS analysis, the forward light scatter gives an assessment of cell size. In PBMC isolates, the relative size difference of monocytes from lymphocytes enables them to appear as two distinct cell populations on the FACS scattergram. However, in a number of patients with acute pancreatitis, particularly those with severe disease, a third population of cells, larger again in cell size than monocytes, and which did not express the surface antigens for lymphocytes or monocytes being tested, were observed (Figure 6.3). In one patient with severe disease who died on the ninth day following admission from multiple organ failure, this large cell population constituted 29.9 % of the PBMC isolate.

6.3.2 PHA-stimulated PBMC ³HTdr uptake

Results were available from only 5 volunteers because of an operator error with the cell harvester. In patients with both mild and severe acute pancreatitis on admission demonstrate a fall in mean DNA synthesis although this is only statistically significant in the severe group compared with volunteers as shown in Figure 6.3. By day 5 of admission, DNA synthesis is returned towards volunteer levels and there was no significant difference between the groups (Figure 6.4).
Figure 6.1

Ratio of CD4 positive to CD8 positive cells in PBMC populations isolated from 6 volunteers and 10 patients with acute pancreatitis on day 1 and day 5 of admission (stratified for mild (n=5) and severe (n=5) disease (Atlanta classification (Bradley, 1993)). Minimum of 5 000 cells examined per episode for each subject by flow cytometry.
Figure 6.2

Ratio of CD4 positive plus CD8 positive cells over CD14 positive cells in PBMC populations isolated from 6 volunteers and 10 patients with acute pancreatitis on day 1 and day 5 of admission (stratified for mild (n=5) and severe (n=5) disease (Atlanta classification (Bradley, 1993)). Minimum of 5 000 cells examined per episode for each subject by flow cytometry. * P<0.01 and ** P=0.03 comparing the volunteer group with the patient group (Mann-Whitney U test).
Figure 6.3

Flow cytometry scattergrams of PBMCs isolated from a patient with (a) mild disease and (b) severe disease (Atlanta classification (Bradley, 1993)). The cell subpopulations labelled A, B and C represent predominately CD3 positive (lymphocytes), CD14 positive (monocytes) and CD3 and CD14 negative (non lymphocyte/monocyte) cells respectively. Minimum of 5 000 cells examined. Note the prominent CD3 and CD14 negative cell population in (b) severe disease compared with (a), 39.1 % v 7.8 % respectively of total cell number.
Figure 6.4

Mean uptake of tritiated thymidine ($^3$HTdr) phytohaemagglutinin-stimulated PBMCs isolated from 5 volunteers and 14 patients with acute pancreatitis on day 1 and day 5 of admission (stratified for mild (n=6) and severe (n=8) disease (Atlanta classification (Bradley, 1993)). Final phytohaemagglutinin concentration, O 0 μg/ml, □ 10 μg/ml and ■ 100 μg/ml. 2 x 10$^5$ cells per well incubated in triplicate under standard conditions for 72 hr before being pulsed with 1 μCi of tritiated thymidine for 4 hr. Error bars represent the standard error of the mean. * P=0.03 comparing the volunteer group with the severe group on day 1 (phytohaemagglutinin concentration, 100 μg/ml)(Mann-Whitney U test).
6.4 Discussion

The findings of the present study demonstrate a shift in the phenotype of PBMCs in patients with acute pancreatitis compared with volunteers represented by an increase in the percentage of monocytes (CD14 positive cells) and a fall in the percentage of T lymphocytes although the CD4 to CD8 ratio was maintained (Figure 6.1). PBMC DNA synthesis as assessed by PHA-stimulated uptake of thymidine is reduced in patients with acute pancreatitis on admission although this only achieved statistical significance in those subsequently shown to have severe disease (Figure 6.4).

A monocytosis in the days following major injury is well recognised (Faist, 1987; Faist, 1988; Miller-Graziano, 1988). A similar pattern of monocytosis is demonstrated in the present study in patients with acute pancreatitis. As monocytes produce the greatest amount of pro-inflammatory cytokines per cell compared with lymphocytes and neutrophils, the finding of the monocytosis may explain in part the increase in IL-6 and IL-8 release observed from PBMCs cultured in standard cell numbers in patients with acute pancreatitis compared with healthy volunteers (as reported in Chapters 3 and 4). However, this is clearly not the whole picture, as on day 5 of admission, the mean percentage of monocytes in patients with mild or severe disease were similar but yet there was a significant difference in both PBMC IL-6 and IL-8 release between these two groups.

The depletion of T lymphocytes is also well reported in patients with major trauma (Faist, 1988), burns (O-Mahony, 1985) and sepsis (Nishijima, 1986; Williams, 1983). The findings of the present study suggest that this phenomenon is also observed in patients with acute pancreatitis and is in agreement with the findings of Widdison and co-workers (Widdison, 1994d). As already mentioned,
Curley and co-workers (Curley, 1993) observed a fall in CD4 positive cells, most marked in those with severe disease although the total lymphocyte count in this study is not reported. In the present study, the ratio of CD4 positive to CD8 positive cells is not significantly different in patients with acute pancreatitis compared with volunteers and again this finding is in accordance with the findings of Widdison and co-workers (Widdison, 1994c). The mechanism for this lymphocyte depletion is not fully known, but exposure to endotoxin is a recognised precipitating factor (Richardson, 1989), a feature observed in acute pancreatitis (Exley, 1992; Windsor, 1993). Furthermore, exogenous steroids (Haynes, 1978) and adrenaline (Crary, 1983) will redistribute circulating T cells and cause them to leave the peripheral blood and enter the spleen and other lymphoid tissue and it is possible that high circulating levels of endogenous hormones in patients with acute pancreatitis could mediate these effects. It is also evident that in a variety of inflammatory conditions, an imbalance occurs in the distribution of T cells between the peripheral blood and the tissues (O-Mahony, 1985). Thus the distribution of lymphocytes within the peripheral blood may not reflect the distribution of lymphocytes in other parts of the body and in particular, at sites of inflammation. Furthermore, the significance of the present results depends on the extent to which the phenotypic markers detected by the monoclonal antibodies used reflect the functional capacity or activity of the T cells they identify. This correlation between phenotype and function may not be exact. For example, a small subset of CD4 helper cells have been shown to be of importance in the generation of suppressor cells (Thomas, 1982) and it is now clear that the two main T cell subsets are both phenotypically and functionally heterogeneous (Takada, 1983). However, the data of the present study do not support the theory that there is an unopposed decrease in the number of CD4 positive helper T lymphocytes.
While the balance of T suppressor to T helper cells appears to be preserved in patients with acute pancreatitis (Figure 6.1), T lymphocyte control of monocyte activity may be impaired as the ratio of such cells to monocytes is altered, (Figure 6.2). Of note, this imbalance is significantly different compared with volunteers in the groups of patients with acute pancreatitis except for those with mild disease on day 5. It is in these groups of patients that PBMC IL-6 and IL-8 release is significantly increased compared with volunteers, while such cytokine release has returned to within 'normal' levels in patients with mild acute pancreatitis on day 5. A possible explanation for these findings could be the reduced production of Type II cytokines from the reduced number of lymphocytes as there was not an apparent alteration in the response of PBMCs in vitro to IL-4 or IL-10 in patients with acute pancreatitis compared with volunteers as reported in Chapter 5. Attempts to quantify IL-4 and IL-10 concentrations in the culture supernatants of PBMCs were unsuccessful. ELISAs for these cytokines are under development but the limit of detection of commercially available kits do not appear to be sensitive enough for such a study (de Wall Malefyt, 1993).

An additional finding of importance to that of the redistribution of lymphocytes and monocytes observed in the present study, is that a greater proportion of the isolated PBMCs from patients with acute pancreatitis (particularly those with severe disease) compared with the healthy volunteer group do not appear to express any the surface antigens being tested (Figure 6.3). An increase in immature lymphocytes and monocytes is likely (Wood, 1987) but in addition, the appearance of immature granulocytes in so called PBMCs obtained by Ficoll-fractionation is also described in patients with burns (Volencac, 1979). This phenomenon would tend to dilute the number of IL-6 and IL-8 producing cells in the standard cell number of plated PBMCs. Thus the real increase in IL-6 and IL-8
from mononuclear cells isolated from patients with acute pancreatitis may be underestimated in the present study.

The interpretation of changes in DNA synthesis as indicated by the PHA-stimulated uptake of thymidine is difficult in the light of the lymphocyte redistribution described above. Furthermore, there is marked inter-individual variation in both the volunteer and patient groups. Reduced DNA synthesis may simply reflect reduced lymphocyte number in the standard PBMC population. However, this may not be the full picture and impaired blastogenesis may also be present. For example, the mean percentage depletion of CD3 positive cells in patients with severe acute pancreatitis on the first day of admission was 38% of the mean volunteer value, while the mean PHA-stimulated (100 μg/ml) uptake of 3HTdr was only 29% of the mean volunteer value. Nevertheless, it is conceivable that lymphocyte function is impaired in patients with acute pancreatitis.
Chapter 7

Effect of interleukin-4 and interleukin-10 on human umbilical vein endothelial cell interleukin-6 and interleukin-8 release

7.1 Introduction

Endothelial cells form a physical but interactive barrier between the blood and the tissues and are important in the pathophysiology of conditions such as multiple organ failure and septic shock (Glauser, 1991; McMillen, 1993). A variety of circulating factors present in major inflammatory conditions including acute pancreatitis such as bacterial lipopolysaccharide, IL-1β and TNFα have been shown to induce functional alterations in endothelial cells in culture including the synthesis and release of pro-inflammatory cytokines such as IL-6 and IL-8 (Gimbrone, 1989; Jirik, 1989; Shalaby, 1989) and alterations in the expression of cell surface structures involved in neutrophil and monocyte adhesion to endothelial cells (Baggiolini, 1989; Bevilacqua, 1993; Zimmerman, 1992). The role of the IL-6 and IL-8 produced by endothelial cells is not clear, but they appear to influence the inflammatory response by interacting with circulating immune cells in order to mediate local cell activation and chemoattraction (Kuijpers, 1992; Mantovani, 1992;
Clearly, if such pro-inflammatory events occur unchecked then this might contribute to micro-circulatory failure and the tissue destruction seen in severe sepsis.

It seems reasonable that there should be mechanisms that down-regulate IL-6 and IL-8 release by endothelial cells. Of considerable interest in this regard are the actions of IL-4 and IL-10, which have been shown to attenuate pro-inflammatory cytokine production from a variety of immune-competent cells including polymorphonuclear cells, monocytes and macrophages (Cassatella, 1993; Essner, 1989; Fiorentino, 1991; Lee, 1990). The data presented in Chapter 5 demonstrates that PBMCs isolated from patients with acute pancreatitis on the first day of admission manifest a similar percentage down-regulation in pro-inflammatory cytokine release in response to IL-4 or IL-10 compared with healthy volunteers. The presence of such regulatory cytokines may favour a balance of cytokine production which diminishes the inflammatory response in vivo, for example, IL-4 promotes IL-1ra production from alveolar macrophages while simultaneously reducing the production of IL-1α or IL-1β (Galve-de Rochemonteix, 1993). However, there is also evidence to suggest that IL-4 and IL-10, (like many other members of the cytokine family) may not act uniformly on all cell types (Smeland, 1989). Indeed, IL-4 has been shown to induce rather than suppress IL-6 production from unstimulated and endotoxin-stimulated human endothelial cells (Colotta, 1992; Howells, 1991), but IL-10 had no effect on lipopolysaccharide stimulated IL-6 production in a murine endothelioma cell line (Sironi, 1993).

The purpose of this study was to examine the potential regulatory role of IL-4 and IL-10 on endotoxin (LPS)-stimulated human umbilical vein endothelial cell (HUVEC) release of IL-6 and IL-8.
7.2 Materials and Methods

7.2.1 Human umbilical vein endothelial cell cultures

HUVECs were obtained and cultured as described in Sections 2.5.1 and 2.5.2 respectively. Initial dose response curves were performed to identify the appropriate working conditions of human AB serum concentration (0-10 %), time of LPS pulse (1-5 hours), LPS-stimulation concentration (0.1-100 ng/ml), and the time of incubation following the LPS pulse (0-48 hours) to optimise IL-6 and IL-8 release.

Confluent HUVECs were then cultured in the presence or absence of LPS at a final concentration of 10 ng/ml with 5% human AB serum for 5 hours. Recombinant human IL-4 or IL-10 were added at the same time at a final concentration ranging from 50 fg/ml to 50 ng/ml. After 5 hours, the cells were washed with E-SFM growth media and further incubated for 12 hours in E-SFM growth media. Supernatants from the cultures were removed after 12 hours and stored at -70 °C for subsequent batch cytokine assay. All cultures were performed at 37 °C in the presence of 5 % CO₂, 95% humidified air.

7.2.2 Interleukin-6 and Interleukin-8 ELISA

IL-6 and IL-8 were assayed using sandwich ELISAs as described in Sections 2.6.2 and 2.6.3 respectively.

7.2.3 Statistical analysis

One-factor analysis of variance was used to compare the effect of IL-4 or IL-10 on LPS-stimulated HUVEC cytokine release with that of LPS-stimulated HUVEC in the absence of IL-4 or IL-10.
7.3 Results

Initial dose response curves were obtained on HUVEC lines to identify the appropriate working conditions of human AB serum concentration (Figure 7.1), time of LPS pulse (Figure 7.2), LPS-stimulation concentration (Figure 7.3), and the time of incubation following the LPS pulse (Figure 7.4). For subsequent experimentation on HUVEC, the human AB serum concentration chosen was 5 %, the time of LPS pulse was 5 hours, the LPS-stimulation concentration was 10 ng/ml, and the time of incubation following the LPS pulse was 12 hours.

Results are expressed as the mean of experiments performed on four HUVEC lines. Spontaneous HUVEC IL-6 and IL-8 release in the presence or absence of 5 % AB serum was undetectable over the study period. IL-4 and IL-10 had no measurable effect on spontaneous IL-6 and IL-8 release from HUVECs following the 12 hour incubation period. In the presence of LPS and human AB serum, the addition of IL-4 to the culture medium led to a dose-dependent increase in the mean IL-6 release by HUVEC, rising from $428 \pm 44 \text{ pg/ml}$ (no IL-4) to $1337 \pm 168 \text{ pg/ml}$ (IL-4: 50 ng/ml; $p=0.023$, one-factor analysis of variance) (Figure 7.5). IL-10 had no effect on mean IL-6 production from HUVEC in the presence of LPS (Figure 7.6). In the presence of LPS and human AB serum, the addition of IL-4 to the culture medium led to an initial dose-dependent increase in the mean IL-8 release by HUVEC rising from $2594 \pm 493 \text{ pg/ml}$ (no IL-4) to $7892 \pm 320 \text{ pg/ml}$ (IL-4; 5 pg/ml; $p=0.001$, one-factor analysis of variance) (Figure 7.7). Similarly, in the presence of LPS and human AB serum, the addition of IL-10 to the culture medium led to a dose-dependent increase in the mean IL-8 release by HUVEC rising from $2594 \pm 493 \text{ pg/ml}$ (no IL-4) to $8359 \pm 712 \text{ pg/ml}$ (IL-4; 50 pg/ml; $p=0.002$, one-factor analysis of variance) (Figure 7.8).
Figure 7.1

Lipopolysaccharide-stimulated (final concentration 100 ng/ml) IL-6 (○) and IL-8 (□) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of human AB serum concentration in the culture medium. Wells were pulsed in triplicate with LPS for 3 hrs in the presence of human AB serum (0-10 %) with subsequent incubation under standard conditions for 24 hr. IL-6 and IL-8 were measured by ELISA. Results of a representative experiment.
Figure 7.2

Lipopolysaccharide-stimulated (final concentration 100 ng/ml) IL-6 (○) and IL-8 (□) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of length of LPS pulse. Wells were pulsed in triplicate with LPS for 1, 3 or 5 hr in the presence of 5 % human AB serum with subsequent incubation under standard conditions for 24 hr. IL-6 and IL-8 were measured by ELISA. Results of a representative experiment.
Figure 7.3

Lipopolysaccharide-stimulated IL-6 (○) and IL-8 (□) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of LPS concentration. Wells were pulsed in triplicate with LPS (final concentration 0.1 to 100 ng/ml) for 3 hr in the presence of 5 % human AB serum with subsequent incubation under standard conditions for 24 hr. IL-6 and IL-8 were measured by ELISA. Results of a representative experiment.
Figure 7.4

Lipopolysaccharide-stimulated IL-6 (○) and IL-8 (□) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of period of incubation. Wells were pulsed in triplicate with LPS (final concentration 10 ng/ml) for 5 hr in the presence of 5% human AB serum with subsequent incubation under standard conditions for 0-48 hr. IL-6 and IL-8 were measured by ELISA. Results of a representative experiment.
Figure 7.5

Lipopolysaccharide-stimulated IL-6 (○) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of IL-4 concentration. Wells were pulsed in triplicate with LPS (final concentration 10 ng/ml) for 5 hrs in the presence of 5 % human AB serum and IL-4 (final concentration 0-50 ng/ml) with subsequent incubation under standard conditions for 12 hr. IL-6 was measured by ELISA. Results are the mean of four HUVEC lines with the standard error of the mean represented by the error bars. * P < 0.03 compared with 0 ng/ml of IL-4 (one-factor analysis of variance).
Figure 7.6

Lipopolysaccharide-stimulated IL-6 (O) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of IL-10 concentration. Wells were pulsed in triplicate with LPS (final concentration 10 ng/ml) for 5 hrs in the presence of 5 % human AB serum and IL-10 (final concentration 0-50 ng/ml) with subsequent incubation under standard conditions for 12 hr. IL-6 was measured by ELISA. Results are the mean of four HUVEC lines with the standard error of the mean represented by the error bars.
Figure 7.7

Lipopolysaccharide-stimulated IL-8 (□) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of IL-4 concentration. Wells were pulsed in triplicate with LPS (final concentration 10 ng/ml) for 5 hrs in the presence of 5 % human AB serum and IL-4 (final concentration 0-50 ng/ml) with subsequent incubation under standard conditions for 12 hr. IL-8 was measured by ELISA. Results are the mean of four HUVEC lines with the standard error of the mean represented by the error bars. * P < 0.03 compared with 0 ng/ml of IL-4 (one-factor analysis of variance).
Figure 7.8

Lipopolysaccharide-stimulated IL-8 (□) concentration in the culture supernatant from human umbilical vein endothelial cell monolayers as a function of IL-10 concentration. Wells were pulsed in triplicate with LPS (final concentration 10 ng/ml) for 5 hrs in the presence of 5% human AB serum and IL-10 (final concentration 0-50 ng/ml) with subsequent incubation under standard conditions for 12 hr. IL-8 was measured by ELISA. Results are the mean of four HUVEC lines with the standard error of the mean represented by the error bars. * P < 0.02 compared with 0 ng/ml of IL-10 (one-factor analysis of variance).
The addition of IL-4 and IL-10 after the 5 hr pulse of LPS (at the start of the 12 hour incubation period had little effect on IL-6 or IL-8 release. In these experiments results are the mean ± standard error of the mean of two HUVEC lines. LPS-stimulated HUVEC release of IL-6 was 470 ± 90 pg/ml, and IL-4 at 50 ng/ml following the LPS pulse enhanced the release of IL-6 to 540 ± 120 pg/ml, and IL-10 at 50 ng/ml following an LPS pulse increased the release of IL-6 to 500 ± 90 pg/ml. LPS-stimulated HUVEC release of IL-8 was 2490 ± 100 pg/ml, and IL-4 at 50 ng/ml following the LPS pulse enhanced the release of IL-8 to 3130 ± 480 pg/ml, and IL-10 at 50 ng/ml following an LPS pulse increased the release of IL-8 to 3065 ± 95 pg/ml. None of these increases were statistically significant.

7.4 Discussion

The anti-inflammatory actions of IL-4 and IL-10 are well recognised (Cassatella, 1993; Essner, 1989; Fiorentino, 1991; Lee, 1990). For example, both in experimental arthritis (Allen, 1993) and in cancer patients receiving immunotherapy (Wong, 1993), the production of the pro-inflammatory cytokine IL-1 is reduced by IL-4 coupled with the selective up-regulation of IL-1ra by resting or activated monocytes. IL-10 protects mice against Staphylococcus enterotoxin B-induced lethal shock (Bean, 1993) and IL-4 and IL-10 exhibit synergy to inhibit cell-mediated immunity in a mouse model of leishmaniasis (Powrie, 1993). IL-6 and IL-8 can be regarded as pro-inflammatory cytokines. Thus, in the present study, the enhancement by IL-4 and IL-10 of the release of the pro-inflammatory cytokines IL-6 and IL-8 by endothelial cells in the presence of endotoxin appears somewhat paradoxical. Both IL-4 and IL-10 up-regulated IL-8 release in the presence of endotoxin. In contrast, IL-4 but not IL-10 up-regulated IL-6 release in the presence of endotoxin. HUVEC pro-inflammatory cytokine production is generally
detectable within hours of an LPS-stimulus in the presence of serum factors (von Asmuth, 1993). The results of the present study agree with those of Sironi and co-workers (Sironi, 1993) who showed that IL-10 does not alter LPS-stimulated IL-6 production in a murine endothelioma cell line. IL-4 had no effect on pro-inflammatory cytokine production in the absence of an LPS stimulus in the present study and this differs from the findings of Howells and co-workers (Howells, 1991) who observed increased IL-6 release in HUVECs not exposed to endotoxin. These differences may, however, be due to different time courses since the incubation period in the present study was just 12 hours compared with 3 days in the study by Howells and co-workers.

The role of pro-inflammatory cytokines in both acute and chronic inflammatory diseases is now well recognised and successful therapeutic strategies have been developed to reduce the influence of such cytokines, for example, the administration of anti-TNFα (Exley, 1990) or anti-IL-6 (Starnes, 1990) in certain models of septic shock. The established role of IL-4 and IL-10 in the down-regulation of leukocyte pro-inflammatory cytokine release has led to the suggestion that IL-4 and IL-10 could be used to treat conditions such as septic shock. The results of the present study indicate that IL-4 and IL-10 augment LPS-mediated IL-6 and IL-8 release from endothelial cells and may indeed provide a means of altering pro-inflammatory cytokine concentration gradients within the local microenvironment. Further studies are required to determine the outcome of treatment with IL-4 or IL-10 under various conditions in vivo. Nevertheless, the findings presented here indicate that IL-4 and IL-10 may be involved in a complex regulation of endothelial cell activation following exposure to endotoxin. An acute inflammatory response requires the sequestration of circulating monocytes, lymphocytes and neutrophils into sites of inflammation. Clearly, the attraction and migration of circulating cells into such sites must be regulated with some precision.
and specificity. The fine-tuning of the cytokines produced by endothelial cells and
the cell-surface molecules expressed by these cells in the local micro-environment
may lend this precision and specificity.
Chapter 8

Serum concentrations of inflammatory mediators in patients with acute pancreatitis and their relationship to organ failure

8.1 Introduction

The central role of TNFα in orchestrating aspects of the early inflammatory response in sepsis is well established (Dinarello, 1993a). Elevated levels of TNFα have been documented in the serum of some groups of patients with sepsis (Damas, 1989) and following exposure to endotoxin (Michie, 1988b). However, TNFα is often undetectable in the sera of patients with acute pancreatitis, even those with severe disease (Banks, 1991a; Exley, 1992). This may be due to the short half life of TNFα, binding of TNFα to other proteins, or difficulties with the various methods of measuring TNFα in serum. Alternatively, it may be that TNFα is not produced in patients with acute pancreatitis or is mainly produced in the tissues and only occasionally spills over into the circulation. TNFα release from PBMCs isolated from patients with acute pancreatitis on admission was found not to be different from healthy volunteers (as discussed in Chapter 3). However, this may actually be an important finding as in a number of patients, 'normal' PBMC TNFα
release may be inappropriate in the presence of an elevated serum TNFα concentration.

The paracrine and endocrine actions of TNFα are exerted by interaction with membrane-bound TNFα receptors on the surface of target cells (Sherry, 1988). It appears that following exposure to substances such as bacterial endotoxin (and indeed TNFα itself) and during adherence, such target cells down-regulate their responsiveness to TNFα by shedding the receptor into the circulation (Lantz, 1994; Leeuwenberg, 1994; Shapiro, 1993). Two such soluble receptors have been identified, a 55 kDa (sTNFR55) and a 75 kDA (sTNFR75) fragment (Tartaglia, 1992). The release of these soluble receptors (which have a longer half life in serum than TNFα itself) appears to reflect the degree of TNFα-induced inflammation (Spinias, 1992). Thus, although TNFα may be undetectable in blood, the presence of such soluble TNFα receptors may be a useful index of TNFα activity. The role of the shed TNFα receptors is not fully understood. On the one hand, they could exert an anti-inflammatory effect; the shedding of surface receptors down-regulating the target cell’s responsiveness and the soluble TNFR may bind TNFα in the circulation thereby reducing its bioavailability (Olsson, 1991). On the other hand, it is not inconceivable that the TNFα-sTNFR complex could be recognised by a different cell surface receptor allowing the induction of a secondary inflammatory response (analogous to the soluble CD14 receptor-LPS-LPS binding protein complex that allows endothelium and other target cells that do not possess the CD14 receptor to respond to the presence of endotoxaemia (Arditi, 1993; Frey, 1992; Haziot, 1993)). However, sTNFR appear to be protective against TNFα mediated cell injury in vitro (Engelmann, 1989; Olsson, 1991; Van Zee, 1992). They also appear to be effective in vivo; the administration of sTNFR to baboons following LPS challenge reduced the typical release of TNFα and also the TNFα inducible
cytokines, IL-1β and IL-6 (Van Zee, 1992). STNFR also improved survival in a lethal endotoxaemia model in mice (Mohler, 1993).

Other cytokines involved in the inflammatory cascade, such as IL-6 are more often detected in the sera of patients with acute pancreatitis (Heath, 1993; Leser, 1991; Viedma, 1992). Both the serum concentration of IL-6 on admission and the peak level of IL-6 have some correlation with the severity of the disease (Heath, 1993; Leser, 1991; Viedma, 1992). One of the many actions of IL-6 is to induce the production of hepatic acute phase proteins, such as C-reactive protein (CRP). Serum CRP concentration is also elevated in patients with acute pancreatitis and correlates with the severity of the disease (Mayer, 1984; Puolakkainen, 1987; Wilson, 1989). The rise in serum CRP concentration lags behind the peak in serum IL-6 concentration by some 24 to 48 hours (Windsor, 1993) and this has been interpreted by some investigators to indicate a cause and effect relationship.

It has been proposed that a prolonged or excessive inflammatory response may be one of the mechanisms whereby patients develop multiple organ failure (Cerra, 1987; Deitch, 1992). The major causes of death in acute pancreatitis stem from either local peripancreatic complications or from multiple organ failure, or a combination of the two (de Beaux, 1995; Mann, 1994). In the present study, the systemic inflammatory response as measured by markers of inflammation (TNFα, sTNFR55, sTNFR75, IL-6 and CRP) in the serum of patients with acute pancreatitis and the relationship of these to the development of local complications and organ failure has been investigated.
8.2 Patients and Methods

8.2.1 Patients

The study population comprised patients with acute pancreatitis (section 2.1.1) admitted to the Royal Infirmary of Edinburgh. The clinical course of the patients was followed prospectively, allowing retrospective categorisation of patients into those with mild or severe disease based on the Atlanta classification (Bradley, 1993). Twenty eight patients with severe disease represent a consecutive series over a 16 month period. Patients with mild disease for comparison (n=30) represent a random selection from 78 patients with mild disease admitted over the same time period. Patients with severe disease were further sub-categorised into those with local pancreatic complications alone (n=18) and those with organ failure (a score of 1 or more; Goris score (Goris, 1985)) (n=10). Six patients with organ failure died during the index admission. The patient characteristics are given in Table 8.1. Venous blood was sampled on the first and second day of admission and the serum stored at -70 °C until subsequent batch analysis for TNFα, sTNFR55, sTNFR75, IL-6 and CRP concentration by indirect enzyme-linked immunosorbant assays (ELISAs).

8.2.2 Cytokine and CRP ELISAs

TNFα and the soluble TNFα receptors were measured by ELISA as described in sections 2.6.1 and 2.6.4 respectively. IL-6 and CRP were also measured by ELISA as described in sections 2.6.2 and 2.6.5 respectively.
Table 8.1

Details of patients with acute pancreatitis studied. Pancreatic complications defined by the Atlanta criteria (Bradley, 1993) and organ failure by the modified Goris score (Goris, 1985).

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8.2.3 Statistical analysis

Results are presented as the median (interquartile range). Initial comparison between patients with mild disease, a local pancreatic complication alone and organ failure was performed using the Kruskal-Wallis test. Thereafter, Mann-Whitney U tests were applied to compare differences between any two groups with significance taken at a p value < 0.05. Where levels of the cytokine being measured were below the limit of detection of the assay, the minimum detection concentration was assigned to the sample and statistical analysis performed using this convention.

8.3 Results

None of the 30 patients with mild disease developed significant complications. In contrast, 18 of the 28 (64 %) with severe pancreatitis developed local complications alone while the remaining 10 (36 %) developed failure of one or more organ systems, with or without an accompanying local pancreatic complication. There was no significant difference in the age (p=0.29) or sex ratio (p=0.57) between those with mild pancreatitis and those with severe pancreatitis.

Serum TNFα concentrations were below the level of detection in 41 of the 58 patients on the first day of admission (29/30 who had mild disease, 8/18 who developed a local pancreatic complication and 4/10 who developed organ failure) and 50 of the 58 patients on the second day of admission (30/30 who had mild disease, 16/18 who developed a local pancreatic complication and 4/10 who developed organ failure). On the first day of admission, median serum TNFα concentration was greater in the group who developed organ failure (p<0.001) and in those who developed a local pancreatic complication (p<0.001) compared with
the group who had mild disease (Figure 8.1). There was no significant difference between the group who developed organ failure and the group who developed a local pancreatic complication alone (p=0.34) (Figure 8.1). On the second day of admission, median serum TNFα concentration was greater in the group who developed organ failure compared with both the group who developed a local pancreatic complication (p<0.006) and the group who had mild disease (p<0.006) (Figure 8.1). There was no significant difference between the group who developed a local pancreatic complication alone and those who had mild disease (p=0.07) (Figure 8.1).

Serum sTNFR55 and sTNFR75 were detectable in all 58 patients. On both day 1 and 2 of admission, median serum sTNFR55 concentration was greater in the group who developed organ failure compared with the group who developed a local pancreatic complication (p<0.003). In both groups with severe disease, median serum sTNFR55 concentration was greater than in the group who had mild disease (p<0.001 in both cases) (Figure 8.2). STNFR75 concentrations showed a similar distribution between the 3 groups to that found for the sTNFR55 receptor (Figure 8.3).

Serum IL-6 concentrations were below the level of detection in 24 of the 58 patients on the first day of admission (20/30 who had mild disease, 3/18 who developed a local pancreatic complication and 1/10 who developed organ failure) and 23 of the 58 patients on the second day of admission (19/30 who had mild disease, 4/18 who developed a local pancreatic complication and 0/10 who developed organ failure). On both day 1 and 2 of admission, median serum IL-6 concentration was greater in the group who developed organ failure compared with the group who developed a local pancreatic complication alone (p<0.03) (Figure
Figure 8.1

Median serum TNFα concentration in the peripheral venous blood on the first and second day of admission in patients with acute pancreatitis categorised by eventual disease outcome: mild disease (○ n=30), local pancreatic complication alone (□ n=18)(Atlanta criteria (Bradley, 1993)); and organ failure (■ n=10)(modified Goris score (Goris, 1985)). Interquartile range represented by the error bars. TNFα measured by ELISA. The dotted line represents the limit of detection of the assay (15 pg/ml).
Figure 8.2

Median serum soluble TNFα receptor (55 kDa fragment) concentration in the peripheral venous blood on the first and second day of admission in patients with acute pancreatitis categorised by eventual disease outcome: mild disease (○ n=30); local pancreatic complication alone (□ n=18)(Atlanta criteria (Bradley, 1993)); and organ failure (■ n=10)(modified Goris score (Goris, 1985)). Interquartile range represented by the error bars. STNFR55 measured by ELISA.
Figure 8.3

Median serum soluble TNFα receptor (75 kDa fragment) concentration in the peripheral venous blood on the first and second day of admission in patients with acute pancreatitis categorised by eventual disease outcome: mild disease (O n=30); local pancreatic complication alone (□ n=18)(Atlanta criteria (Bradley, 1993)); and organ failure (■ n=10)(modified Goris score (Goris, 1985)). Interquartile range represented by the error bars. STNFR75 measured by ELISA.
8.4), which in turn was greater than in the group who had mild disease (p<0.001) (Figure 8.4).

CRP was detectable in the serum of all patients. On the first day of admission, there was no significant difference in median serum CRP levels between patients who developed organ failure, a local pancreatic complication or had mild disease (p=0.15) (Figure 8.5). By the second day of admission, median serum CRP concentration was greater in the group who went on to develop organ failure compared with those who developed a local pancreatic complication (p=0.02) or those who had mild disease alone (p=0.003) (Figure 8.5). However, median serum CRP level was not significantly different in the group who developed a local pancreatic complication compared with the group who had mild disease (p=0.80) (Figure 8.5).

8.4 Discussion

The present study demonstrates that during the first two days of admission, concentrations of markers of inflammation in the sera of patients with acute pancreatitis are highest in those who subsequently develop organ failure.

TNFα has been implicated as a key mediator in the development of multiple organ failure (Casey, 1993; Deitch, 1992). However in the present study, (in keeping with previous reports (Banks, 1991a; Exley, 1992)) TNF was detected in only a minority of patients on the first day of admission (41 of 58) and in only 8 patients by the second day of admission. This finding may be accounted for by the short half life of serum TNFα and the fact that in the present study, blood for TNFα estimation was sampled only once per day. Other authors have suggested that
Figure 8.4

Median serum IL-6 concentration in the peripheral venous blood on the first and second day of admission in patients with acute pancreatitis categorised by eventual disease outcome: mild disease (☐ n=30), local pancreatic complication alone (☐ n=18)(Atlanta criteria (Bradley, 1993)); and organ failure (■ n=10)(modified Goris score (Goris, 1985)). Interquartile range represented by the error bars. IL-6 measured by ELISA. The dotted line represents the limit of detection of the assay (40 pg/ml).
Figure 8.5

Median serum C-reactive protein concentration in the peripheral venous blood on the first and second day of admission in patients with acute pancreatitis categorised by eventual disease outcome: mild disease (○ n=30), local pancreatic complication alone (□ n=18)(Atlanta criteria (Bradley, 1993)); and organ failure (■ n=10)(modified Goris score (Goris, 1985)). Interquartile range represented by the error bars. C-reactive protein measured by ELISA.
TNFα appears transiently and repetitively in the circulation and thus the peak concentration of TNFα could easily be missed by infrequent blood sampling (Dofferhoff, 1992). In contrast, sTNF receptors were detected in all patients, with a stepwise increase in the median concentration of both sTNFR55 and sTNFR75 being observed between patients who had mild disease, those who developed a local pancreatic complication alone, and those who developed organ failure (Figures 8.2 and 8.3). Spinas and coworkers (Spinas, 1992) demonstrated a significant correlation between peak serum TNFα and sTNFR55 in human volunteers after intravenous administration of Escherichia coli endotoxin suggesting that sTNFR55 may reflect the degree of TNFα-induced inflammation. If this assumption is correct, the finding in the present study of elevated sTNF receptors on admission in patients who subsequently develop severe acute pancreatitis would support the contention that TNFα is an important central mediator of a systemic inflammatory response with progression to multiple organ failure early in the course of the disease.

In the present study, the serum levels of sTNFR on admission in patients with acute pancreatitis was most elevated in those who subsequently developed organ failure. Increased serum levels of sTNFR are reported in patients with sepsis and correlated with the simultaneously obtained APACHE II and multiple organ failure score as well as with mortality (Ertel, 1994; Froon, 1994). This correlation is perhaps not surprising in patients with established organ failure as the serum concentration of sTNFR correlates with the serum creatinine concentration, reflecting the degree of existing renal failure complicating the sepsis (Froon, 1994). However, in the present study, the serum levels of sTNFR were measured in patients with acute pancreatitis shortly following admission and the vast majority of these patients had no overt renal failure as manifest by a rise in serum creatinine at the time of venous sampling. While the administration of sTNFR was demonstrated
to be protective against TNFα mediated cell injury in vitro (Engelmann, 1989; Olsson, 1991; Van Zee, 1992), and also in vivo in a baboon model (Van Zee, 1992) and a mouse model of intravenous endotoxin challenge, the potential role of such therapy in patients with acute pancreatitis is less promising as in many patients, the major TNFα challenge would appear from the present study to have occurred prior to admission. Nevertheless, sTNFR therapy may have a part to play in the minimisation of secondary inflammatory responses.

IL-6 has also been implicated in the pathogenesis of multiple organ failure (Hoch, 1993). In the present study IL-6 was detectable more often than TNFα, being present in the serum of 34 patients on day 1 and 35 on day 2 of admission, with a stepwise increase in the median concentration of IL-6 between patients progressing to mild disease, a local pancreatic complication alone or organ failure (Figure 8.4). Furthermore, by day 2 of admission, IL-6 was detectable in all 10 patients who later developed organ failure. Patel and co-workers (Patel, 1994) found that serum IL-6 levels in patients with sepsis syndrome from an intra-abdominal septic focus, were greater on enrolment in those patients who subsequently died when compared with survivors. Furthermore, the non-survivors had a greater incidence of organ failure relative to survivors. I have demonstrated prolonged up-regulation of IL-6 release from peripheral blood mononuclear cells isolated from patients with acute pancreatitis who develop severe disease compared with patients with mild disease (Chapter 4), which demonstrates the possible contribution of leucocyte activation to the increase in serum IL-6 concentration.

In the present study, serum CRP concentrations were unhelpful on the first day of admission in predicting disease outcome (Figure 8.5). However, by the second day following admission, median serum CRP concentration was significantly elevated in the group of patients developing organ failure compared
with patients with mild disease or a local pancreatic complication alone. This finding concurs with other authors who report an elevation of serum CRP concentration in patients with acute pancreatitis who subsequently develop severe disease (Mayer, 1985; Puolakkainen, 1987; Wilson, 1989). This is consistent with the concept of a systemic inflammatory response contributing to the development of organ failure in critical illness (Cerra, 1987).

Patients dying from acute pancreatitis, appear to do so as a consequence of multiple organ failure (de Beaux, 1995; Tran, 1992). While such organ failure is usually associated with pancreatic inflammation and necrosis, development of a local pancreatic complication alone in the absence of organ failure is rarely fatal (de Beaux, 1995). The present study has shown that severe acute pancreatitis is associated with increased levels of serum markers of a systemic inflammatory response and that within this group of patients, those who develop organ failure show a greater concentration of these markers compared with those who develop local pancreatic complications alone. The serum markers of inflammation which were measured may have derived from the tissues, endothelial cells or leukocytes but the source of these mediators cannot be identified from the present study. Nevertheless, the findings reflect the host systemic response to pancreatic inflammation and the level of the response in the present study did relate to the development of organ failure. While there is evidence to suggest that the host response to pancreatic inflammation may have a significant genetic predetermination (Rinderknecht, 1994), further study of the mechanisms leading to a systemic inflammatory response may allow early identification of patients at risk and thereby aid the development of therapeutic intervention to modify the biological response in patients with acute pancreatitis.
A randomised double-blind study of glutamine supplementation of total parenteral nutrition in patients with severe acute pancreatitis: effect on peripheral blood mononuclear cell pro-inflammatory cytokine release and proliferative response

9.1 Introduction

Glutamine is the most abundant amino acid both in the plasma and in the intracellular free amino acid pool (Wallace, 1992). The glutamine molecule has a 5 carbon chain with 2 amino moieties and accounts for some 30% of all of the amino acid nitrogen that is transported in the plasma. Despite an intense traffic of glutamine between organs, the circulating concentration of glutamine (500-900 μM) is usually maintained at a fairly constant level. In the postabsorptive state, glutamine is synthesised de novo by a number of cells, principally skeletal muscle, the lungs and the liver and is therefore not an essential amino acid. Glutamine accounts for more than 50% of the intracellular free amino acid pool in skeletal muscle and this serves as a major source of this amino acid during periods of
metabolic stress (Herskowitz, 1990). Furthermore, branched chain amino acids (isoleucine, leucine, valine) can be metabolised to glutamine in skeletal muscle. The lungs also release glutamine in the post absorptive state by the uptake of precursor nitrogen from the circulation in the form of ammonia and glutamate. The liver plays an important role in glutamine homeostasis because it can either generate or consume glutamine.

Glutamine is essential for a wide variety of physiological processes. It is the principal fuel source for the intestinal mucosa and other rapidly dividing cells, in particular, the growth and function of immune cells including lymphocytes and macrophages (Alexander, 1993; Sarantos, 1993) as well as pneumatocytes and vascular endothelial cells. In conditions of excess glutamine utilisation, such as sepsis, trauma, major surgery or severe acute pancreatitis, endogenous glutamine production may not be adequate and glutamine depletion occurs (Lacey, 1990; Steininger, 1989). Intracellular glutamine stores may be depleted by more than 50% while plasma levels fall by up to 30%. Exogenous glutamine may be required to satisfy the body's glutamine requirements, but the glutamine depletion is compounded by the fact that many of these patients are being fed parenterally rather than enterally, and traditional parenteral feeding does not contain glutamine. This is because of its relative insolubility as well as its long term instability in solution where it may degrade to ammonia and pyroglutamic acid.

Patients with severe acute pancreatitis can develop local pancreatic complications related to pancreatic necrosis and develop distant organ dysfunction (Bradley, 1993). Infection of the necrotic pancreas is thought to be a key step in the progression to a persistent systemic inflammatory response and multiple organ dysfunction (Beger, 1986; Widdison, 1993). An intact T-cell response and gut mucosal barrier play an important roles in the defence of the host against bacterial
invasion, and T-cell DNA synthesis (as determined by thymidine incorporation in vitro) correlates well with host resistance to sepsis in vivo (Moss, 1988). T-cells are dependent on glutamine for optimal growth and function (Ardawi, 1988) and glutamine depletion may be partly responsible for T-cell suppression seen in these severely stressed patients (Curley, 1993; Widdison, 1996). T-cells also exert an important regulatory role on monocyte pro-inflammatory cytokine release (Fiorentino, 1991; Lee, 1990), and T-cell suppression may contribute to the prolonged increase in pro-inflammatory cytokine release (IL-6 and IL-8) observed in patients with severe acute pancreatitis as described in Chapter 4. Clinical susceptibility to sepsis may also be enhanced by abnormal production of pro-inflammatory cytokines (O'Riordain, 1992).

Recently, the development of glutamine dipeptides has allowed glutamine to be introduced into total parenteral nutrition regimens which are stored prior to administration to patients. These dipeptides, are very stable in solution but are rapidly broken down in vivo to allow immediate bio-availability of the constituent amino-acids. The clinical administration of these compounds is well tolerated, even in patients with severe sepsis (Karner, 1989). Van der Hulst and coworkers (van der Hulst, 1993) demonstrated that glutamine given via the parenteral route can prevent the deterioration of gut integrity and preserve mucosal structure in patients with inflammatory bowel disease or intestinal malignancy. O'Riordain and coworkers (O'Riordain, 1994) demonstrated that glutamine given via the parenteral route can restore T cell blastogenesis in patients undergoing colorectal resection. While glutamine may improve gut barrier function and T cell blastogenesis, it is not clear whether glutamine would increase or decrease pro-inflammatory drive in patients with acute pancreatitis. The aims of this study then, were to perform a double blind randomised controlled study comparing the effect of glutamine supplemented total parenteral nutrition versus conventional total parenteral nutrition on T-cell
proliferative response and pro-inflammatory cytokine release (TNFα, IL-6 and IL-8) by peripheral blood mononuclear cells in patients with severe acute pancreatitis.

9.2 Patients and Methods

9.2.1 Patients

Patients over the age of 18 years with severe acute pancreatitis were included in the study. Severe acute pancreatitis for the purpose of this study was defined as a modified Glasgow score (Blamey, 1984) of 3 or more in a patient requiring total parenteral nutrition because of persisting failure of the gastrointestinal tract 7 days after the onset of illness. Patients with respiratory failure (requiring ventilatory support), renal failure (creatinine >200 μmol/l) or hepatic failure (bilirubin >100 μmol/l in the absence of choledocholithiasis) were excluded until resolution of organ failure. In addition, patients receiving systemic steroids were excluded. The study protocol was approved by the Ethical Committee (Subcommittee for Surgery and Orthopaedic Surgery) of the Lothian Health Board. Full written informed consent was obtained from all patients before inclusion to the study.

9.2.2 Study protocol

The 14 study patients were commenced on conventional total parenteral nutrition for a minimum of 3 days prior to randomisation to receive either conventional or glutamine supplemented total parenteral nutrition which ran continuously for 7 subsequent days. Following the study period, total parenteral nutrition was stopped or continued with a conventional regimen depending on the clinical condition of the patient. Peripheral blood was taken on day 0 (before the commencement of the study total parenteral nutrition), on day 4 and on day 7 (at the
end of the study total parenteral nutrition) for the isolation of blood mononuclear cells. In vitro peripheral blood mononuclear cell cultures were performed to determine unstimulated and phytohaemagglutinin-stimulated T-cell DNA synthesis and the spontaneous release of TNFα, IL-6 and IL-8 into the supernatant. The clinicians responsible for patient management and the laboratory investigator were blinded to which total parenteral nutrition was used.

9.2.3 Total parenteral nutrition formulae

Conventional total parenteral nutrition was based on an amino acid solution (Vamin 18 EF, Pharmacia & Upjohn GmbH, Erlangen, Germany) providing 0.25 g nitrogen per kg body weight per day with lipid emulsion (Intralipid 20 %, Pharmacia & Upjohn) and glucose (Baxter, Newbury, U.K.). Electrolytes, trace elements and vitamins were added to maintain requirements. The calorie to nitrogen ratio was 2060 kcal to 17.5 g nitrogen and the glucose to lipid calories was 57% to 43% respectively. The total parenteral nutrition supplemented with glutamine consisted of an isocaloric, isonitrogenous and isovolumetric solution, but Vamin 18 EF in the conventional regime was replaced with glutamine-supplemented amino-acid solution (Glamin, Pharmacia & Upjohn). This regimen, based on the amino-acid profile of Vamin 18 EF additionally provided 0.22 g glutamine per kg body weight per day in the form of dipeptide glycy1-glutamine, and glutamine in this regimen represented 17% of the total administered nitrogen.

9.2.4 PBMC proliferation protocol

PBMCs were incubated in triplicate at 2 x 10^5 cells per well in the presence or absence of phytohaemagglutinin (final concentration 100 μg/ml). Subsequent incubation, pulse with tritiated thymidine, storage and cell harvest are described in Section 2.4, Chapter 2.
9.2.5 PBMC pro-inflammatory cytokine release protocol

PBMCs were incubated in triplicate at $2 \times 10^5$ cells per well. Following 24 hr incubation, the supernatants were removed and stored in aliquots at -70 °C for subsequent batch cytokine assay.

9.2.6 TNFα, IL-6 and IL-8 ELISAs

Supernatants and serum were assayed for TNFα, IL-6 and IL-8 by ELISAs as described in Sections 2.6.1, 2.6.2 and 2.6.3, Chapter 2 respectively.

9.2.7 Statistical analysis

The Mann-Whitney U tests was used to compare patients characteristics and the change in DNA synthesis and cytokine release over the study period between groups. Two-tailed tests were used on all occasions. P values of less than 0.05 were considered statistically significant.

9.3 Results

Fourteen patients with severe acute pancreatitis were recruited to the study, 7 in each group. One patient in the glutamine supplemented total parenteral nutrition group was withdrawn as a result of central line sepsis on day 3 of the study protocol and was excluded from further analysis or comparison. The patients characteristics at entry to the study are shown in Table 9.1. The aetiology of the acute pancreatitis in the glutamine total parenteral nutrition group was gallstones in 2 patients, alcohol in 1 patient, idiopathic in 2 patients and post endoscopic retrograde cholangiopancreatography in 1 patient and in the conventional total parenteral nutrition group, gallstones in 2 patients, alcohol in 3 patients and idiopathic in 2 patients. Local pancreatic complications (pancreatic necrosis, pseudocyst or abscess
Table 9.1

Characteristics of patients with severe acute pancreatitis at entry to the study. Values are median (interquartile range) P values were calculated using the Mann-Whitney U test. * as measured by thymidine incorporation: see Patients and methods; Section 9.2.

<table>
<thead>
<tr>
<th></th>
<th>Glutamine TPN (n=6)</th>
<th>Control TPN (n=7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>51 (45-58)</td>
<td>53 (44-56)</td>
<td>1</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>4:2</td>
<td>4:3</td>
<td>0.74</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>75 (59-81)</td>
<td>75 (72-100)</td>
<td>0.48</td>
</tr>
<tr>
<td>Duration of illness prior to randomisation (days)</td>
<td>18 (10-27)</td>
<td>10 (9-19)</td>
<td>0.25</td>
</tr>
<tr>
<td>T- cell response * PHA 0 μg/ml (counts per min)</td>
<td>1851 (1493-2264)</td>
<td>2382 (921-3646)</td>
<td>0.67</td>
</tr>
<tr>
<td>T- cell response * PHA 100 μg/ml (counts per min)</td>
<td>43888 (19121-62534)</td>
<td>41832 (36776-61767)</td>
<td>0.67</td>
</tr>
<tr>
<td>PBMC tumour necrosis factor release (pg/ml)</td>
<td>130 (12.5-422)</td>
<td>25.4 (12.5-183)</td>
<td>0.39</td>
</tr>
<tr>
<td>PBMC interleukin-6 release (pg/ml)</td>
<td>228 (40-5356)</td>
<td>459 (40-3495)</td>
<td>0.66</td>
</tr>
<tr>
<td>PBMC interleukin-8 release (ng/ml)</td>
<td>42.5 (20.8-86.4)</td>
<td>27.3 (6.9-54.8)</td>
<td>0.57</td>
</tr>
</tbody>
</table>
as defined by the Atlanta classification (Bradley, 1993)) were present in 4 patients in the glutamine total parenteral nutrition group and 5 patients in the conventional total parenteral nutrition group. No patient died from the disease during the index admission.

9.3.2 PBMC proliferation

While median unstimulated T-cell DNA synthesis increased by 472 counts per min in the glutamine total parenteral nutrition group over the study period compared with a decrease of 416 cpm in the conventional total parenteral nutrition group, this change was not significantly different between the two groups (Figure 9.1). The median phytohaemagglutinin stimulated T-cell DNA synthesis increased in both the glutamine and conventional total parenteral nutrition groups (3099 and 216 cpm respectively) but this change was not significantly different between the two groups (Figure 9.2).

9.3.2. PBMC pro-inflammatory cytokine release

There was no significant change in median peripheral blood mononuclear cell TNFα release or IL-6 release over the study period in the glutamine total parenteral nutrition group compared with the conventional total parenteral nutrition group (Figure 9.3 and 9.4 respectively). In contrast, the median peripheral blood mononuclear cell IL-8 release decreased by 17.7 ng/ml in the glutamine total parenteral nutrition group over the study period compared with an increase of 43.3 ng/ml in the conventional total parenteral nutrition group and this change was significantly different between the two groups; p=0.045 (Figure 9.5).
Figure 9.1

Median change in unstimulated T-cell DNA synthesis on day 4 and day 7 of the study period compared with baseline (day 0) in patients with severe acute pancreatitis randomised to receive either glutamine supplemented total parenteral nutrition (■) or isonitrogenous, isocaloric conventional total parenteral nutrition (□). Box and whisker plots represent the interquartile range and the range respectively. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 72 hr before being pulsed with 1 μCi of tritiated thymidine for 4 hr.
Figure 9.2

Median change in phytohaemagglutinin stimulated (final concentration 100 μg/ml) T-cell DNA synthesis on day 4 and day 7 of the study period compared with baseline (day 0) in patients with severe acute pancreatitis randomised to receive either glutamine supplemented total parenteral nutrition (■) or isonitrogenous, isocaloric conventional total parenteral nutrition (□). Box and whisker plots represent the interquartile range and the range respectively. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 72 hr before being pulsed with 1 μCi of tritiated thymidine for 4 hr.
Figure 9.3

Median change on day 4 and day 7 of the study period compared with baseline (day 0) in spontaneous tumour necrosis factor-α concentration in the culture supernatant from peripheral blood mononuclear cells isolated from patients with severe acute pancreatitis randomised to receive either glutamine supplemented total parenteral nutrition (■) or isonitrogenous, isocaloric conventional total parenteral nutrition (□). Box and whisker plots represent the interquartile range and the range respectively. $2 \times 10^5$ cells per well incubated in triplicate under standard conditions for 24 hr. TNFα was measured by ELISA.
Figure 9.4

Median change on day 4 and day 7 of the study period compared with baseline (day 0) in spontaneous IL-6 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from patients with severe acute pancreatitis randomised to receive either glutamine supplemented total parenteral nutrition (■) or isonitrogenous, isocaloric conventional total parenteral nutrition (□). Box and whisker plots represent the interquartile range and the range respectively. 2 x 10⁵ cells per well incubated in triplicate under standard conditions for 24 hr. IL-6 was measured by ELISA.
Figure 9.5

Median change on day 4 and day 7 of the study period compared with baseline (day 0) in spontaneous IL-8 concentration in the culture supernatant from peripheral blood mononuclear cells isolated from patients with severe acute pancreatitis randomised to receive either glutamine supplemented total parenteral nutrition (■) or isonitrogenous, isocaloric conventional total parenteral nutrition (□). Box and whisker plots represent the interquartile range and the range respectively. 2 x 10^5 cells per well incubated in triplicate under standard conditions for 24 hr. IL-8 was measured by ELISA. * P=0.045 comparing the glutamine group with the conventional group; Mann-Whitney U test.
9.4 Discussion

Patients with severe acute pancreatitis are recognised to suffer glutamine depletion and the development of sepsis both within the necrotic pancreas and at distant sites may adversely affect survival of the patient. Glutamine depletion has been implicated in the development of T-cell suppression seen in such patients. This study has shown that the administration of total parenteral nutrition supplemented with glutamine (as the glycyl-glutamine dipeptide) tends to improve T-cell DNA synthesis in patients with severe acute pancreatitis. However, this difference failed to reach statistical significance compared with a conventional total parenteral nutrition (Figure 9.1 and 9.2). In a previous study (O'Riordain, 1994), it was demonstrated that total parenteral nutrition supplemented with glutamine significantly improves T-cell DNA synthesis in patients with colorectal surgery undergoing elective resection compared with both preoperative values and a group receiving conventional total parenteral nutrition. In the present study, small patient numbers associated with substantial inter-individual variation may partly explain the lack of statistical significance. The ability of T cells to respond to a mitogenic stimulus correlates well with host resistance to sepsis, (Moss, 1988) and experimental in vivo enhancement of T-cell response is associated with reduced susceptibility to infection (Horgan, 1990). Glutamine supplementation reduces mortality in septic rats (Ardawi, 1991) and Ziegler et al (Ziegler, 1992) have shown a reduction in the rate of clinical infection in patients undergoing bone marrow transplantation who received glutamine supplemented total parenteral nutrition. These papers suggested that glutamine supplementation may reduce the incidence of clinical infection by enhancing immune function, but specific immunologic parameters were not examined in either of these studies. In the present study, there was no mortality and few clinical infections to allow meaningful comparison
between the glutamine supplemented and conventional total parenteral nutrition groups and these were not the stated aims of the study.

Glutamine can play an important role in lymphocyte metabolism for the generation of energy and as a precursor of protein and nucleotide synthesis within the rapidly dividing cell (Brand, 1989). Lymphocyte activation in vivo results in a large increase in protein synthesis which corresponds with a significant increase in thymidine uptake in vitro. Although glutamine may be synthesised in the body, it becomes "conditionally essential" when demand outstrips supply within conditions of increased use. It has been speculated that one of the reasons why intracellular free glutamine in skeletal muscle becomes depleted under these conditions is to provide glutamine for the cells of the immune system (O'Riordan, 1994). In addition to the direct effects on the T-cell, it is also possible that glutamine may mediate beneficial effects indirectly. Glutamine may help reduce T-cell suppression by reversing pathophysiological processes which lead to such suppression. Loss of gut barrier function has been implicated as a major cause of sepsis (Deitch, 1991; Wilmore, 1988). By maintaining gut barrier function (van der Hulst, 1993), glutamine may well prevent the translocation of endotoxin and other mediators of sepsis, thus preventing the T-cell suppression. Another possible mechanism by which glutamine may prevent the T-cell suppression associated with disease is via glutathione. Glutathione is an important anti-oxidant, and tissue glutathione levels are known to be reduced in acute pancreatitis (Braganza, 1995). A decrease in glutathione levels is associated with a decreased phytohaemagglutinin response in an animal model (Robinson, 1993) and glutamine has been shown to restore glutathione levels in these situations (Hong, 1992).

In addition to its role in lymphocyte function, glutamine also plays an important role in macrophage/monocyte metabolism. The present study
demonstrated that there was no significant change in peripheral blood monocyte TNFα and IL-6 release in either group over the study period (Figure 9.3 and 9.4 respectively). In contrast, IL-8 release was reduced in the glutamine supplemented total parenteral nutrition group while it was increased in the conventional total parenteral nutrition group (Figure 9.5). Monocytes are terminally differentiated cells with a low rate of DNA synthesis. Although glutamine may be required for nucleic acid synthesis in the lymphocyte, Souba and coworkers (Souba, 1990) have suggested that in times of stress, glutamine also may support monocyte production of the cytokine mediators of the catabolic response. Indeed glutamine is required for the production of interleukin-1 by cultured macrophages (Wallace, 1992) and it seems likely that it is required for the production of other pro-inflammatory cytokines as well. However, glutamine may have an indirect effect to mediate a reduction in pro-inflammatory cytokine release. Restoration of T-cell function will improve the lymphocyte mediated regulation of monocyte pro-inflammatory cytokine release. Furthermore, glutamine improves gut barrier function and if this is associated with an attenuation of portal endotoxaemia, this might reduce the stimulus to monocyte pro-inflammatory cytokine release. TNFα is recognised as an important central mediator of the systemic inflammatory response seen in sepsis. However, its role in acute pancreatitis is less clear. The detection of tumour necrosis factor in the serum of patients with acute pancreatitis, even in those with severe disease, is not a consistent finding as discussed more fully in Chapter 8. IL-6 is a key cytokine as a signal to the hepatocyte to induce an acute phase protein response and serum levels of IL-6 are typically elevated in patients with acute pancreatitis. However, in the present study, glutamine supplemented total parenteral nutrition had no significant effect on TNFα or IL-6 release from peripheral blood mononuclear cells. Perhaps a more important cytokine as far as damage to distant organs and the production of distant organ failure is IL-8. IL-8 has a multiplicity of actions, but it is an important cytokine in the context of acute
pancreatitis as it can prime circulating neutrophils as well as stimulate endothelial cells, thereby promoting neutrophil sequestration in uninjured end-organs, setting the stage for neutrophil-mediated end-organ injury (Biffl, 1995; Botha, 1996). Indeed, IL-8 has been implicated in the acute lung injury seen in acute pancreatitis (Donnelly, 1993). Thus, the reduction in interleukin-8 release from peripheral blood mononuclear cells observed in the present study may be of benefit to patients with severe acute pancreatitis. In this context, we have observed that resolution of IL-8 release from peripheral blood mononuclear cells back towards levels found from healthy volunteers is associated with the patients clinical recovery (Chapter 4).

The present study has demonstrated potential benefits of glutamine supplemented total parenteral nutrition over conventional regimens in patients with established severe acute pancreatitis. The administration of glutamine (either via the enteral route where possible, or the parenteral route) earlier in the course of the disease in patients with predicted severe disease may have more marked immunological effect with an improvement in clinical outcome in such patients.
Chapter 10

Discussion

Acute pancreatitis remains a common and potentially lethal disease. The greater the magnitude of the systemic inflammatory response (as part of the disease pathophysiology) the greater is the likely severity of the illness with progression to multiple organ dysfunction. The hypothesis of this thesis is that pro-inflammatory cytokine release from peripheral blood mononuclear cells is increased in patients with acute pancreatitis early in the course of the disease (as part of the systemic inflammatory response), and this increased cytokine release contributes to the severity of the illness. I have examined aspects of the immune system relating to this hypothesis in patients with acute pancreatitis with a view to the possibility of modulating the systemic inflammatory response and thus the outcome for the patient.

IL-6 and IL-8 release from peripheral blood mononuclear cells isolated from patients with acute pancreatitis at the time of admission is increased compared with healthy volunteers (Chapter 3). Severe disease (as defined by the Atlanta classification (Bradley, 1993)) is characterised by prolonged increased release of such cytokines at least until day five of admission (Chapter 4). Products of the
cyclo-oxygenase pathway are recruited to minimise such increased release of IL-6 and IL-8 (Chapter 5). Moreover, peripheral blood mononuclear cells from patients with acute pancreatitis appear to remain sensitive in vitro to the down-regulatory effect of IL-4 and IL-10, (cytokines produced mainly from T lymphocytes)(Chapter 5). However, both the number of circulating T lymphocytes and the in vitro phytohaemagglutinin-stimulated proliferative response appears to be diminished in patients with acute pancreatitis (especially in those with severe disease) compared with healthy volunteers (Chapter 6). These findings would suggest a potential therapeutic benefit for the administration of regulatory cytokines, such as IL-4 and IL-10, to patients with acute pancreatitis in an attempt to control the systemic inflammatory response. The timing of such intervention is likely to be as soon as possible after the diagnosis of the illness, perhaps targeting the group of patients at risk of severe disease to minimise potential adverse effects of therapy in those who will have mild disease anyway. However, the administration of IL-4 or IL-10 themselves may have an adverse effect on the overall release of IL-6 and IL-8 as demonstrated in Chapter 7 where in vitro IL-6 and IL-8 release from endothelial cells is significantly increased by IL-4 and IL-10. Furthermore, TNFα induced inflammation, as suggested by elevated serum levels of soluble TNFα receptors in patients with acute pancreatitis on admission, (especially in those who subsequently developed organ failure) would imply that significant inflammatory events have occurred in patients prior to presentation to hospital and thus before therapeutic intervention could be implemented (Chapter 8). Nevertheless, in patients with established severe acute pancreatitis, glutamine supplementation of total parenteral nutrition led to a significant reduction in IL-8 release from peripheral blood mononuclear cells and a trend towards improved un-stimulated and phytohaemagglutinin-stimulated proliferative responses compared with a conventional total parenteral nutrition regimen (Chapter 9).
Measurement of cytokine release by peripheral blood mononuclear cells in disease states and extrapolation of such release to the pathophysiology of disease is complicated by intra- and inter-subject variation. Gene polymorphism in the control mechanisms of cytokine production of human monocytes is recognised (Danis, 1995). Furthermore, the change in cytokine production rather than the absolute level of cytokine production may be more important for the individual in determining outcome, but in most human studies, the individual’s resting or normal cytokine production is usually not known. An alternative approach, (as employed in this thesis) is to study groups with similar severity of disease and use the mean values obtained from each group for comparison. In a recent study, peripheral blood mononuclear cells were isolated from 10 patients (including one with acute pancreatitis) who were critically ill with sepsis over a three day study period (Rogy, 1996). Five of the subjects died. While LPS-stimulated TNFα, IL-1β and IL-6 release were generally higher in the non-survivors than survivors, there was no significant difference between the two study groups. McKay and co-workers (McKay, 1996) studied LPS-stimulated monocytes isolated from patients with acute pancreatitis, stratified for 'uncomplicated course' (n=10) and 'systemic complications' (n=16) of whom five died during the index admission. Monocyte TNFα release was significantly greater one day following admission but not by day 3 or day 5 in the systemic complication group compared with the uncomplicated group. Monocyte IL-1β release, in contrast, only became significantly greater in the systemic complication group by day 5. Monocyte IL-6 release was significantly greater on day 1 and day 3 but not day 5 while IL-8 release was only significantly greater on day 3 in the systemic complication group compared with the uncomplicated group. These findings vary from the findings of this thesis. In the present work, pro-inflammatory cytokine release was increased in patients with acute pancreatitis compared with controls. However, there was no significant difference in peripheral blood mononuclear cell IL-6 and IL-8 release on day 1
between those patients with mild or severe disease. In contrast, IL-6 and IL-8 release remained significantly elevated by day 5 in the severe group whereas levels of release returned to 'normal' values in the mild group. A number of factors may explain these different findings. Firstly, McKay and co-workers studied isolated monocytes rather than blood mononuclear cells. Secondly, the uncomplicated group in the McKay study had evidence of potential severe disease, all study patients having an APACHE II score greater than 5 and thus representing a sicker population than the mild group of the present study. While monocyte pro-inflammatory cytokine release may be increased in patients with acute pancreatitis, monocyte phagocytic activity is significantly impaired in those patients with predicted severe disease compared with mild disease (Liras, 1996). Although monocyte phagocytic activity had improved by day 5 in both the mild and severe groups, the activity in the severe group was reported to be still below the level observed in healthy volunteers. Neutrophil phagocytic activity mirrored these results. In contrast, granulocyte elastase activity and fluorescence with acridine orange were greater in patients with predicted severe disease, indicative of excessive neutrophil activation, which persisted by day 5 of admission (Liras, 1996).

The role of TNFα in patients with acute pancreatitis remains unclear. TNFα is often undetectable in the sera of such patients although soluble TNFα receptors are usually detected and relate on admission to subsequent disease activity. Similar findings were reported in a study which recruited 146 patients with sepsis syndrome admitted to an intensive care unit (Goldie, 1995). The median APACHE II score on admission in this study was 23 and the 30 day mortality in the study was 49 %. TNFα was detectable in the serum on admission to the intensive care unit in only 14 %. However, soluble TNFα receptors were detectable in all patients and were significantly higher in non-survivors compared with survivors. Nevertheless, studies in experimental models of acute pancreatitis have suggested a primary role
for TNFα. In one study (Hughes, 1995), severe acute pancreatitis was induced in germ-free rats, (which have no source of endogenous endotoxin), by ductal infusion of artificial bile. Serum TNFα levels remained low in the sham group but were significantly elevated in both normal rats and germ-free rats with acute pancreatitis and there was with no significant difference in TNFα concentration between the latter two groups. These findings would suggest that the rise in serum TNFα was related to the onset of acute pancreatitis rather than due to subsequent endotoxaemia related to the disease. In another study (Fink, 1995), acute pancreatitis was induced in two strains of mice by two different methods (caerulein hyper-stimulation or feeding a choline deficient, ethionine enriched diet) and total pancreatic mRNA for TNFα and the 55 kDa and 75 kDa TNFα receptors measured. There was no constitutive expression of TNFα mRNA within pancreatic tissue, but it was induced rapidly in the pancreata of all animals during the progression of acute pancreatitis. All normal pancreata demonstrated a 55 kDa to 75 kDa receptor mRNA ratio of 5:1. During the progression of both forms of acute pancreatitis, mRNA for the 55 kDa receptor was down-regulated to zero while the 75 kDa receptor mRNA was up-regulated five fold. The precise role of such mRNA expression for TNFα and its receptors in the pathophysiology of acute pancreatitis in these models is not clear. However, in a human model of inflammation, local production of TNFα enhanced neutrophil function at the site of inflammation, with increased cytotoxicity and enhanced ability to respond to weak environmental signals (Yee, 1994).

The increased IL-6 and IL-8 release from peripheral blood mononuclear cells isolated from patients with acute pancreatitis compared with healthy volunteers is believed to contribute to the elevation in serum levels of these pro-inflammatory cytokines in patients with acute pancreatitis and also other conditions associated with sepsis syndrome (Goldie, 1995) including major trauma (Botha, 1996). The role of these cytokines when they appear in the circulation has not been fully
elucidated but their propagation of a state of systemic leucocyte activation has been proposed by Rot (Rot, 1992). In a study of 33 patients at risk of multiple organ failure following major trauma, plasma from these patients was tested for the priming effect on normal neutrophils and for the presence of platelet-activating factor (PAF) and IL-8 (Botha, 1996). Plasma sampled at 3, 6, 12 and 24 hr after injury significantly primed normal neutrophils to release increased amounts of superoxide. The priming effect of the plasma taken at 3 hours could be inhibited by a PAF antagonist, but not thereafter suggesting other mediators such as complement fragments, IL-6 or IL-8 was responsible for subsequent neutrophil priming. Indeed, plasma IL-8 levels were raised at 6 and 12 hours after injury and were significantly higher in the 6 patients who developed multiple organ failure. Other studies have demonstrated that IL-8 can stimulate normal endothelial cells to an activated phenotype (Biffl, 1995). Thus, pro-inflammatory molecules such as IL-8 can prime circulating neutrophils as well as endothelial cells, thereby promoting neutrophils sequestration in uninjured end organs and setting the stage for distant organ damage (Wang, 1995).

While increased pro-inflammatory cytokine release has been demonstrated in patients with acute pancreatitis, the biological effect of such cytokines is tempered by the balance between pro-inflammatory and anti-inflammatory mediators (Kubes, 1993). IL-4 and IL-10 are cytokines thought to exert predominately anti-inflammatory effects. For example, in a mouse model, the role of TNFα in T-cell-mediated inflammation depended on the prevailing pro-inflammatory/anti-inflammatory cytokine balance (Hernandez-Pando, 1994). Joyce and co-workers (Joyce, 1994) demonstrated that IL-10 reduced the pro-inflammatory potential of TNFα by down-regulation of monocyte TNFα receptor expression, increasing soluble TNFα receptor levels and inhibiting the release of TNFα itself. In a recent abstract, IL-10 levels in the sera of patients with acute
pancreatitis appear to inversely reflect the severity of the disease (Galloway, 1996a). IL-10 concentration was elevated in patients with mild disease while levels recorded in the severe group were similar to those seen in healthy human volunteers. These relatively low values in severe acute pancreatitis would support the concept of reduced anti-inflammatory cytokine production allowing unbalanced pro-inflammatory activity. In contrast, another study, demonstrated that phytohaemagglutinin-stimulated peripheral blood mononuclear cells isolated from patients following major trauma (n=8) or burns (n=16) produced greater amounts of IL-4 and IL-10 compared with healthy volunteers (O'Sullivan, 1995). However, pro-inflammatory cytokines such as TNFα, IL-6 and IL-8 were not measured, and it may be that the final ratio of cytokine production are more important with regard to biological action. IL-4 plasma levels have also been reported in major trauma victims (DiPiro, 1995). IL-4 plasma levels were significantly greater in those patients (both on admission and maximal values) who developed sepsis. However, two observations in this paper (DiPiro, 1995), may have relevance to acute pancreatitis. A low admission IL-4 level was associated with a greater incidence of nosocomial infection, and patients over the age of 30 years had significantly lower IL-4 levels in response to sepsis (risk of infection and increasing age are risk factors in acute pancreatitis). Another study reported that the intra-peritoneal administration of IL-10 reduced the severity of acute pancreatitis in a rat model (Rongione, 1995).

The severity of acute pancreatitis is related to the magnitude of the systemic inflammatory response associated with the disease. The sequential release of TNFα, IL-1β, IL-6, IL-8 and other inflammatory molecules such as PAF are incriminated in the pathogenesis of this response. It thus follows that strategies to inhibit either the production or the function of such pro-inflammatory mediators could abrogate the response. A variety of antibodies to cytokines, soluble cytokine
receptors and receptor antagonists have been identified or developed and their use in both animal models and patients with sepsis syndrome have been described (Christman, 1995; Debets, 1994). An alternative approach is to reduce the serum levels of inflammatory molecules by continuous haemofiltration (Wakabayashi, 1996). An anti-TNFα polyclonal antibody attenuated the physiological and biochemical changes associated with acute pancreatitis in a rat model (Grewal, 1994). The calcium channel blocker, diltiazem, improved survival from 40 % to 80 % in a rat model of bile-induced pancreatitis (Hughes, 1996). Calcium has been suggested as a signal for TNFα release and also for TNFα action at distant sites. Calcium channel blockade reduced serum TNFα levels although whether this was a direct effect on TNFα production or mediated indirectly through alteration in pancreatic blood flow or anti-protease activity is not clear from this study (Hughes, 1996).

IL-1 receptor antagonism (IL-1ra) has been investigated in rat and mice models of acute pancreatitis. IL-1ra given before or shortly after the induction of acute pancreatitis by intra-peritoneal injection reduced the pancreatic inflammation and necrosis as well as significantly diminishing the expected rise in serum TNFα and IL-6 in a dose-response effect (Norman, 1995a). The administration of IL-1ra also reduced the multiple organ failure and mortality in these animal models (Norman, 1995b; Tanaka, 1995).

The beneficial effects of PAF antagonists on experimental acute pancreatitis have been discussed in the Introduction (Section 1.10). A further report demonstrated that an intra-peritoneal injection of a PAF antagonist 30 minutes after the induction of acute pancreatitis in a microembolic rat model of acute pancreatitis ameliorated the acute lung injury typical of this model (Galloway, 1996b). A phase II trial of the same PAF antagonist as referred to above in patients with acute
pancreatitis has demonstrated the agent to be safe and well tolerated (Kingsnorth, 1995). Furthermore, there was a significant reduction in the incidence of organ failure and in total organ failure score at the end of the 72 hour medication period compared with placebo. PAF antagonist treatment also reduced serum IL-8 although the changes in serum IL-6 were not significant.

These studies discussed above point to the potential of bio-modulation of the systemic inflammatory response (in conditions such as acute pancreatitis) by blocking, through a variety of mechanisms, key steps in the initiation and propagation of inflammation. However, a number of difficulties are evident that currently limit such intervention in current clinical acute pancreatitis. The timing of intervention is important. In the majority of studies using animal models of the disease, the immuno-modulatory compounds were given at the time of, or shortly after, the induction of acute pancreatitis. In the clinical situation, several hours if not days go by prior to the patient seeking medical attention and the diagnosis being confirmed. In this respect, the finding of significant TNFα induced inflammation as indicated by raised levels of soluble TNFα receptors in the sera of patients with acute pancreatitis who subsequently developed severe disease and organ failure (Chapter 8), suggest that the window of opportunity for such immuno-therapy may have been missed. However, one small group of patients that afford rapid diagnosis are those patients who develop acute pancreatitis following endoscopic retrograde cholangio-pancreatography. Nevertheless, strategies of immuno-modulation may also be applicable to minimise the effect of secondary events that would add to the state of systemic inflammation. A further limitation of immuno-therapy, particularly with regard to the use of monotherapy, is the level of redundancy within the human immune system. It is recognised that the triggering signals and the subsequent actions of cytokines and other inflammatory mediators overlap to a large extent so that a number of inflammatory events would require to be abrogated.
Furthermore, the immune system is primarily present to aid with healing and repair following injury or infection and tampering with the system could alter the balance of inflammation in a deleterious manner to the host. This was the recent view of a drug company seeking to embark on clinical trials of an IL-1 receptor antagonist in acute pancreatitis. Despite the encouraging results in animal studies, (Norman, 1995a; Norman, 1995b; Tanaka, 1995) the company were dissuaded from mounting such a clinical trial (Pancreatitis Experts Meeting, Amsterdam, March 1996).

Immunotherapy is not without its adverse effects. It would therefore seem logical to target those patients at risk of severe disease. However, current methods of disease prediction are inaccurate and require 24 if not 48 hours of physical and biochemical measurements to make the assessment; the time period over which immunotherapy is likely to be most effective.

An alternative approach is the use of nutritional agents that have an influence on the immune system. For example, glutamine, along with other amino-acids such as arginine are under study in this respect. In the present study, I was able to demonstrate reduced IL-8 release from peripheral blood mononuclear cells in patients with established severe disease with the use of glutamine supplemented total parenteral nutrition compared with a conventional regimen (Chapter 9). The median time to randomisation in this study was approximately two weeks following admission (Table 9.1, Page 168). There is a case for considering the administration of glutamine (along with other nutritional substances) earlier in the course of the disease, and for employing the more physiological enteral route where possible. A preliminary report has demonstrated encouraging findings with early enteral nutrition on markers of the systemic inflammatory response, organ failure and time in the intensive care unit in patients with acute pancreatitis (Kanwar, 1997). Another abstract has reported the benefits of glutamine supplementation of both enteral and
parenteral nutrition in 156 critically ill adult patients including patients with acute pancreatitis (Griffiths, 1996). Glutamine supplementation reduced both the post-intervention intensive care unit and total hospital costs per survivor, and in the case of parenteral feeding, also improved survival compared with conventional regimens. Clearly, further research in nutritional pharmacy is required but the findings to date are encouraging.

While the systemic inflammatory response of acute pancreatitis can occur solely in the presence of pancreatic necrosis, endotoxaemia from the intestines and infection of the pancreas or distant organs can significantly contribute to the response. A number of factors predispose to endotoxin or bacterial translocation across the gut. Reduced intestinal transit time and bacterial overgrowth (Leveau, 1996), along with reduced blood flow and increased intestinal permeability (Wang, 1996) are described in experimental acute pancreatitis. Serum LPS binding protein (which binds LPS and facilitates its presentation to monocytes and macrophages) is increased in patients with acute pancreatitis (Erwin, 1996). Furthermore, LPS binding protein correlated with the severity of the disease, higher levels found in those patients with severe disease, becoming significant by 72 hours following admission compared with mild disease. A number of strategies to reduce infection in acute pancreatitis have been tried including selective decontamination of the gut and systemic anti-microbial agents. In one study (Luiten, 1995), patients with severe acute pancreatitis were randomised to standard treatment or standard treatment with selective decontamination of the gut. Mortality in the decontamination group was significantly reduced 11/50 (22 %) versus 18/52 (35 %). The difference was mainly caused by a reduction in late mortality due to a significant reduction in Gram negative infection of the pancreas. In both groups of patients, all Gram negative aerobic pancreatic infection was preceded by colonisation of the digestive tract by the same bacteria. A number of studies
investigating the effect of systemic antibiotic therapy in both experimental and clinical acute pancreatitis have been reported (Pederzoli, 1993; Widdison, 1994a; Foitzik, 1996; Johnson, 1996). Reduction in the number of septic complications, particularly of the pancreas is a constant finding although disappointingly, this has not translated into a reduction in mortality. The most effective anti-microbial agents in this respect are those with good penetration into necrotic tissue.

Acute pancreatitis remains a potentially lethal disease. Nevertheless, our understanding of the pathophysiology of the disease (Formela, 1995) and prediction of severity (Hedström, 1996) is improving while potential therapeutic agents are under investigation (Skaife, 1996). What is also becoming evident, is the genetic predisposition in some patients to develop severe disease, sometimes with the rapid onset of multiple organ failure and death within days of admission (Rinderknecht, 1994). Genetic variation in an individual's immunological response to pancreatic inflammation is likely as observed in patients with sepsis (Guillou, 1993; Wakefield, 1993). Furthermore, genetic variation at the level of the pancreatic acinar cell itself in response to pancreatic injury, in particular, the ability to activate acinar cell apoptotic programs and regulate the appearance of stem cells may also influence the outcome for the patient (Iovanna, 1996).

Nevertheless, the future for patients with severe acute pancreatitis is encouraging as the fruits of research in a variety of areas related to the disease become realised. Further research into the pathophysiology of the disease will pave the way to the development of specific treatments for a condition that to date, relies on supportive therapy alone. More accurate, early disease prediction will allow the identification of high risk groups, perhaps making use of the immunological and genetic markers discussed in this thesis. Advances in the care of the critically ill
and of organ support will also benefit those patients who develop organ failure and who previously succumbed from their illness.
Appendix 1

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Appendix 2

Scientific presentations and papers resulting from this thesis work

12.1 Presentations

Presentations from the work presented in this thesis have been read at a number of local, national and international scientific meetings. Papers have been read at

1. Caledonian Society of Gastroenterology including one entitled 'Interleukin-6 release from peripheral blood mononuclear cells in acute pancreatitis' which was awarded the Astra Research Prize (February, 1995).

2. Surgical Research Society, two of which have been presented in the Plenary Session for the Patey Prize entitled 'Serum soluble TNF receptor concentrations are associated with disease severity in acute pancreatitis' (January 1996) and 'Regulatory effect of IL-4 and IL-10 on human umbilical vein endothelial cell cytokine production' (July 1994).


4. Royal College of Surgeons of Edinburgh.

5. Pancreatic Society of Great Britain and Ireland.
6. European International Hepato-pancreatico-biliary Association Congress,
   (Athens, May 1995).
7. European Pancreatic Club. One of these presentations entitled 'Activation of
   the cyclo-oxygenase pathway as a regulatory mechanism of blood
   mononuclear cell interleukin-8 release in patients with acute pancreatitis' was
   awarded the Clinical Science Paper Prize (Barcelona, June 1995).
8. European Society for Parenteral and Enteral Nutrition (Geneva, September
   1996)
9. Overseas meeting of the Royal College of Surgeons of Edinburgh, (Hong
   Kong, October 1996).

12.2 Published papers

5. de Beaux AC, O'Riordain MG, Ross JA, Jodozi L, Carter DC, Fearon KCH.
   Glutamine supplemented total parenteral nutrition reduces blood mononuclear
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4. de Beaux AC, Ross JA, Maingay JP, Fearon KCH, Carter DC. Pro-
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   patients with acute pancreatitis: correlation of IL-6 and IL-8 release with
3. de Beaux AC, Goldie AS, Ross JA, Carter DC, Fearon KCH. Serum
   concentrations of inflammatory mediators related to organ failure in patients

   Extended abstract in Gastroenterology Digest October 1996:3-4.


Permission has been received from the editors of the above journals to allow reproduction of the published papers at the end of this thesis.

### 12.3 Published abstracts


1. de Beaux AC, Ross JA, Fearon KCH, Carter DC. Down-regulation of IL-6 production from peripheral blood mononuclear cells from patients with acute pancreatitis by IL-4. *Br J Surg* 1994;81:1816
Proinflammatory cytokine release by peripheral blood mononuclear cells from patients with acute pancreatitis


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Proinflammatory cytokine release was measured from peripheral blood mononuclear cells (PBMCs) isolated from six volunteers and, on admission, from 16 patients with acute pancreatitis. Tumour necrosis factor (TNF) release in patients did not differ significantly from that of volunteers, whereas both interleukin (IL) 6 and IL-8 release in patients was raised when compared with that in the volunteer group (mean±s.e.m.) IL-6 20±7(4-6) versus 9±3(1-7) ng/ml, P = 0.03; IL-8 283±40 versus 128±22 ng/ml, P = 0.009. When variation in white cell count was accounted for, IL-6 and IL-8 release but not that of TNF was significantly greater in patients with severe disease than in those with mild disease. These results point to a complex upregulation of proinflammatory cytokine release from PBMCs in patients with acute pancreatitis, components of which relate to the clinical progress of the disease.

Acute pancreatitis is a common and potentially fatal disease which is characterized by a systemic inflammatory response that may progress to multiple organ failure and death. It has been proposed that organ dysfunction results from excessive leucocyte activation. Barrie and co-workers have noted that the pulmonary sequelae of experimental acute pancreatitis in sheep are prevented by leucocyte depletion before the induction of pancreatitis. Similarly leucocyte depletion reduces acute lung injury in experimental acute pancreatitis in rats. In other models of pancreatitis, pronounced margination of leucocytes within the capillaries and venules of the liver and lungs is an early event in progressive organ dysfunction and failure. Research has focused on neutrophil activity, but other cellular systems may be important in pathogenesis of the disease. The mechanisms promoting intravascular leucocyte activation are not clear. Proinflammatory mediators may be released into the circulation from the focus of the acutely inflamed pancreas. Endotoxaemia is a recognized feature of the disease and may also mediate intravascular events.

The evidence for leucocyte activation in acute pancreatitis in humans has largely been accrued through indirect means by measurement of surrogate markers of inflammation in the serum of patients. Neutrophil elastase released during phagocytosis is rapidly complexed in the circulation with α1-protease inhibitor. The serum concentration of this complex is raised in a number of inflammatory conditions, including acute pancreatitis, and is thought to be a crude marker of neutrophil activation. Other leucocyte products, including proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL) 6, have been measured in patients with acute pancreatitis. TNF is a short-lived cytokine, produced early in the disease process, and is thus often undetectable in the serum of patients following admission. However, the level of IL-6 appears to be more stable and is raised in the serum of patients with acute pancreatitis, correlating to some degree with the severity of disease.

This paper investigates whether in vitro proinflammatory cytokine release by peripheral blood mononuclear cells (PBMCs) is associated with disease severity in vivo. To examine leucocyte activation directly, the study investigated the spontaneous and endotoxin-stimulated release of proinflammatory cytokines from PBMCs isolated from patients with acute pancreatitis. Cytokine release in vitro was then related to the clinical course of the patients and to the serum concentration of the corresponding cytokine. To investigate the influence of serum factors on cytokine release, PBMCs were incubated in both autologous serum and fetal calf serum (FCS). PBMCs are a mixed cell population comprised largely of monocytes and lymphocytes. These cell types participate in intercellular signalling which can significantly modify cell function in mixed cultures in comparison with that in mononuclear cells. Thus coincubation of mononuclear cells is important as it may more accurately reflect their actions in vivo.

Patients and methods

Sixteen consecutive patients with acute pancreatitis were studied. The diagnosis of acute pancreatitis was based on the presence of appropriate clinical or radiographic evidence accompanied by a serum amylase concentration greater than 1000 units/l (Phadebas; Pharmacia Diagnostics, Uppsala, Sweden; normal range 70–300 units/l). Peripheral blood was taken from patients on the first day of admission for isolation of PBMCs, acquisition of serum and white cell counting. The progress of the patient was evaluated with regard to the development of pancreatic complications as defined by the 1992 Atlanta Convention of multiple organ failure as defined by the Goris score. No patient was receiving steroids, non-steroidal anti-inflammatory drugs or other immunosuppressive medication on admission. Six control subjects were healthy volunteers, taking no medication and with no known pancreatic pathology. Written informed consent was obtained from patients and the protocol was approved by the local ethics committee.

Isolation and culture of peripheral blood mononuclear cells

PBMCs were cultured in 96-well flat-bottomed tissue culture plates at 2×10⁶ cells per well in 200 μl RPMI 1640 supplemented with penicillin 50 units/ml, streptomycin 50 μg/ml, glutamine 2 m mol/l and 5 per cent FCS (ICN Flow Laboratories, Irvine, UK) or 5 per cent autologous serum. In lipopolysaccharide (LPS)-stimulated cultures (Escherichia coli 0127:B8; Sigma Chemicals, Poole, UK) the final concentration of LPS was 10⁻³.
5 μg/ml. Supernatants from the cultures were removed after 24 h and stored at –70°C for subsequent batch cytokine assay. All cultures were performed at 37°C in the presence of 5% per carbon dioxide.

Assay for interleukins 6 and 8 and tumour necrosis factor (TNF)

IL-6, IL-8 and TNF were measured by enzyme-linked immunosorbent assay (ELISA). The capture antibody was mouse monoclonal anti-human IL-6 antibody (Boehringer Mannheim, Lewes, UK) or rabbit polyclonal anti-human IL-8 (AMS Biotechnology UK, Witten, UK). The detecting antibodies were goat polyclonal anti-IL-6 or anti-IL-8 antibody (R&D Systems, Oxon, UK) followed by polyclonal sheep anti-goat immunoglobulin G horseradish peroxidase-labelled antibody (Sigma Immunochimicals, Poole, UK) with tetramethylbenzidine (Boehringer Mannheim) as substrate. The limits of sensitivity of the assays were 40 pg/ml for IL-6 and 200 pg/ml for IL-8. Intra-assay and interassay variation was less than 5%.

The capture antibody for TNF was mouse monoclonal anti-human TNF and the detecting antibody was peroxidase-conjugated mouse monoclonal anti-human TNF Fab fragments (Boehringer Mannheim), used according to the manufacturer’s instructions. The limit of sensitivity of the assay was 15 pg/ml. Intra-assay and interassay variation was less than 4%. The spontaneous cytokine release was not significantly greater in patients with pancreatitis than in controls (3.5 ± 2.2 versus 2.3 ± 1.5, P = 0.04). The IL-8 release was not significantly enhanced in either the pancreatitis or control group from PBMCs cultured in media supplemented with autologous serum compared with FCS (1.4 ± 0.9 versus 1.2 ± 0.8, P = 0.36).

TNF and IL-6 were detectable on the first day of admission in the serum of seven patients, five of whom developed severe disease as described above (Fig. 1). However, there was no correlation between the serum TNF and IL-6 concentration and spontaneous PBMC

Table 1: Spontaneous and lipopolysaccharide-stimulated tumour necrosis factor, interleukin 6 and interleukin 8 release from peripheral blood mononuclear cells isolated from six healthy volunteers and during the first day of admission in 16 patients with acute pancreatitis

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>TNF (pg/ml)</th>
<th>IL-6 (ng/ml)</th>
<th>IL-8 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous in FCS Control</td>
<td>1356(376) 69(4-5)</td>
<td>139(374) 10(6-13)</td>
<td>238(26) 23(4-6)</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>2053(741) 93(7-17)</td>
<td>2425(441) 20(7-4-6)</td>
<td>283(40) 20(7-4-6)</td>
</tr>
<tr>
<td>Spontaneous in AS Control</td>
<td>2053(741) 93(7-17)</td>
<td>2425(441) 20(7-4-6)</td>
<td>283(40) 20(7-4-6)</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>3554(825) 11(5-9)</td>
<td>3554(825) 24(2-5)</td>
<td>427(35) 24(2-5)</td>
</tr>
<tr>
<td>LPS stimulation in FCS Control</td>
<td>2596(839) 11(5-9)</td>
<td>3554(825) 24(2-5)</td>
<td>427(35) 24(2-5)</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>3994(1199) 17(7-26)</td>
<td>3994(1199) 17(7-26)</td>
<td>419(56) 17(7-26)</td>
</tr>
<tr>
<td>LPS stimulation in AS Control</td>
<td>4799(708) 35(8-5-5)</td>
<td>4799(708) 35(8-5-5)</td>
<td>419(56) 35(8-5-5)</td>
</tr>
</tbody>
</table>

Cells were incubated in the presence of 5% per cent fetal calf serum (FCS) or 5% per cent autologous serum (AS). Values are mean(s.e.m.). TNF, tumour necrosis factor; IL, interleukin; LPS, lipopolysaccharide. *P<0.05 (TNF versus control, Mann-Whitney U test).

Fig. 1 Serum tumour necrosis factor (TNF) and interleukin (IL) 6 concentration in patients with acute pancreatitis measured on the first day of admission. #: Patients with mild disease (n = 5); O, patients with mild disease (n = 11)
cytokine release ($r = 0.10$, $P = 0.71$ and $r = 0.22$, $P = 0.42$ for TNF and IL-6 respectively). IL-8 was detected in the serum of only two patients. TNF, IL-6 and IL-8 were undetectable in the serum of volunteers.

The spontaneous release of TNF, IL-6 and IL-8 from PBMCs isolated from patients with acute pancreatitis on the first day of admission, and cultured at a standard cell concentration of $10^6$ per ml, is depicted in Fig. 2. PBMC release of TNF, IL-6 and IL-8 was not significantly different between the 11 patients with mild and the five with severe pancreatitis. As an estimation of cytokine production per unit of blood, allowing for the variation in white cell count between patients, cytokine production from the standard cell concentration was multiplied by the mononuclear cell count (monocyte count plus lymphocyte count) measured on the first day of admission; these results are depicted in Fig. 3. PBMC IL-6 and IL-8 release

<table>
<thead>
<tr>
<th>Mild disease</th>
<th>Severe disease</th>
</tr>
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<tbody>
<tr>
<td><strong>a</strong></td>
<td><strong>b</strong></td>
</tr>
<tr>
<td>TNF (ng/ml)</td>
<td>IL-8 (ng/ml)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1000</td>
<td>25</td>
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<td>6000</td>
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<tr>
<td>12000</td>
<td>7000</td>
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</table>

**Fig. 2** Spontaneous release of a tumour necrosis factor (TNF), b interleukin (IL) 6 and c IL-8 from peripheral blood mononuclear cells (PBMCs) in patients with acute pancreatitis on the first day of admission, comparing patients with severe disease ($n = 5$) with those with mild disease ($n = 11$). Points represent cytokine release from PBMCs plated at a standard concentration of $10^6$ cells/ml. There were no significant differences between patients with mild and severe disease.

per unit of blood in patients with severe disease was significantly increased compared with that in mild disease. However, there was no significant difference in TNF release from PBMCs between the two groups (Fig. 3).

Discussion
This study demonstrates increased release of IL-6 and IL-8 from PBMCs in the blood of patients with acute pancreatitis compared with that in healthy volunteers (Table 1). While there was no significant difference in proinflammatory cytokine release per cell when comparing patients with mild or severe disease (Fig. 2), making allowance for the white cell count revealed that IL-6 and IL-8 release per unit of blood was significantly greater in those with severe disease (Fig. 3). This effect was not observed for TNF release (Fig. 2) despite the observation that TNF plays an important early role in the cascade of inflammatory events. Although blood was withdrawn from patients as soon after admission as feasible, the disease process, as manifested by patient symptoms, had been present for hours and in some cases days before admission. It is thus likely that significant release of TNF from PBMCs had already occurred, as indicated by raised serum TNF levels in a number of patients (Fig. 1). It is perhaps of greater significance, with regard to the pathogenesis of acute pancreatitis and its complications, that PBMCs in patients were still able to mount a similar TNF response to that in healthy volunteers despite the raised serum TNF concentration.

Endotoxaemia is a feature of acute pancreatitis. Foulis and co-workers detected endotoxin in the serum of 15 of 16 patients with acute pancreatitis. More recently a significant fall in the serum levels of endogenous anti-endotoxin core antibodies was observed in 28 of 33 cases, implying exposure to endotoxin. Monocyte activation follows exposure to bacterial LPS. While monocytes are able to recognize LPS directly, activation is potentiated when LPS forms a complex with LPS binding protein, which then interacts with the monocyte CD14 receptor. The LPS binding protein is present in human serum but, being an acute-phase protein, its serum concentration is raised in patients with acute pancreatitis. The concentration of LPS binding protein in FCS is not known. However, the presence of LPS binding protein is a consistent finding in other animals and its presence in FCS is probable. In the present study incubation with endotoxin led to a reduced release of TNF, IL-6 and IL-8 from PBMCs in both patient and control groups. While endotoxaemia is unlikely to be the primary event in the pathogenesis of acute pancreatitis, its presence could contribute to the state of monocyte activation and subsequent development of the systemic inflammatory response syndrome associated with the disease.

Culture of isolated PBMCs in either autologous serum or a preliminary study: her presence of LPS binding protein is found in patients with acute pancreatitis. The presence of LPS binding protein in FCS was greater in response to incubation with autologous serum than with FCS. This finding suggests that I-cadherin contributes to the state of PBMC activation with regard to TNF and IL-6 secretion. Damaged acinar cells and the leucocyte infiltrate associated with the disease are known to secrete a wide variety of proinflammatory agents, including complement factors, oxygen-derived free radicals, cytokines, platelet activating factor and leukotrienes. All these agents affect endothelial cells and promote circulating leukocyte activation. Differences in endotoxin concentration between FCS and autologous serum are unlikely to account for this observation, as the LPS stimulation concentration used in this study induced maximal cytokine release in preliminary studies.

An increase in serum IL-6 concentration early in the course of acute pancreatitis is well documented. While a number of immunocompetent cells secrete IL-6 in vivo, the increased secretion of IL-6 by PBMCs observed in the present patients may contribute significantly to raised serum IL-6 concentrations. Studies have reported that both the concentration on admission and the peak level of serum IL-6 correlate with disease severity. Faist et al. have shown that monocyte IL-6 secretion is increased twofold in patients with sepsis. In an intervention study using a mouse burn-sepsis model, IL-1β and indomethacin administration reduced the production of TNF, IL-1 and IL-6 by cultured splenocytes towards normal control levels with a corresponding improvement in the survival rate. These results suggest that in inflammatory conditions such as acute pancreatitis excessive production of IL-6 from PBMCs may be altered by therapeutic intervention and could conceivably influence outcome. Alternatively, IL-6 may simply be a marker of more important proinflammatory mediators.

IL-8 was detected in the serum on the first day of admission in only two patients in the present study. The IL-8 ELISA detection limit of 200 pg/ml and the blood’s capacity for inactivating IL-8 (principally by means of irreversible binding with red blood cells) render serum IL-8 measurement early in the course of an acute inflammatory disease a poor reflection of cellular or PBMC IL-8 production. Similar upregulation of IL-8 release from monocytes has been observed in a study simulating ischaemia-reperfusion. The role of IL-8 in neutrophil-endothelial cell interaction, through chemotaxis and potentiation of the action of the intercellular adhesion molecule 1 on endothelial cells, is well known. In addition IL-8 can induce neutrophil shape change and the shedding of l-selectin from the neutrophil surface, events that appear essential for neutrophil migration. However, the role of IL-8 in the propagation of multiple organ failure is not clear. Both Rotter and Tanaka et al. independently proposed that a sudden release of IL-8 can result in premature activation of circulating neutrophils, including the release of l-selectin and activation of the integrin CD11-CD18 ligand. These events serve to prevent neutrophil emigration by inducing intravascular neutrophil aggregation and stiffening of the cell membrane. These prematurely activated neutrophils are carried in the circulation to sequester in the next microcirculation they encounter. This sequence of events would account for the leucocyte sequestration in pulmonary capillaries observed in animal models of acute pancreatitis and for the beneficial effect of leucocyte depletion in preventing pulmonary complications in such models.

The results of the present study reveal increased proinflammatory cytokine release from PBMCs in patients with acute pancreatitis early in the course of the disease. Further research is required to identify whether this upregulation results from an imbalance in the production of inflammatory mediators or a loss of sensitivity to
downregulatory signals, and to determine whether early therapeutic intervention can alter the progress of the disease.

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References
Interleukin-4 and Interleukin-10 Increase Endotoxin-Stimulated Human Umbilical Vein Endothelial Cell Interleukin-8 Release


ABSTRACT

The aim of this study was to determine the effect of interleukin-4 (IL-4) and interleukin-10 (IL-10) on interleukin-8 (IL-8) release from endothelial cells. Confluent monolayers of human umbilical vein endothelial cells (HUVECs) were incubated in the absence or presence of 10 ng/ml of bacterial lipopolysaccharide (LPS), with 5% human AB serum and recombinant human IL-4 or IL-10 over a dose range from 50 fg/ml to 50 ng/ml (final concentration). IL-4 and IL-10 had no effect on HUVEC IL-8 release in the absence of LPS. In the presence of LPS, IL-4 and IL-10 enhanced IL-8 release by approximately 300% compared with LPS-stimulated cells alone, IL-8 release increasing from 2594 ± 493 pg/ml (no IL-4 or IL-10) to 7892 ± 320 pg/ml (IL-4, 5 pg/ml; p = 0.001) and 8359 ± 712 pg/ml (IL-10, 50 pg/ml; p = 0.002). IL-8 release in response to IL-4 or IL-10 plateaued above 5 and 50 pg/ml, respectively. This study suggests that IL-4 and IL-10 may be involved in the complex regulation of endothelial cell cytokine production during the response to endotoxin.

INTRODUCTION

Endothelial cells form a physical but interactive barrier between the blood and the tissues and are important in the pathophysiology of conditions, such as multiple-organ failure and septic shock. A variety of circulating factors present in major sepsis such as bacterial lipopolysaccharide, interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α), have been shown to induce functional alterations in endothelial cells in culture, including the synthesis and release of proinflammatory cytokines, such as IL-6 and IL-8, and alterations in the expression of cell surface structures involved in adhesion. The role of the IL-6 and IL-8 produced by endothelial cells is not clear, but they appear to influence the inflammatory response by interacting with circulating immune cells to mediate local cell activation and chemoattraction. Clearly, if such proinflammatory events occur unchecked, then this might contribute to microcirculatory failure and the tissue destruction seen in severe sepsis. It seems reasonable that the body should possess mechanisms that downregulate IL-6 and IL-8 release by endothelial cells.

Of considerable interest in this regard are the actions of IL-4 and IL-10, which have been shown to attenuate proinflammatory cytokine production from a variety of immunocompetent cells, including polymorphonuclear cells, monocytes, and macrophages. Paradoxically, however, IL-4 has been shown to induced rather than suppress IL-6 production from unstimulated and endotoxin-stimulated human endothelial cells, but IL-10 had no effect on lipopolysaccharide (LPS)-stimulated IL-6 production. The purpose of this study was to examine whether the same pertains with regard to the effects of IL-4 and IL-10 on the release of IL-8 from endotoxin-stimulated human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Culture media and reagents

RPMI 1640, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from ICN Flow Laboratories (Irvine, UK) and lipopolysaccharide (Escherichia coli 0127:B9), human AB serum (heat inactivated: 56°C for 30 minutes), hydrocortisone, sodium citrate, and sodium acetate from Sigma Chemicals (Poole, UK). Endothelial serum-free (E-SFM) growth and plating medium and recombinant human epidermal growth factor (rEGF) were obtained from GIBCO BRL (Paisley, UK). Endothelial cell growth factor (ECGF), collagenease H, and tetramethylbenzidine (TMB) were purchased from Boehringer Mannheim (Lewes, UK). Recombinant human IL-4, IL-10, and recombinant human endothelial cell-derived IL-8...
were obtained from Genzyme (West Malling, UK). Sulfuric acid was obtained from BDH, Ltd. (Poole, UK).

**Human umbilical vein endothelial cell cultures**

HUVECs were obtained by a modification of the method of Jaffe et al. In brief, human umbilical veins were obtained fresh, cannulated, and treated with collagenase. The vein was flushed with RPMI 1640, and the cells obtained were washed first in RPMI and then in E-SFM growth media. Cells were then cultured in 75 cm² plastic flasks (Costar, High Wycombe, UK) in E-SFM plating media supplemented with penicillin (50 IU/ml), streptomycin (50 μg/ml), hydrocortisone (1 μg/ml), ECGF (50 μg/ml), and rEGF (10 ng/ml). Primary cultures were detached by trypsin-EDTA, washed in RPMI supplemented with 10% FBS, and then washed in E-SFM growth media before being plated on to 96-well flat-bottomed tissue culture plates (Costar, High Wycombe, UK) in E-SFM plating media supplemented as before and the cells grown to confluence. All HUVEC cell lines were characterized by the expression of von Willebrand factor and were used for experiments between the second and fourth passages.

**Experiment protocol**

Confluent HUVECs were incubated in the present or absence of LPS at a final concentration of 10 ng/ml with 5% human AB serum for 5 h. IL-4 or IL-10 were added to the cultures at a final concentration of 50 fg/ml to 50 ng/ml. After 5 h, the cells were washed with E-SFM growth media and incubated for a further 12 h in E-SFM growth media. Supernatants from the cultures were removed after this period and stored at -70°C for subsequent batch cytokine assay. All cultures were performed at 37°C in the presence of 5% CO₂ and 95% humidified air.

**Interleukin-8 Enzyme-linked immunosorbent assay (ELISA)**

IL-8 was assayed using a sandwich ELISA. Supernatants were diluted 1:5 and incubated in duplicate in 96-well ELISA plates (Costar, High Wycombe, UK) coated with rabbit polyclonal anti-human IL-8 (AMS Biotechnology UK, Ltd., Witeney, UK). After incubation, wells were incubated sequentially with goat polyclonal anti-IL-8 antibody (British Biotechnology, Oxford, UK), polyclonal rabbit IgG horseradish peroxidase-labeled antibody (Sigma Immunochromicals, Poole, UK), and the peroxidase substrate TMB in sodium citrate/acetate buffer (pH 4.9). The reaction was stopped with 1 M H₂SO₄, and the plates read at 450 nm using the MR5000 ELISA plate reader (Dynatech, Billinghamurst, UK). Concentrations in the samples were calculated using AssayZap computer software (BioSoft, Cambridge, UK). The limit of sensitivity of the assay was 40 pg/ml.

**Statistical analysis**

One-factor analysis of variance was used to compare the effect of IL-4 or IL-10 on LPS-stimulated HUVEC IL-8 release with that of LPS-stimulated HUVEC in the absence of IL-4 or IL-10.

**RESULTS**

A dose-response curve for the endotoxin-stimulated release of IL-8 from HUVECs was established using LPS over the range 0.1-100 ng/ml and AB serum over the range 0-10%. The optimal LPS concentration to induce IL-8 release was 10 ng/ml with 5% AB serum. HUVECs did not respond to a LPS stimulus (0.1-100 ng/ml) in the absence of AB serum. The results of a representative experiment comparing LPS concentration, length of pulse, and length of further incubation time with HUVEC IL-8 release is shown in Fig. 1.

Results are expressed as the mean of experiments performed on four HUVEC lines. Spontaneous HUVEC IL-8 release in the presence or absence of 5% AB serum was undetectable. IL-4 and IL-10 had no detectable effect on spontaneous IL-8 release from HUVECs following the 12 h incubation period. In the presence of LPS and AB serum, the addition of IL-4 to the culture medium (Fig. 2) led to an initial dose-dependent increase in the mean IL-8 release by HUVEC, rising from 2594 ± 493 pg/ml (no IL-4) to 7892 ± 320 pg/ml (IL-4; 5 pg/ml; p = 0.001, one-factor analysis of variance). Similarly, in the presence of LPS, the addition of IL-10 to the culture medium (Fig. 3) led to a dose-dependent increase in the mean IL-8 release by HUVEC, rising from 2594 ± 493 pg/ml (no IL-10) to 8359 ± 712 pg/ml (IL-10; 50 pg/ml; p = 0.002, one-factor analysis of variance). The addition of IL-4 and IL-10 after the pulse of LPS (at the start of the 12 h incubation period) had little effect on IL-8 release. In these experiments results are the mean ± standard error of the mean of two HUVEC lines. LPS-stimulated HUVEC release of IL-8 was 2490 ± 100 pg/ml, and IL-4 at 50 ng/ml following an LPS pulse enhanced the release of IL-8 to 3130 ± 480 pg/ml, and IL-10 at 50 ng/ml following an LPS pulse.
puzzle increased the release of IL-8 to 3065 ± 95 pg/ml. None of these increases were statistically significant.

DISCUSSION

The antiinflammatory actions of IL-4 and IL-10 are well recognized.\(^{10-13}\) For example, both in experimental arthritis\(^{11}\) and in cancer patients receiving immunotherapy,\(^{19}\) the production of the proinflammatory cytokine IL-1 is reduced by IL-4. In addition, IL-10 protects mice against Staphylococcus enterotoxin B-induced lethal shock,\(^{20}\) and IL-4 and IL-10 exhibit synergy to inhibit cell-mediated immunity in a mouse model of leishmaniasis.\(^{21}\) IL-8 can be regarded as a proinflammatory cytokine. Thus, in the present study, the enhancement by IL-4 and IL-10 of IL-8 release by endothelial cells in the presence of endotoxin appears somewhat paradoxical.

IL-8 is a potent chemoattractant for circulating leukocytes to sites of acute inflammation.\(^{19,22,23}\) Endothelial surface-bound IL-8 is known to promote the integrin-mediated adhesion between neutrophils and endothelial cells.\(^{18,24}\) In addition, IL-8 induces neutrophil shape change and the shedding of L-selectin from the surface of neutrophils adhered to endothelial cells, events that are essential for neutrophil emigration.\(^{25}\) However, Gimbrone and coworkers\(^{25}\) have shown that although certain inflammatory stimuli render cultured human endothelial cells hyperadhesive for neutrophils, this state is transient and reversible. The mechanism for the attenuation of endothelial cell-neutrophil interaction was attributed in part to the secretion by activated endothelial cells of a soluble leukocyte adhesion inhibitor, identified as IL-8γγ, the extended amino-terminal IL-8 variant.\(^{25}\) Furthermore, recombinant human IL-8 inhibited neutrophil adhesion to IL-1-activated endothelial monolayers, protecting the monolayers from neutrophil-mediated damage.\(^{25}\) One explanation for the observations reported by Gimbrone and coworkers is that IL-8, like all chemoattractants, is an ineffective promoter of neutrophil invasion if the stimulus is distributed equally between apical and basal compartments, thereby destroying the concentration gradient. Under normal conditions of blood flow through a vessel, the IL-8 released by endothelial cells from the luminal surface may be bound to antibodies and red cells and removed. However, if neutrophils come into contact with soluble blood-borne IL-8, they may undergo shape change and shedding of the L-selectin receptor before their initial contact with endothelial cells, thus losing their ability to adhere to the endothelium and migrate.\(^{25}\) IL-4 and IL-10 may therefore exert an antiinflammatory action on endothelial cells by inducing shedding of surface-bound IL-8 or by the secretion of intracellular IL-8. The further synthesis of IL-8 de novo may not occur because IL-4 in particular has been shown not to induce IL-8 messenger RNA expression.\(^{26}\)

The role of proinflammatory cytokines in both acute and chronic inflammatory diseases is now well recognized, and successful therapeutic strategies have been developed to reduce the influence of such cytokines, for example the administration
of anti-TNFα or anti-IL-6 antibodies in certain models of septic shock. The established role of IL-4 and IL-10 in the downregulation of leukocyte proinflammatory cytokine release has led to the suggestion that IL-4 and IL-10 could be used to treat such conditions as septic shock. The results of the present study indicate that IL-4 and IL-10 augment LPS-mediated IL-8 release from endothelial cells and may indeed provide a means of altering IL-8 concentration gradients within the local microenvironment. Further studies are required to determine the outcome of treatment with IL-4 or IL-10 under various conditions in vivo.

The findings presented here, together with the earlier work on endothelial cell IL-6 release, indicate that IL-4 and IL-10 may be involved in a complex regulation of endothelial cell activation following exposure to endotoxin. An acute inflammatory response requires the sequestration of circulating monocytes, lymphocytes, and neutrophils into sites of inflammation. Clearly, the attraction and migration of circulating cells into such sites may be regulated with some precision and specificity. The fine-tuning of the cytokines produced by endothelial cells and the cell surface molecules expressed by these cells in the local microenvironment may lend this precision and specificity.

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REFERENCES


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Serum concentrations of inflammatory mediators related to organ failure in patients with acute pancreatitis

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Leucocyte activation and proinflammatory cytokine release (tumour necrosis factor (TNF) and interleukin 6 (IL-6)) are thought to contribute to the induction of a systemic inflammatory response, an acute-phase response and multiple organ failure in patients with acute pancreatitis. The serum concentration of TNF, soluble TNF receptors (sTNFRs), IL-6 and C-reactive protein (CRP) in 58 patients with acute pancreatitis was assessed during the first 2 days of admission. Thirty patients had mild disease and 28 severe disease, of whom 18 developed local pancreatic complications alone (Atlanta classification) and ten developed organ failure (a Goris score of 1 or more). TNF was detected in only 17 patients on the first day of admission, while soluble TNF receptors were detected in all patients and IL-6 in 34. On the first and second days of admission there was a progressive and significant ($P<0.03$) increase in the median concentration of sTNFR$_55$, sTNFR$_75$ and IL-6 in patients eventually classified into those with mild disease, a local pancreatic complication alone, or organ failure. This pattern was also evident in CRP levels from the second but not the first day of admission. These findings suggest that proinflammatory cytokines or their soluble receptors may be more accurate early predictors of outcome than CRP. Moreover, markers of inflammation in the sera of patients with acute pancreatitis are highest in those who subsequently develop organ failure.

Acute pancreatitis remains a common and potentially fatal disease in which some patients develop extensive pancreatic inflammation and necrosis, a systemic inflammatory response and multiple organ failure. It has been proposed that the systemic sequelae of pancreatitis arise from excessive leucocyte activation with the release of secondary mediators such as proinflammatory cytokines, including tumour necrosis factor (TNF) and interleukin (IL) 6. The central role of TNF (a potent cytokine produced mainly by activated macrophages) in orchestrating aspects of the early inflammatory response in sepsis is well established. Increased levels of TNF have been documented in the serum of some groups of patients with sepsis and after exposure to endotoxin. However, TNF is often undetectable in the sera of patients with acute pancreatitis, even those with severe disease. This may be because of the short half-life of TNF, binding of TNF to other proteins, or difficulties with the various methods of measuring TNF in serum. Alternatively, it may be that TNF is not produced in patients with acute pancreatitis or is mainly produced in the tissues and only occasionally spills over into the circulation.

The paracrine and endocrine actions of TNF are exerted by interaction with membrane-bound TNF receptors on the surface of target cells. It appears that after exposure to substances such as bacterial endotoxin (and indeed TNF itself), such target cells downregulate their responsiveness to TNF by shedding the receptor into the circulation. Two such soluble receptors have been identified: a 55-kDa (sTNFR$_55$) and a 75-kDa (sTNFR$_75$) fragment. The release of these soluble receptors (which have a longer half-life in serum than TNF itself) appears to reflect the degree of TNF-induced inflammation. Thus, although TNF may be undetectable in blood, the presence of such soluble TNF receptors may be a useful index of TNF activity.

Other cytokines involved in the inflammatory cascade, such as IL-6, are more often detected in the sera of patients with acute pancreatitis. Both the serum concentration of IL-6 on admission and the peak level of IL-6 have some correlation with the severity of the disease. One of the many actions of IL-6 is to induce the production of hepatic acute-phase proteins, such as C-reactive protein (CRP). The serum concentration of CRP is also increased in patients with acute pancreatitis and correlates with the severity of the disease. The rise in serum CRP concentration lags behind the peak in serum IL-6 concentration by some 24-48 h and has been interpreted by some investigators to indicate a cause and effect relationship.

It has been proposed that a prolonged or excessive inflammatory response may be one of the mechanisms whereby patients develop multiple organ failure. The major causes of death in acute pancreatitis stem from either local peripancreatic complications or multiple organ failure, or a combination of the two. In the present study, the systemic inflammatory response as measured by markers of inflammation (TNF, sTNFR$_55$, sTNFR$_75$, IL-6 and CRP) in the serum of patients with acute pancreatitis have been investigated and related to the development of local complications and organ failure.

Patients and methods

Patients with acute pancreatitis admitted to the Royal Infirmary, Edinburgh were studied. The diagnosis of acute pancreatitis was based on the presence of appropriate clinical or radiographic evidence accompanied by a serum amylase concentration greater than 1000 units/l (Phadebas; Pharmacia Diagnostics, Uppsala, Sweden; normal range 70-300 units/l). The clinical course of the patients was followed prospectively, allowing retrospective categorization of patients into those with mild or severe disease based on the Atlanta classification. Twenty-eight patients with severe disease represented a consecutive series over a 16-month period. Thirty of 78 patients with mild disease admitted over the same time period were randomly selected for comparison.
Patients with severe disease were further subcategorized into those with local pancreatic complications alone \((n = 18)\) and those with organ failure (a Goris score\(^2\) of 1 or more; \(n = 10\)). Six patients with organ failure died during the index admission. Gallstones were suspected as the aetiology in 26 patients, alcohol in 18 and the presence of pancreatic cancer in one. Pancreatitis complicated endoscopic retrograde cholangiography in two patients and the aetiology was not identified in 11. Venous blood was sampled on the first and second day of admission and the serum stored at \(-70^\circ\text{C}\) until subsequent batch analysis for TNF, sTNFR\(_{55}\), IL-6 and CRP concentration by indirect enzyme-linked immunosorbent assay (ELISA).

Assay of cytokines and C-reactive protein

TNF was measured with an ELISA using a murine monoclonal antihuman TNF antibody and peroxidase-conjugated Fab fragments of a murine monoclonal antihuman TNF antibody (Boehringer Mannheim, Lewes, UK). A standard curve was constructed with recombinant human TNF (British Biotechnology, Abingdon, UK) and calibrated with an international standard (NIBSC, Potters Bar, UK). The lower limit of detection of the assay was 15 pg/ml.

Serum sTNFR\(_{55}\) and sTNFR\(_{75}\) were detected by ELISA, with monoclonal and polyclonal anti-sTNFR\(_{55}\) and anti-sTNFR\(_{75}\) antibodies. Purified sTNFR\(_{55}\) and sTNFR\(_{75}\) were used to construct standard curves. The lower limit of detection of the assay was 150 pg/ml for both receptors.

The ELISA for IL-6 used a murine monoclonal antihuman IL-6 antibody (Boehringer Mannheim) as the capture antibody and a goat polyclonal (non-enzyme labelled) antihuman IL-6 (R&D Systems, Oxford, UK) followed by a peroxidase-conjugated sheep anti-goat immunoglobulin G antibody (Sigma Immunochemicals, Poole, UK) to detect bound IL-6. A standard curve was constructed with recombinant human IL-6 (British Biotechnology) and calibrated with an international standard (NIBSC). The lower limit of detection of the assay was 30 pg/ml.

CRP was detected by sandwich ELISA, with rabbit antihuman CRP and peroxidase-conjugated rabbit antihuman CRP (DAKO, High Wycombe, UK). A standard curve was constructed with a human serum CRP calibrator (DAKO). The lower limit of detection of the assay was 1 mg/l.

Statistical analysis

Results are presented as the median (interquartile range). Initial comparison among patients with mild disease, a local pancreatic complication alone and organ failure was performed using the Kruskal–Wallis test. Thereafter, Mann–Whitney \(U\) tests were applied to compare differences between any two groups; significance was assumed when \(P<0.05\). Where levels of the cytokine being measured were below the limit of detection of the assay, the minimum detection concentration was assigned to the sample and statistical analysis was performed using this convention.

Results

None of the 30 patients with mild disease developed significant complications. In contrast, 18 of 28 with severe pancreatitis developed local complications alone, while the remaining ten developed failure of one or more organ systems, with or without an accompanying local pancreatic complication. There was no significant difference in the age \((P=0.29)\) or sex ratio \((P=0.57)\) between those with mild pancreatitis and those with severe pancreatitis.

Serum concentrations of TNF were below the level of detection in 41 of 58 patients on the first day of admission (29 of 30 with mild disease, eight of 18 who developed a local pancreatic complication and four of ten who developed organ failure). On the first day of admission, the median serum concentration of TNF was greater in patients who developed organ failure \((P<0.001)\) and in those who developed a local pancreatic complication \((P<0.001)\) than in those with mild disease (Table 1). There was no significant difference between patients who developed organ failure and those who developed a local pancreatic complication alone \((P=0.34)\). On the second day of admission, the median serum concentration of TNF was greater in patients who developed organ failure than in those who developed a local pancreatic complication \((P<0.006)\) and in those who had mild disease \((P<0.006)\) (Table 1). There was no significant difference between patients who developed a local pancreatic complication alone and those who had mild disease \((P=0.07)\).

Serum sTNFR\(_{55}\) and sTNFR\(_{75}\) were detectable in the 58 patients. On both days 1 and 2 of admission, the median serum concentration of sTNFR\(_{55}\) was greater in patients who developed organ failure than in those who developed a local pancreatic complication \((P<0.003)\). In both patients who developed an organ complication alone (\(C; n=18\)) and organ failure (\(\bullet; n=10\)). Interquartile range represented by the error bars.

| Table 1 | Concentration of serum tumour necrosis factor (TNF) and serum C-reactive protein (CRP) in patients with acute pancreatitis on the first and second day of admission |
| --- | --- | --- |
| Mild disease \((n=30)\) | Pancreatic complication alone \((n=18)\) | Organ failure \((n=10)\) |
| **Serum TNF (pg/ml)** | | |
| Day 1 | 15.0 (15.0–22.4) | 17.8 (15.0–23.8) | 22.6 (15.0–75.1) |
| Day 2 | 15.0 (15.0–22.4) | 15.0 (15.0–22.4) | 21.1 (15.0–51.4) |
| **Serum CRP (mg/l)** | | |
| Day 1 | 31.4 (10.0–61.5) | 11.6 (7.8–36.5) | 36.0 (14.9–58.9) |
| Day 2 | 36.1 (22.8–84.4) | 33.0 (19.7–121.1) | 99.9 (79.4–136.2) |

Values are median (interquartile range).

groups with severe disease, the median serum concentration of sTNFR_55 was greater than that in patients who had mild disease (P<0.001 in both cases) (Fig. 1). Serum concentrations of sTNFR_55 showed a similar distribution among the three groups to that found for the sTNFR_75 receptor (Fig. 2).

Serum concentrations of IL-6 were below the level of detection in 24 of 58 patients on the first day of admission (20 of 30 with mild disease, three of 18 who developed a local pancreatic complication and one of ten who developed organ failure) and in 23 of 58 on the second day (19 of 30 with mild disease and four of 18 who developed a local pancreatic complication). On both days 1 and 2 of admission, the median serum concentration of IL-6 was greater in patients who developed organ failure than in those who developed a local pancreatic complication alone (P<0.03), which in turn was greater than in those with mild disease (P<0.001) (Fig. 3).

CRP was detectable in the serum of all patients. On the first day of admission, there was no significant difference in the median serum levels of CRP among patients who developed organ failure or a local pancreatic complication and those who had mild disease (P=0.15) (Table 1). By the second day of admission, the median serum concentration of CRP was greater in patients who developed organ failure than in those who developed a local pancreatic complication (P=0.02) or those who had mild disease alone (P=0.003) (Table 1). However, the median serum CRP level in patients who developed a local pancreatic complication was not significantly different from that in those with mild disease (P=0.80) (Table 1).

Discussion

The present study demonstrates that during the first 2 days of admission, concentrations of markers of inflammation in the sera of patients with acute pancreatitis are highest in those who subsequently develop organ failure.

TNF has been implicated as a key mediator in the development of multiple organ failure. In the present study, however (in keeping with previous reports), TNF was detected in only 17 patients on the first day of admission and in eight by the second day. This finding may be accounted for by the short half-life of serum TNF and the fact that blood for TNF estimation was sampled only once per day. Other authors have suggested that TNF appears transiently and repetitively in the circulation and thus the peak concentration of TNF could easily be missed by infrequent blood sampling. In contrast, soluble TNF receptors were detected in all patients, with a progressive increase in the median concentration of both sTNFR_55 and sTNFR_75 being observed among patients who had mild disease, those who developed a local pancreatic complication alone and those who developed organ failure (Figs 1 and 2). Spinas and co-workers demonstrated a significant correlation between peak serum TNF and sTNFR_75 in human volunteers after intravenous administration of Escherichia coli endotoxin, suggesting that sTNFR_75 may reflect the degree of TNF-induced inflammation. If this assumption is correct, the finding in the present study of increased soluble TNF receptors on admission in patients who subsequently develop severe acute pancreatitis would support the contention that TNF is an important central mediator of a
Inflammatory mediators are thought to be important in the pathogenesis of the systemic sequelae of acute pancreatitis. Two such mediators are endotoxin and tumour necrosis factor-alpha (TNF-α). Endotoxin, released from Gram-negative organisms following bacterial lysis, is a potent stimulus for the release of proinflammatory cytokines from leukocytes and endothelial cells. In acute pancreatitis, reduced intestinal perfusion resulting in increased intestinal permeability might allow endotoxin to cross the gut wall and enter the circulation. Alternatively, endotoxin might enter the circulation from other infective foci (e.g. a pancreatic abscess). TNF-α, a major proinflammatory cytokine, is considered to be important in orchestrating the early events in the inflammatory response and may contribute to the induction of organ failure in severe inflammatory states.

ENDOTOXIN

The outermost layer of the bacterial cell surface consists of lipopolysaccharide and is chemically unique for each strain of bacteria. The term, endotoxin, refers to the impure extract of lipopolysaccharide found in combination with different proteins. Patients with pancreatitis are prone to develop hypoxia, hypotension, renal failure, hypocalcaemia and disseminated intravascular coagulation, all of which can be produced experimentally by endotoxin. It has been established for some time now that endotoxaemia occurs in a proportion of patients with acute pancreatitis (1, 2). Foulis et al. (1) studied 26 attacks of acute pancreatitis and detected endotoxin in 13 of the attacks. Furthermore, endotoxaemia was found in six of the seven patients defined as having a severe attack. In this study, blood was sampled only once per day and endotoxaemia was said to be present if endotoxin was detected on two consecutive days. The release of endotoxin is known to be episodic and endotoxin is rapidly cleared from the circulation. Thus, detection of endotoxaemia in patients with acute pancreatitis may not only be related to disease severity, but also to the timing and frequency of blood sampling. In another study (2), endotoxin was detected in only 51% of patients with predicted severe disease when blood samples were taken on days 1, 3 and 7 following admission. To address this question further, Kivilaakso et al. (3) measured the levels of antienterobacterial common antigen antibody. The enterobacterial common antigen is a component of the cell wall of Gram-negative bacteria. It is common to all enteric bacteria and is spatially linked to lipopolysaccharide. At the time of admission, patients with severe acute pancreatitis were found to have significantly depressed antienterobacterial common antigen antibody titres when compared to both patients with mild disease and to controls. Furthermore, there was a significant rise in the titre during the course of the illness in those patients who survived. This rise was not seen in those patients who died. The exact mechanism for these changes in antienterobacterial common antigen antibody remains to be delineated. However, they suggest significant exposure to enterobacterial common antigen from the portal circulation, with either binding to circulating antibody and subsequent removal of the resulting complexes (fall in titre), or the synthesis of new antibody (rise in titre).
Table I. Fall in IgM and IgG antiendotoxin antibody response in patients with predicted mild and severe pancreatitis (*p < 0.001 and **p < 0.05 Fisher's exact two-tailed test). Reproduced with permission (4)

<table>
<thead>
<tr>
<th></th>
<th>IgM antibody</th>
<th>IgG antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild pancreatitis (n = 10)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Severe pancreatitis (n = 23)</td>
<td>23*</td>
<td>9**</td>
</tr>
</tbody>
</table>

ANTIENDOTOXIN ANTIBODIES

In our study (4), we investigated naturally occurring, circulating antibodies that react with the core glycolipid region of endotoxin. We found that the serum concentration of IgM antiendotoxin antibodies fell (defined as less than the 10th centile of the normal population or a greater than 50% reduction below the median value) in all patients with predicted severe pancreatitis (Table I). This change was independent of any alteration in the total serum immunoglobulin concentration. In contrast, the serum IgG antiendotoxin antibody concentration fell in only nine of the 23 patients with predicted severe pancreatitis, while a significant fall in IgG class antiendotoxin antibody concentration occurred in all seven patients who developed multiple organ failure (of whom five died). This suggests that depletion of the IgG antiendotoxin antibody pool is physiologically important. A possible explanation for this association is that low antibody titres allow increased exposure to free endotoxin. This results in enhanced activation of systemic responses, such as the cytokine cascade, which are linked to the development of multiple organ failure. These findings are similar to those in our more recent study of sepsis syndrome due to infection, in which a significantly higher mortality rate (5) was found in patients, who had depleted IgG antiendotoxin core antibody upon admission to the intensive care unit.

TUMOUR NECROSIS FACTOR-ALPHA (TNF-α)

Endotoxin has a number of actions that can contribute to the inflammatory response. It is known to activate the complement pathway and C3 catabolism has been demonstrated in patients with acute pancreatitis (1). It can stimulate immune competent cells to release a vast array of inflammatory mediators, including cytokines, free radicals and bioactive lipids. One of the major cytokine mediators, that can potentially be released by endotoxin, is TNF-α. It is secreted predominantly by activated monocytes and macrophages, but lymphocytes, fibroblasts and other cells also produce TNF-α.

The central role of TNF-α in orchestrating an early inflammatory response in patients with sepsis syndrome is well established. Elevated levels of TNF-α have been documented in the serum of patients with sepsis (6) and following exposure to endotoxin (7). However, TNF-α is often undetectable in the sera of patients with acute pancreatitis, even in those with severe disease (2, 8, 9). This may be due to the short serum half-life of TNF-α, the binding of TNF-α to other proteins, difficulties with the various methods of measuring TNF-α in serum, or that TNF-α is mainly produced in the tissues and only occasionally spills over into the circulation. TNF-α is also broken down by neutrophil elastase, which is elevated in patients with acute pancreatitis (10).

Soluble TNF-α receptors

TNF-α exerts its paracrine and endocrine action by interaction with membrane-bound TNF-α receptors on the surface of target cells. Following exposure to substances, such as bacterial endotoxin and TNF-α itself, such target cells downregulate their responsiveness to TNF-α by shedding the receptor into the circulation. Two such soluble receptors have been identified: a 55 kDa (sTNFR55) and a 75 kDa (sTNFR75) fragment (11). The biological function of these shed receptors is not clear, but they may bind to circulating TNF-α and therefore reduce its bioavailability.

We studied the serum concentration of TNF-α and soluble TNF receptors in 58 non-consecutive patients with acute pancreatitis on the first day of admission (12). Thirty of these patients had mild disease and the remainder had severe disease as defined by the Atlanta classification (13), of whom 18 subsequently developed a local pancreatic complication and 10 developed organ failure, as defined by the Goris score (14). As found by others, TNF-α was detected in a minority of patients (29%) (Table II). In contrast, soluble TNF-α receptors were detected in all patients. There was a stepwise and statistically significant (p < 0.01 Mann-Whitney U test) increase in the median concentration of both sTNFR55 and sTNFR75 at hospital admission in patients eventually

Table II. Number of patients with acute pancreatitis with detectable TNF-α and soluble TNF receptors on admission. Severity of disease was determined by the Atlanta classification (12) and organ failure determined by the Goris score (13). Reproduced with permission (12)

<table>
<thead>
<tr>
<th></th>
<th>Mild pancreatitis (n = 30)</th>
<th>Severe pancreatitis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Local complication (n = 18)</td>
<td>Organ failure (n = 10)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>
| sTNFR             | 30                         | 18                  | 10
TNF-α and integrity of antibody function in acute pancreatitis. It has also been demonstrated in pneumocytes of the lung tissue of patients with acute pancreatitis dying from acute respiratory distress syndrome (18). However, a cause-and-effect relationship has not been established for TNF-α.

Both endotoxin and TNF-α are biologically active compounds that have been implicated as key mediators in the development of sepsis syndrome and progression to multiple organ failure. In patients with acute pancreatitis, detection of such mediators in the serum is variable, even in patients with severe disease. Nevertheless, the depletion of serum antibodies to endotoxin and the release of soluble TNF-α receptors into the circulation suggest that both endotoxin and TNF-α are involved in the systemic inflammatory response in acute pancreatitis.

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REFERENCES


